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Peer Review File

Single-cell landscape of innate and acquired drug resistance in acute myeloid leukemia

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REVIEWER COMMENTS

Reviewer #1, expertise in single cell omics, CyTOF and AML (Remarks to the Author):

SUMMARY
Overall, this manuscript describes a tour-de-force in primary patient AML sample profiling, integrating bulk and single cell high-parameter technologies with 2 powerful and functional precision medicine techniques. Applying this many analyses to 21 relapsed and refractory patients is powerful and represents a highly valuable resource.

Overall impact:
The analyses they perform, primarily to understand molecular markers and mechanisms of resistance to venetoclax, are interesting and impactful, despite the large body of literature devoted to this question, this large scale, highly integrated dataset significantly advances our understanding of this frequently-addressed question. The authors leverage their unique dataset to investigate this question and provide two possible solutions/treatment strategies to overcome venetoclax resistance. Given the very widespread use of venetoclax in AML patients, mechanisms of resistance represent a critical area of research. Developing resistance is nearly inevitable for a vast majority of patients. Strategies to overcome that resistance represent an important research priority.

Methods: the technologies that are integrated in this trial (TUPRO) are highly innovative and impactful. This is a stellar line-up of assays.

Our major concerns lie mainly in the description of the methods and of the results. There are many areas that need clarification. However, as written and performed, these are impactful and interesting analyses and results. Some problems with data reporting and explanation weaken the impact and strength of the results.

Results:
Overall, the results are interesting and compelling. However, there are several areas where the data reporting is inadequate or presentation of the data is unclear/misleading:
1. Table S1:
a. It is not clear how many lines of therapy each patient had prior to sampling. Column O/"Treatment before TUPRO sampling" lists drug regimens, but it is unclear if this was the only regimen or if patients had multiple lines of therapy. Likewise, it is unclear what is meant by "Treatment response before TUPRO" (column P), does a "yes" imply CR followed by relapse or does a "yes" mean some reduction of disease burden? Several samples are listed as "relapse" but have a "no" for treatment response before TRUPRO, suggesting perhaps that the treatment listed in columns N/O are non-first line. Later in the manuscript (Figure 2C, lines 205-206), prior exposure to Venetoclax is listed in aggregated patient data, but it would be very important to understand when the patients were exposed to venetoclax if not immediately prior to TUPRO sampling
b. Column T lists responses such as VGPR, SD, PR. These treatment response terms are not standard for AML and should be defined (and referenced).
2. Figure 1 (and Tables 6 and 7): identify AML cells with CyTOF and scRNA seq and bulkify these cells based on AML cell "groups" (AML cell subsets). It is not clear how the authors

define AML cells by CyTOF or by scRNA seq. It would be important to define that and explain what cells contribute to bulkification in the scRNA seq data and in the CyTOF data.
3. Figure 1C: compare concordance of blast % measurements by different technologies, (table S10 and Figure S1). The manuscript lists that the various technologies are concordant with each other. Figure S1B lists concordance. On review of table S10, none of these technologies appear to be concordant with the pathological blast count, which would be the gold-standard. CyTOF shows some concordance, but the other ones have minimal concordance with pathology. This issue needs more clarity.
4. Figure 1D: It is not clear to me what is being correlated in 1D what is being compared to what? Marker expression correlation between samples? Why would that be correlated?). The figure legend does not help with this determination. Results, lines 169-170 Figure 1D: "Blood and BM taken at same visit from same patient showed "highly similar composition (% AML blasts) in CyTOF and PCY" Why would we expect the blood and BM blasts percentage to correlate? It only rarely correlates within the same patient. I think I do not understand what is being correlated. What two things are being compared?
5. Figure 2/Table S9 (PCY drug responses). Figure 2 represents data from which of the tested drug concentrations? Table 9: Data in sheet one is averaged across replicates, but for which concentration is the data representing (each drug is used in 2-3 doses)?
a. Lines 518-520: In the text, AUC values for PCY data are described but AUC values are not found in the manuscript or supplemental information. (Can you calculate an AUC with only 2 drug concentrations tested?) Also, it is unclear if the authors are averaging across different drug concentrations (if that is the case, that would need some justification) or averaging across replicates receiving the same concentration. As written, it is unclear how PCY data is calculated using the different drug concentrations.
6. Lines 187-196 discuss a subset of samples with low sensitivity to common AML treatments. These samples are said to be increasingly sensitive to a list of drugs, with reference to Figure S2B. However, Figure S2B does not identify these samples or display their sensitivity of resistance to drugs.
7. Supplementary Figure S3A is difficult to see and difficult to interpret. It is difficult to see how the absolute changes in response to venetoclax measured by 4i DRP correlate with the PCY measurements, as claimed by the text (lines 210-212). This claim is not supported by the data as it is presented in this figure. In contrast, Figure 2D demonstrates this to be the case for the 4 features that are displayed there.
8. Lines 222-223 claim that lower levels of BCL2 are correlated with venetoclax resistance, but the correlations (r2 values) are very very weak. The protein levels (the actual effector molecule) are not significantly different neither by strength of the r2 nor by significance testing nor by visualization of the whisker plots. This is particularly concerning in light of the overwhelming prior evidence that BCL2 downregulation is a bone fide resistance mechanism to venetoclax and therefore calls into question the quality of the data.
9. Figure 3 and S6, deciphering molecular features of innate and (presumably) acquired venetoclax resistance is very interesting.
10. Figure 3D: the description of how effect size was calculated is unclear (lines 746-751): Does 3D represent genes? Proteins? Both? Please clarify how these values were calculated to get these data.
11. Figure 3E (CD36 expression related to ven resistance): which specific cell cluster and cell types (annotated by scROSHI) are associated with VEN resistance? More specifically, is there a common cell cluster across samples that showed the strongest association between CD36

expressions and VEN drug resistance response? tSNE plots like Figure3C for CD36 expression, BCL2 expression, cell cluster annotations, and cell type annotations (scROSHI annotations) would be informative here Also, a plots that compares CD36 expression in different clusters (like in Figure S4C) would be informative.
12. Figure 4/Lines 261-263: refer to "associations between innate and acquired resistance" being correlated. It would be helpful to explain what is being associated and include a color legend (at least in the figure legend to figure 4A).
a. Figure S7B is more intuitive and interesting than Figure 4A.
b. Can authors report how many associated genes were significant in the comparison of innate and acquired VEN? And what are the genes that were associated with the VEN naïve drug response?
13. Figure 4C: How is the "pathway score" measured?
14. Figure 5 is very interesting and well-done overall. However, Figure panel 5J misses the mark: the only 2 AML cell subtypes that are shown have high CD36 expression. It would be important to show additional AML cell types (especially immature cells) to substantiate the claim that these two cell types have higher CD36 levels relative to other (defined) AML cell types.
15. Figure 5A (association of CD36 levels with survival in TCGA): does this same result hold up in the Beat AML?
16. Figure 5J: please define VST (VST-normalization)
17. Line 166: How was blast content enumerated from scDNA-seq (only those cells with CNVs detected?).
18. What marker genes are used for cell type annotation in scROSHI (what reference is used for these annotations)?
Minor issues:
19. Many tables are not labeled. We were only able to infer which table by the order in which it was listed. This becomes a problem for tables 6 and 7 where the data basically look identical. It would be useful to include the name of the table within the table file.
20. The color scale for ven-resistance in Figure S4 C is difficult to decipher.
21. An issue with word choice: The term "acquired" venetoclax resistance is possibly misleading. Are these all patients who were at one point sensitive to venetoclax and then went on the become resistant? The distinction between venetoclax-exposed and naïve patients is an important one but it seems misleading and possibly incorrect to designate venetoclax-exposed patients as having acquired venetoclax-resistance. Some of these patients may have been innately resistant as well. Unless these venetoclax-exposed patients are known to have responded to venetoclax in the past and developed resistance (which should detailed in table S1), the term should be changed.
22. Typo line 287 "to wards" (means to be towards)
23. Line 576 references Figure S2, but we think they mean Figure S4

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3, expertise in venetoclax and AML (Remarks to the Author):

The authors have submitted a manuscript that reflects a tremendous effort at characterizing ex vivo leukemia samples from patients with relapsed and refractory acute myelogenous leukemia. They have performed a significant multilayered, multi-omic profile of these leukemia samples down to the single cell level. The study summarizes a method of single cell ex vivo drug profiling, along with bulk and single cell DNA and RNA protein profiling, with added clinical annotations.

They show a high throughput ex vivo analysis of drug sensitivity of up to 79 drugs and drug combinations using automated evaluation using pharmacoscopy to assess cell death, as well as blast identification using multiple methods such as cytof and DNA content. Using 21 patients with relapsed refractory AML the submitted paper has a great deal of information regarding the characteristics of these AML blasts, ranging from expression profile, to drug sensitivity. I lament while reading the paper, that much of this information is included in the supplemental section with just a snapshot in the main manuscript.

While there is a plethora of data included within the manuscript, the overall study reads as a "proof of concept" or “feasibility study” of this impressive technology and its potential application for patients with relapsed and refractory AML, with the intent to find new therapies that may be potentially applicable to these patients in a highly individualized manner. The implications of such a technique, if validated and reproducible, or tremendous for patients who have few options after 1 or 2 lines of therapy for newly diagnosed AML. However little as conveyed as to the real world, in real-time applicability for patients in clinic. For example: what is the turnaround time for these assays? Can this be conducted alongside a clinical lab? How to the authors see the implementation of this powerful method going forward? Have the authors considered using this technique in patients in morphologic remission but with detectable minimal residual disease?

Several prior studies have demonstrated ex vivo sensitivity testing for patient samples with relapsed refractory AML using different techniques. This is one of the few papers that integrates not only drug sensitivity, but clinical information including karyotype, baseline mutations, as well as expression profiling by single cell RNA seq and cytof based proteomics. This is one of the major strengths of this paper. The authors do not provide a more detailed description of the patient's prior therapies within their analysis set, or a structured analysis of groups of patients will be treated with similar therapies to determine whether a given set of therapies that are associated with similar mechanisms of relapse or resistant. These would be helpful and provide some context on how this technique functions across therapies. More samples, possibly from trial of uniformly treated patients may also be important. Additionally, p53 mutated AML, which represents a major unmet need in the current treatment of AML is represented by only 3 patients– a potential missed opportunity to learn more about this difficult subset.
There is little in terms of new discovery within this paper, but important confirmation of

observations (using different approaches) regarding potential mechanisms of resistance to venetoclax including up regulation of oxidative phosphorylation, down-regulation of effectors of apoptosis, and up regulation of CD36.

One of the realities that has become very apparent clinically in patients with AML is the tremendous clonal heterogeneity and hierarchy at the time of diagnosis which changes based on the treatment paradigm and which inevitably gives rise to resistant subclones that form the basis for relapsed disease. The authors allude to this within their introduction. However in reviewing the techniques implemented here and the single cell data provided in these relapsed patients, there is less focus on this heterogeneity and clonal hierarchy at the time of relapse, including clonal dominance, or potential drivers of future resistance. It may be interesting to include this information if it can be derived from the available data.

In addition to the above comments, below are some comments by section from the results:

1. Single cell molecular and functional profiling of relapsed refractory AML samples.

A. In terms of feasibility it appears that only 10 visits or 32% were able to be analyzed by all of the technologies listed. Is this is something that can be optimized in the clinical setting, to make sure a complete characterization is facilitated? How much sample would be needed in a prospective manner to be able to perform all of the testing demonstrated.

B. Figure 1D is not well explained within the text, nor the figure Legend. The order in which each figures explained also seems to be backwards. Please consider rewording sentences to describe this figure and reordering the subfigures so it is easier for the reader to understand what you are trying to show.

2. Prior venetoclax treatment Shames the ex vivo drug response landscape.

A. 79 drug and drug combinations tested ex vivo on the samples is quite impressive.

3. Multimodal recovery of innate and acquired venetoclax resistance mechanisms

A. Observing acquired venetoclax resistance in patients treated with prior venetoclax is intuitive and clinically very apparent. The question is the mechanism of this resistance which has been previously reported to by several of the authors cited, including altered dependency to BH 3 family of proteins, as well as modulation of expression of various effectors of intrinsic apoptosis. Much of this is confirmatory findings from prior studies, with additional observation of CD36 up regulation correlating strongly with venetoclax resistance. Any new findings within this data to explain this dynamic resistance. Any followup ex vivo testing of the resistant samples treated with Venetoclax based combinations with other partners found in the screen (ie. Bcl-2i + bcl-xli, etc.) It would be interesting to see here further characterization of BH 3 family proteins including MCL–1, and BCL–XL as drivers of resistance in these patients and whether he is or present as intrinsic resistance among those patients who are naïve to venetoclax. Any analysis to correlate surface markers (besides CD36) and morphology with Ven resistance? There is recent data suggesting that erythroid AML is resistant to Ven. Did this show up in your screen?

Correlation between an erythroid gene signature (RNA seq) and ven resistance?

B. Furthermore the data regarding Bcl-2 expression by Cytof among patients who were exposed are naïve to venetoclax seems to show very little difference, whereas the single cell RNA shows a more significant difference. How would you explain this?

4. Global transcriptional analysis limits oxidative hospitalization, proliferation, and mitochondrial metabolism to venetoclax resistance.

A. This is a very interesting section demonstrating the potential importance of oxidative phosphorylation related genes as a mechanism or resistance to venetoclax. There were also comments on enrichment for proteins involvement mitochondrial complex assembly which may have been increased as well. From the paper in the description, it is not clear what is the hypothesis behind this. How does the increase in oxidative Phosphorylation genes and mitochondrial protein genes promote resistance to venetoclax?

B. The polo-like kinase inhibitor volasertib seem to provide significant activity in samples that were venetoclax resistance. How was this chosen, or was it just part of the panel? Any rationale behind why PLK1 inhibitors would be particularly active here? Any other classes of drugs that could provide activity in this population based on your analysis?

Reviewer #4, expertise in AML functional precision medicine (Remarks to the Author):

In this manuscript Wegmann et al report on single cell genomics and ex vivo drug testing of rrAML patient samples. Overall this is a well written manuscript that leverages 21 rrAML patient samples to compare bulk and single cell DNA, RNA, and protein level data with ex vivo drug testing. The team focused on venetoclax exposure and resistance mechanisms in this cohort but this approach could be used for any promising novel agent and demonstrates a comprehensive approach to better understanding how to best utilize some of the novel therapeutics that have recently entered the armamentarium for AML therapy. Importantly the team demonstrated that a subset of venetoclax naïve patients were intrinsically venetoclax resistant and also outlined potential mechanisms by which ven naïve and ven expsosed patients acquired resistance.
The expansion of technology allowing for single cell resolution of data at the DNA, RNA, and protein levels has the potential to provide a wealth of information to guide investigations into chemotherapy resistance. Here, Wegmann et al have provided a useful repository of key data exploring potential mechanisms by which AML blasts are surviving upfront chemotherapy and the use of targeted agents. This data is well annotated and novel for the field and appropriate conclusions are drawn from the extensive genomic data and ex vivo response testing that was done.

Major Critiques:
A particular strength of this manuscript is that 7 of the 21 rrAML patients included in this cohort had their leukemia blast burden quantified and profiled at multiple points during

treatment and that both blood and bone marrow were collected. I was disappointed to see that there was not more data presented on clonal evolution over time for those patients with serial samples. Figure 1D shows high correlation over time points for CyTOF data but the manuscript would be improved if differences in ex vivo responses and in single cell profiling was elaborated on. In addition, information on clinical events between paired sample collections should be provided.
Figure 2 – would strongly advise changing the appearance of the blast cell in Fig 2 panel A to have the appearance of a blast rather than that of a band cell.
Figure 2C, 2D and throughout the remainder of the manuscript would change to VEN exposed rather than experienced.
For Figure 5G data from patient TP031 is not clearly called out in the text.
Lastly, it would have been nice to see more work testing CD36 blockade in AML beyond the automated microscopy to better delineate how this extensive testing can potentially translate to clinically useful therapeutics. Ideally, PDX models would be tested with a CD36 blocking antibody to improve the pre-clinical evaluation of these potentially promising results. If further preclinical evaluations are planned as a part of future directions it would be useful to the reader to explicitly state this.

Minor Critiques
Line 287 - towards rather than to wards
Figure 5G legend refers to left and right though these panels are oriented up and down.

Single-cell landscape of innate and acquired drug resistance in acute myeloid leukemia (NCOMMS-24-13462)

Response to reviewers

We greatly appreciate the positive and constructive feedback provided by all reviewers. Our response is highlighted in blue text.

Reviewer #1

Expertise in single cell omics, CyTOF and AML (Remarks to the Author):

SUMMARY
Overall, this manuscript describes a tour-de-force in primary patient AML sample profiling, integrating bulk and single cell high-parameter technologies with 2 powerful and functional precision medicine techniques. Applying this many analyses to 21 relapsed and refractory patients is powerful and represents a highly valuable resource.

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Methods: the technologies that are integrated in this trial (TUPRO) are highly innovative and impactful. This is a stellar line-up of assays.

Our major concerns lie mainly in the description of the methods and of the results. There are many areas that need clarification. However, as written and performed, these are impactful and interesting analyses and results. Some problems with data reporting and explanation weaken the impact and strength of the results.

We thank the reviewer/s for their positive and constructive feedback and their detailed suggestions on how to improve our manuscript.

Results:

Overall, the results are interesting and compelling. However, there are several areas where the data reporting is inadequate or presentation of the data is unclear/misleading:

a. It is not clear how many lines of therapy each patient had prior to sampling. Column O/"Treatment before TUPRO sampling" lists drug regimens, but it is unclear if this was the only regimen or if patients had multiple lines of therapy. Likewise, it is unclear what is meant by "Treatment response before TUPRO" (column P), does a "yes" imply CR followed by relapse or does a "yes" mean some reduction of disease burden? Several samples are listed as "relapse" but have a "no" for treatment response before TRUPRO, suggesting perhaps that the treatment listed in columns N/O are non-first line. Later in the manuscript (Figure 2C, lines 205-206), prior exposure to Venetoclax is listed in aggregated patient data, but it would be very important to understand when the patients were exposed to venetoclax if not immediately prior to TUPRO sampling

We have updated the clinical information presented in **Table S1**. Specifically, we have

1) Added a column specifying how many lines of prior treatments a patient received.
2) Removed the column "Treatment response before Tupro", since this annotation was not clearly defined except for follow-up samples.
3) Double-checked and amended the annotations for the "relapse / refractory" column - patient TP035 was reclassified as relapse, and we removed this annotation for any follow-up samples.
4) Re-defined the definition of venetoclax exposure. Previously, we defined venetoclax exposure as having received venetoclax at any time during the course of their treatment. However, when checking the previous treatment lines, we realized that this definition was inconsistent since we identified two additional patients who received venetoclax in earlier treatment lines. To avoid confounding effects introduced by differences in treatments following venetoclax, we therefore decided to re-define "Venetoclax exposed" as receiving venetoclax at the time of sampling and exclude any patient with venetoclax in previous treatment lines from the analysis of innate versus treatment-related venetoclax resistance.

In addition, we now provide a description of each of the columns in **Table S1** in the table caption.

b. Column T lists responses such as VGPR, SD, PR. These treatment response terms are not standard for AML and should be defined (and referenced).

We have amended this. The responses after TuPro are now defined according to the ELN 2022 recommendations (Döhner et al. 2022), Table 8, page 1361)

2. Figure 1 (and Tables 6 and 7): identify AML cells with CyTOF and scRNA seq and bulkify these cells based on AML cell "groups" (AML cell subsets). It is not clear how the authors define AML cells by CyTOF or by scRNA seq. It would be important to define that and explain what cells contribute to bulkification in the scRNA seq data and in the CyTOF data.

We thank the reviewer for this helpful comment. We now provide additional information on how the AML blasts were defined in each technology:

1) **Figure S1** now includes an additional panel highlighting the CyTOF marker profiles defining each cell type.
2) We provide the list of marker genes used by scRNA-seq in **Table S14**.
3) We have extended the methods section for scRNA-seq (**lines 555-563**) and CyTOF (**lines 666-674**):

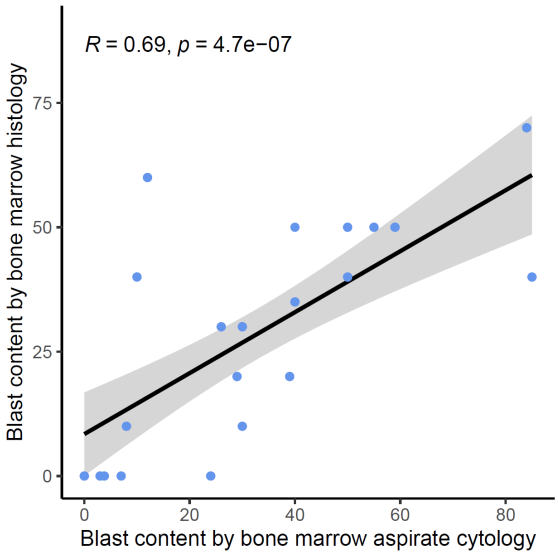
   "Each cell in the dataset was assigned a label using the scROSHI [74] workflow. Briefly, cells are classified based on expression of cell-type specific genes (**Table S14**) using a two-step procedure that takes into account the hierarchical nature of cell types. In the first step, a "major cell type" (e.g. myeloid blast, T-cell, B-cell) is assigned. In the second step, this assignment is refined and cell subtypes are assigned within each major cell type. The gene lists for the major cell types were generated based on unsupervised clustering and marker identification (Seurat: FindMarkers [75,76]) of the first three samples in this study (Sample IDs: DOROFEG, UBADAFA, DOROBOF). The markers for non-malignant cell subtypes were obtained from CIBERSORT [77], and the markers for AML subtypes were based on van Galen et al. [4]"

   "The main cell types present in each sample, including B-cells, T/NK-cells, myeloid cells, granulocytes, platelets and AML cells were identified using a random forest classifier trained on manually gated cell populations in Cytobank. Specifically, CD34 positive AML cells were defined as CD34 high CD45 low. We also observed a population of cells that was negative for most of the tested markers. This population may represent an atypical type of AML cell and was labeled as "putative AML" (see **Figure S1a**). Because this population likely includes both malignant and nonmalignant myeloid progenitors, we also considered those cells as AML, however, we only included samples with > 5% blasts by pathology in the downstream analyses."

3. Figure 1C: compare concordance of blast % measurements by different technologies, (table S10 and Figure S1). The manuscript lists that the various technologies are concordant with each other. Figure S1B lists concordance. On review of table S10, none of these technologies appear to be concordant with the pathological blast count, which would be the gold-standard. CyTOF shows some concordance, but the other ones have minimal concordance with pathology. This issue needs more clarity.

We thank the reviewer for this comment. The clinical gold-standard would indeed be the cytological blast count on the bone marrow aspirate or peripheral blood. However, in our original analysis, for bone marrow samples, we used the average between bone marrow cytology and histology as the pathology readout. Since the histology readout can be variable (Hodes et al. 2019), we have amended this and present the updated analysis in a clearer format in **Figure S1b**. This analysis highlights good concordance between all the TuPro technologies and pathology (all correlations > 0.7). Note that these correlations are as high as the correlation observed between

the pathology bone marrow aspirate and bone marrow histology readouts (**Reviewer Figure 1**). The higher concordance between TuPro technologies compared to TuPro technologies and pathology likely can be explained by differences in sample preparations: All TuPro nodes measured aliquots of the same cell suspension prepared by a density gradient centrifugation, while pathology employs different sample preparation strategies.



***Reviewer Figure 1**: Correlation between blast content measured by two different pathology readouts.*

4. Figure 1D: It is not clear to me what is being correlated in 1D what is being compared to what? Marker expression correlation between samples? Why would that be correlated?). The figure legend does not help with this determination. Results, lines 169-170 Figure 1D: “Blood and BM taken at same visit from same patient showed “highly similar composition (% AML blasts) in CyTOF and PCY” Why would we expect the blood and BM blasts percentage to correlate? It only rarely correlates within the same patient. I think I do not understand what is being correlated. What two things are being compared?

The plots in the original Figure 1D showed the following:

- Left panel: Correlation between marker expression in AML cells from CyTOF - the idea here is that cells from the same patient have more similar protein expression, regardless of the time pint or the sampling site
- Middle panel: Correlation between cell type composition from CyTOF - we found that blood and bone marrow samples from the same patient taken at the same time have similar cell type composition, primarily driven by the blast content: Samples with high blast content in blood also tend to have high blast content in BM in this cohort.
- Right panel: Correlation between ex vivo response profiles from PCY

However, as the reviewer pointed out, the correlation between blood and bone marrow cell type composition is unexpected and in this cohort, was mostly driven by a few samples that had very high blast content in both sample types. We have therefore removed the middle panel, and instead

added example correlation plots for each of the categories to make it more clear what is being correlated (see **Figure 1d** below). We think this has improved the clarity of the Figure, and we hope the reviewer agrees.

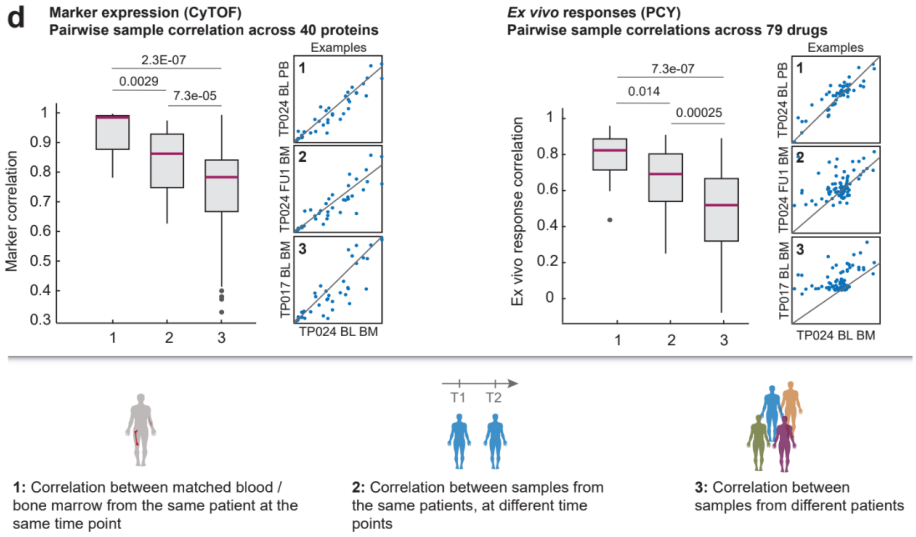


***Figure 1d**: Correlation (Spearman) of molecular (CyTOF marker expression across 40 proteins, left) and functional (PCY ex vivo responses across 79 drugs, right) profiles between 1) pairs of matched blood / bone marrow samples from the same patient taken at the same visit (CyTOF n=15; PCY n=14), 2) pairs of samples from the same patient taken at different visits (CyTOF n=26; PCY n=26), 3) pairs of samples from different patients (CyTOF n=820; PCY n=663). Only samples with > 5% blast content by pathology are included in this analysis. Small scatterplots show an example pairwise comparison for each category. P values from two-sided, two-sample Wilcoxon test. Box plots indicate the median (horizontal line) and 25% and 75% ranges (box) and whiskers indicate the 1.5x interquartile range above or below the box. Outliers beyond this range are shown as individual data points.*

5. Figure 2/Table S9 (PCY drug responses). Figure 2 represents data from which of the tested drug concentrations? Table 9: Data in sheet one is averaged across replicates, but for which concentration is the data representing (each drug is used in 2-3 doses)?

We apologize for the confusion. The data presented in **Figure 2** represents aggregated values across all concentrations ("PCY score"). We have adjusted the labeling of the colorbar in **Figure 2** and updated the Figure legend, which now reads as follows:

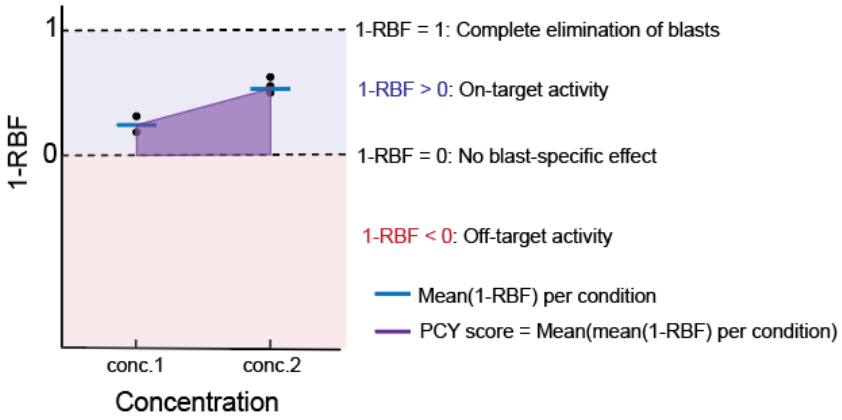
"Heatmap showing the PCY-based ex vivo responses (PCY scores) of 38 rrAML samples with blast content > 5% by pathology to 79 drugs and drug combinations. Relative blast fraction (RBF) is the fraction of AML cells after 24 h of drug treatment relative to the mean fraction of AML cells after 24 h of control treatment. PCY score represents aggregated 1-RBF values across replicate wells and drug concentrations (see Methods). Thus, positive values indicate on-target reduction in AML cells. Samples and drugs are ordered by hierarchical clustering (Euclidean distance, complete linkage), a cluster of samples characterized by ex vivo resistance to common AML treatments is highlighted in red."

a. Lines 518-520: In the text, AUC values for PCY data are described but AUC values are not found in the manuscript or supplemental information. (Can you calculate an AUC with only 2 drug concentrations tested?) Also, it is unclear if the authors are averaging across different drug concentrations (if that is the case, that would need some justification) or averaging across replicates receiving the same concentration. As written, it is unclear how PCY data is calculated using the different drug concentrations.

We have updated the naming and now call the concentration-aggregated drug response values "PCY score" throughout the manuscript. Historically, we used to call these scores AUCs, since the PCY score corresponds to the area under the concentrations (**see Reviewer Figure 2**). However, we agree with the reviewer that these might be confusing as the calculation and meaning differs from more commonly used dose-response assays measuring bulk viability.

We have now clarified the methods section and describe the calculation of the PCY score in more detail (**lines 511-514**):

"The responses were then averaged (mean) across replicate wells per condition (where a condition describes one drug at one and concentration), and responses per drug were further aggregated into a PCY score by taking the mean across these condition averages per drug. Thus, PCY score = mean per drug(mean per condition(1-RBF))."



***Reviewer Figure 2**: Graphical explanation of PCY score calculation.*

6. Lines 187-196 discuss a subset of samples with low sensitivity to common AML treatments. These samples are said to be increasingly sensitive to a list of drugs, with reference to Figure S2B. However, Figure S2B does not identify these samples or display their sensitivity of resistance to drugs.

We thank the reviewer for pointing out the opportunity to better clarify this. We have now highlighted the resistant samples in **Figure 2a**. Additionally, we now added specific drug-drug

correlations from the global correlation analysis in **Figure S2b-c** to better showcase the altered drug sensitivities across samples.

7. Supplementary Figure S3A is difficult to see and difficult to interpret. It is difficult to see how the absolute changes in response to venetoclax measured by 4i DRP correlate with the PCY measurements, as claimed by the text (lines 210-212). This claim is not supported by the data as it is presented in this figure. In contrast, Figure 2D demonstrates this to be the case for the 4 features that are displayed there.

We have simplified the presentation of the data in **Figure S3a**. The new panel now just shows the mean and standard error of the mean absolute log2 fold changes across all 4iDRP measurements:

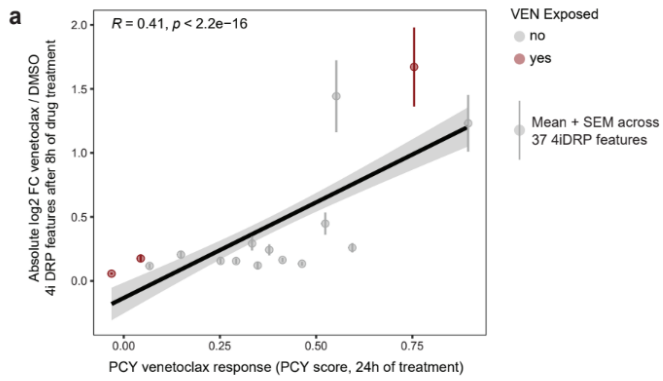


Figure S3a: *Absolute log2 fold changes (FC) comparing VEN to DMSO control across 4i DRP features versus VEN PCY scores. Each dot corresponds to the mean absolute FC across 37 4iDRP features for a single sample, lines correspond to the standard error of the mean, and colors mark VEN exposure at the time of sampling.*

8. Lines 222-223 claim that lower levels of BCL2 are correlated with venetoclax resistance, but the correlations (r2 values) are very very weak. The protein levels (the actual effector molecule) are not significantly different neither by strength of the r2 nor by significance testing nor by visualization of the whisker plots. This is particularly concerning in light of the overwhelming prior evidence that BCL2 downregulation is a bone fide resistance mechanism to venetoclax and therefore calls into question the quality of the data.

Following this comment made by both reviewer 1 and 3, we performed an in-depth analysis of the CyTOF data. We realized that a group of cells labeled “putative AML” likely corresponds to nonmalignant myeloid progenitors, but also more mature, CD34 negative AML blasts that are particularly abundant in venetoclax resistant samples. We therefore decided to re-do all analyses presented in **Figure 1d and Figures 2-4**, with the following changes:

- We now exclude samples with low blast content (<5%) by pathology (cytology on blood or bone marrow aspirate, bone marrow histology for cases where no cytology readout was available)

- For the bulkified CyTOF marker profiles, we now include both the CD34 positive AML and the putative AML cell fractions

With these changes, we now get much better consistency between RNA and protein levels for Bcl-2 (**Figure 3b**) as well as other markers included in the CyTOF panel (**Figure S6a-b, previously S7a-b**).

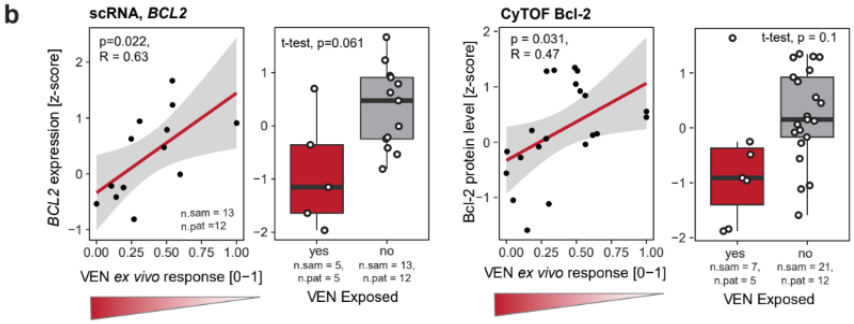


Figure 3b: *RNA and protein levels of the VEN target Bcl-2 measured by scRNA-seq and CyTOF, averaged across all AML blasts per sample. Scatterplots show Bcl-2 levels as a function of innate resistance in VEN naive samples. Regression lines with 95% confidence bands, Pearson's R and corresponding P values are indicated. Box plots as in Figure 1d compare Bcl-2 levels between VEN naive and exposed samples, P values from two-tailed Welch's t-test.*

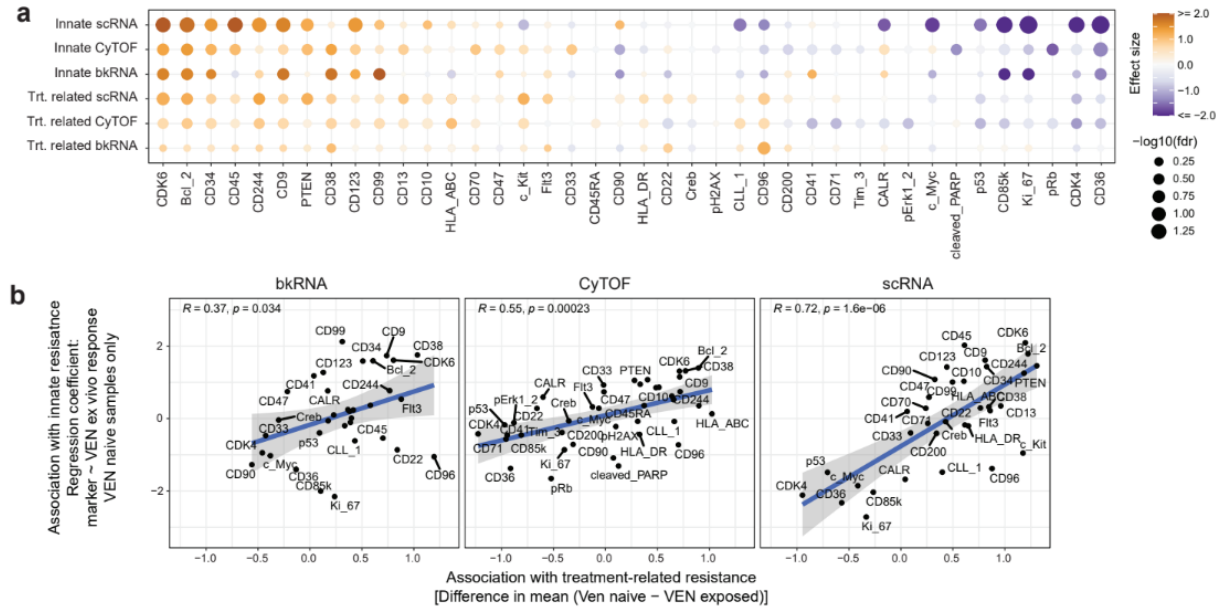


Figure S6a-b: *Association of protein / RNA levels with innate and treatment-related resistance to VEN for all markers in the CyTOF cancer panel. Effect size represents the slope of the regression line for innate resistance and the difference in mean between VEN naive and VEN exposed samples for acquired resistance. P values obtained from a linear regression and a two-sided Welch's t-test, respectively.*

9. Figure 3 and S6, deciphering molecular features of innate and (presumably) acquired venetoclax resistance is very interesting.

We thank the reviewer for highlighting this.

10. Figure 3D: the description of how effect size was calculated is unclear (lines 746-751): Does 3D represent genes? Proteins? Both? Please clarify how these values were calculated to get these data.

We apologize for the lack of clarity - **Figure 3d** is based on scRNA-seq data and thus represents gene expression in AML cells. We have added a title to the figure panel for clarification, and also added more details in the figure legend explaining the calculation of effect size:

"Association of known genes involved in VEN resistance with innate or treatment-related VEN resistance. Values are derived from bulkified scRNA-seq AML cell transcriptomes. For treatment-related resistance, effect size corresponds to the delta mean gene expression between samples from patients that were or were not exposed to venetoclax, and P value was calculated using a two-sided Welch's t-test. For innate resistance, the effect size and P value are obtained from a linear regression modeling gene expression as a function of venetoclax PCY score (see panels a-b)."

11. Figure 3E (CD36 expression related to ven resistance): which specific cell cluster and cell types (annotated by scROSHI) are associated with VEN resistance? More specifically, is there a common cell cluster across samples that showed the strongest association between CD36 expressions and VEN drug resistance response? tSNE plots like Figure3C for CD36 expression, BCL2 expression, cell cluster annotations, and cell type annotations (scROSHI annotations) would be informative here Also, a plots that compares CD36 expression in different clusters (like in Figure S4C) would be informative.

We thank the reviewer for these suggestions. We have reworked **Figure S4** to better address these interesting questions. Specifically:

- We now show a t-SNE colored by the scROSHI annotation of AML subtype (**Figure S4c**)
- We have added a panel showing the AML subtype composition to **Figure S4d**, as well as a panel showing CD36 expression per cluster.

From the new Figure S4, it becomes clear that *CD36* expression is overall negatively correlated with both venetoclax response and *BCL2* expression. Blast maturation likely plays a role in this, since the clusters that mostly contain cells from venetoclax resistant samples (clusters 6, 7 and 8) also contain elevated fractions of more mature AML subtypes, while the most sensitive clusters (2 and 4) mostly contain HSC progenitor-like cells.

We have also added a t-SNE colored by CD36 expression to **Figure 3e,** highlighting the mutually exclusive expression of *BCL2* and *CD36* across all AML cells in the cohort (there are only 988 cells / 2% that express both *CD36* and *BCL2*).

12. Figure 4/Lines 261-263: refer to “associations between innate and acquired resistance” being correlated. It would be helpful to explain what is being associated and include a color legend (at least in the figure legend to figure 4A).

We thank the reviewer for this helpful comment. We have simplified Figure 4a and removed the color entirely since it was just showing the average of the x- and y-axis for each point. To make it more clear what is shown on each axis, we have added example plots explaining the calculation for the *RBX1* gene and we have extended the figure legend:

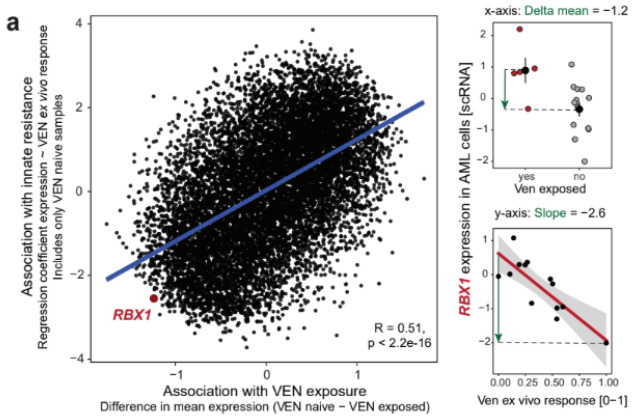


Figure 4a: *Comparison of associations between gene expression (bulkified scRNA-seq in AML cells) with innate and treatment-related VEN resistance, respectively. The x-axis corresponds to the delta mean gene expression between samples from patients that were or were not exposed to venetoclax at the time of sampling. The y-axis corresponds to the slope of a linear regression modeling gene expression as a function of venetoclax ex vivo response (PCY score). The RBX1 gene is highlighted and example plots are shown on the right.*

a. Figure S7B is more intuitive and interesting than Figure 4A.
Figure S7b (now **Figure S6b**) shows a subset of the data points (only genes included in the CyTOF marker panel) shown in Figure 4a. Because the remainder of Figure 4 relates to the full transcriptome, we feel that the current panel is a better fit for this figure.

b. Can authors report how many associated genes were significant in the comparison of innate and acquired VEN? And what are the genes that were associated with the VEN naïve drug response?

There were 44 genes significantly associated with innate VEN responsiveness at an FDR cutoff of 0.05. None of the comparisons done for the “treatment-related resistance” (VEN exposed) reached this significance cutoff. These results are now provided in the source data for Figure 4a.

13. Figure 4C: How is the “pathway score” measured?

Pathway scores were calculated using a rank-based single-sample scoring method called singscore (Foroutan et al. 2018). We have added a brief description in the main text (**lines 256-259**), added a reference to the figure legend, and extended the corresponding methods section (**lines 595-603**).

Main text:
"In addition, we calculated a pathway activation score (singscore [40], reflecting the relative expression level of all genes in a pathway) for the terms "nuclear division" and "OXPHOS", and these pathway scores were negatively correlated with VEN PCY scores (**Figure 4c**)."

Methods:
"Pathway activation scores were calculated from bulkified expression profiles using a rank-based method as implemented in the singscore R package (Foroutan et al. 2018), with all parameters left at their default values. This method works by first ranking all genes per sample by increasing expression level. A directed pathway score (assuming that a pathway is more active the higher the corresponding genes are expressed) for a given gene set is then defined as the average, mean-centered rank of all genes in that gene set. Pathway scores thus reflect the relative mean percentile rank of the target gene sets within each sample, and a sample with a high score has higher relative expression of the genes in a given pathway. "

14. Figure 5 is very interesting and well-done overall. However, Figure panel 5J misses the mark: the only 2 AML cell subtypes that are shown have high CD36 expression. It would be important to show additional AML cell types (especially immature cells) to substantiate the claim that these two cell types have higher CD36 levels relative to other (defined) AML cell types.

We thank the reviewer for this compliment. We have updated **Figure 5j**, and we now split the "AML" cell type into the three main subtypes identified by scROSHI (based on reference gene sets from van Galen et al, 2019). Consistent with literature (Zhang et al. 2020), CD36 expression was low in immature cell types and highest in monocyte-like AML.

15. Figure 5A (association of CD36 levels with survival in TCGA): does this same result hold up in the Beat AML?

We have performed the same analysis on the BEAT-AML cohort (excluding pediatric AML), and we observe the same trend. We have added the Kaplan-Meyer curves to a new **Supplementary Figure 7**.

Supplementary Figure 7

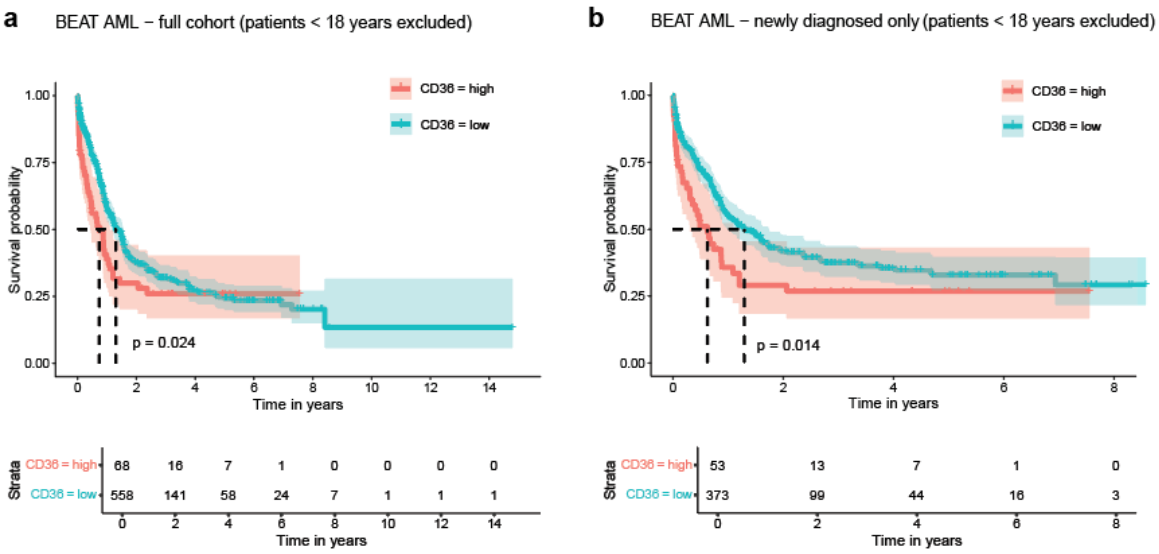


Figure S7: *Kaplan-Meier curves for patients in the BEAT-AML cohort, stratified by CD36 expression level (bulk RNA-seq). P value from log-rank test is indicated.* ***a,*** *including all samples from patients > 18 years old,* ***b,*** *only including samples from newly diagnosed patients > 18 years old. Patient numbers per 2-year interval are indicated in the bottom panel.*

16. Figure 5J: please define VST (VST-normalization)

We apologize for the lack of clarity. We have added the definition to the figure legend:

> "**j**: Expression of CD36 across cell types. Values represent average expression (variance-stabilizing transformation (VST, (Love et al. 2014)) of normalized counts) per cell type and sample derived from scRNA-seq data."

17. Line 166: How was blast content enumerated from scDNA-seq (only those cells with CNVs detected?).

Yes. We provide a description of how the blasts were defined by different technologies in the legend of **Figure S1**. We have now added a reference to this Figure when describing the blast fraction concordance in the main text (**lines 148-152**).

18. What marker genes are used for cell type annotation in scROSHI (what reference is used for these annotations)?

We have added a more detailed description of the cell typing done by scRNA-seq in the methods section (**lines 555-563**), and we now provide the list of reference gene sets in **Table S14**:

> ""Each cell in the dataset was assigned a label using the scROSHI [74] workflow. Briefly, cells are classified based on expression of cell-type specific genes (**Table S14**) using a two-step procedure that takes into account the hierarchical nature of cell types. In the first step, a "major cell type" (e.g. myeloid blast, T-cell, B-cell) is assigned. In the second step,

this assignment is refined and cell subtypes are assigned within each major cell type. The gene lists for the major cell types were generated based on unsupervised clustering and marker identification (Seurat: FindMarkers [75,76]) of the first three samples in this study (Sample IDs: DOROFEG, UBADAFA, DOROBOF). The markers for non-malignant cell subtypes were obtained from CIBERSORT [77], and the markers for AML subtypes were based on van Galen et al. [4]"

Minor issues:

19. Many tables are not labeled. We were only able to infer which table by the order in which it was listed. This becomes a problem for tables 6 and 7 where the data basically look identical. It would be useful to include the name of the table within the table file.

We have added a header to each supplementary table.

20. The color scale for ven-resistance in Figure S4 C is difficult to decipher.

We agree with the reviewer. We have changed the color scheme and we hope that it is now easier to read.

21. An issue with word choice: The term "acquired" venetoclax resistance is possibly misleading. Are these all patients who were at one point sensitive to venetoclax and then went on the become resistant? The distinction between venetoclax-exposed and naïve patients is an important one but it seems misleading and possibly incorrect to designate venetoclax-exposed patients as having acquired venetoclax-resistance. Some of these patients may have been innately resistant as well. Unless these venetoclax-exposed patients are known to have responded to venetoclax in the past and developed resistance (which should detailed in table S1), the term should be changed.

We thank the reviewer for this suggestion. It is true that we do not know if these patients were initially sensitive to venetoclax. In addition, we also re-defined venetoclax exposure as receiving venetoclax right before or at the time of TuPro sampling. Therefore, we re-labeled "acquired resistance" to "treatment-related resistance".

22. Typo line 287 "to wards" (means to be towards)

Fixed.

23. Line 576 references Figure S2, but we think they mean Figure S4

We thank the reviewer for their attention to detail. This is fixed now.

Reviewer #2

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for their valuable input.

Reviewer #3

Expertise in venetoclax and AML (Remarks to the Author):

The authors have submitted a manuscript that reflects a tremendous effort at characterizing ex vivo leukemia samples from patients with relapsed and refractory acute myelogenous leukemia. They have performed a significant multilayered, multi-omic profile of these leukemia samples down to the single cell level. The study summarizes a method of single cell ex vivo drug profiling, along with bulk and single cell DNA and RNA protein profiling, with added clinical annotations.

They show a high throughput ex vivo analysis of drug sensitivity of up to 79 drugs and drug combinations using automated evaluation using pharmacoscopy to assess cell death, as well as blast identification using multiple methods such as cytof and DNA content. Using 21 patients with relapsed refractory AML the submitted paper has a great deal of information regarding the characteristics of these AML blasts, ranging from expression profile, to drug sensitivity. I lament while reading the paper, that much of this information is included in the supplemental section with just a snapshot in the main manuscript.

We thank the reviewer for this positive and constructive feedback. We agree that there would be many additional angles to explore. We highlight findings from our own analyses, but also present this data as an extensive resource with the hope that this data can be further analyzed to answer additional questions and be of use to the research community.

While there is a plethora of data included within the manuscript, the overall study reads as a "proof of concept" or “feasibility study” of this impressive technology and its potential application for patients with relapsed and refractory AML, with the intent to find new therapies that may be potentially applicable to these patients in a highly individualized manner. The implications of such a technique, if validated and reproducible, or tremendous for patients who have few options after 1 or 2 lines of therapy for newly diagnosed AML. However little as conveyed as to the real world, in real-time applicability for patients in clinic. For example: what is the turnaround time for these assays? Can this be conducted alongside a clinical lab? How to the authors see the implementation of this powerful method going forward? Have the authors considered using this technique in patients in morphologic remission but with detectable minimal residual disease?

We thank the reviewer for these comments. We have so far focused the manuscript mostly on cohort-level exploratory analysis. However, as part of the TumorProfiler study (Irmisch et al. 2021), this cohort was also analyzed in the context of a "Fast Diagnostic Loop", where the data generated by all the technologies were delivered to the clinic for discussion in a pre-molecular tumor board (pre-MTB) with a turnaround time of 4 weeks (2 weeks from sampling to data generation, another 2 weeks to generate a report). This turnaround time is similar to existing comprehensive genetic tests like FoundationOne Heme, and could potentially be shortened by e.g. fully automating report generation or prioritizing very urgent samples. Thus, these tests could in principle be run in addition to established clinical diagnostics. We have added a paragraph regarding the clinical applicability (**lines 128-135**):

"The turnaround time from sampling to completion of analysis across all nodes was maximally 2 weeks, and 30/31 patient visits (97%) were discussed within 4 weeks of sampling in a "pre-molecular tumor board (pre-MTB)" consisting of members of each technology node as well as clinical hemato-oncology experts. Because this trial was designed as an observational study with feasibility as the primary outcome, the pre-MTB results were not used for actual treatment decisions. Nevertheless, the rapid availability of results underline the feasibility of multi-modal tumor profiling for guiding treatment decisions."

Future trials:
While there are currently no trials in planning utilizing the full spectrum of Tupro technologies for AML, we are starting a randomized, interventional clinical trial for rrAML to investigate the clinical benefit of PCY-guided functional precision medicine (RAPID-01 trial, **NCT06138990**). Besides AML, the TuPro study also included metastatic melanoma and ovarian cancer (manuscripts in preparation). For melanoma, which represents the biggest cohort (106 patients), there is also a manuscript in preparation which analyzes the clinical utility of the TuPro in more detail. For ovarian cancer, the TuPro enabled a follow-up randomized interventional trial investigating the clinical benefit of multi-omics and functional profiling for neo-adjuvant therapy selection in homologous-repair proficient ovarian cancers (OV Precision trial, **NCT06466382**).

MRD:
Not all technologies used in the current setup are suitable for the analysis of minimal residual disease. For instance, all bulk technologies rely on a minimal fraction of blast cells (>5%, ideally >20% for bulk RNA and proteotyping). Functional assays (PCY, 4iDRP) require sufficient numbers of viable blast cells in order to make robust measurements of blast-specific drug responses. However, in an MRD setting, these technologies could still be used if samples were enriched for blasts during processing. scRNAseq and CyTOF are in principle able to detect even very small subsets of cells, if a sufficiently large number of cells are measured. However, since AML resembles normal hematopoiesis and expresses very similar markers, it is challenging to distinguish leukemic clones from normal hematopoietic precursors based on gene / protein abundance alone. Ideally, one would perform a combined assessment of mutational status and gene / protein abundance in the same single cells. This could be achieved by using adapting the scRNA-seq protocol such that commonly mutated genes are specifically enriched and sequenced

(van Galen et al. 2019), or by combining scDNA-seq with sequencing-based immunophenotyping e.g. **MissionBio Tapestri scMRD panel for AML**, (Robinson et al. 2023).

Several prior studies have demonstrated ex vivo sensitivity testing for patient samples with relapsed refractory AML using different techniques. This is one of the few papers that integrates not only drug sensitivity, but clinical information including karyotype, baseline mutations, as well as expression profiling by single cell RNA seq and cytof based proteomics. This is one of the major strengths of this paper. The authors do not provide a more detailed description of the patient's prior therapies within their analysis set, or a structured analysis of groups of patients will be treated with similar therapies to determine whether a given set of therapies that are associated with similar mechanisms of relapse or resistant. These would be helpful and provide some context on how this technique functions across therapies. More samples, possibly from trial of uniformly treated patients may also be important. Additionally, p53 mutated AML, which represents a major unmet need in the current treatment of AML is represented by only 3 patients– a potential missed opportunity to learn more about this difficult subset.
There is little in terms of new discovery within this paper, but important confirmation of observations (using different approaches) regarding potential mechanisms of resistance to venetoclax including up regulation of oxidative phosphorylation, down-regulation of effectors of apoptosis, and up regulation of CD36.

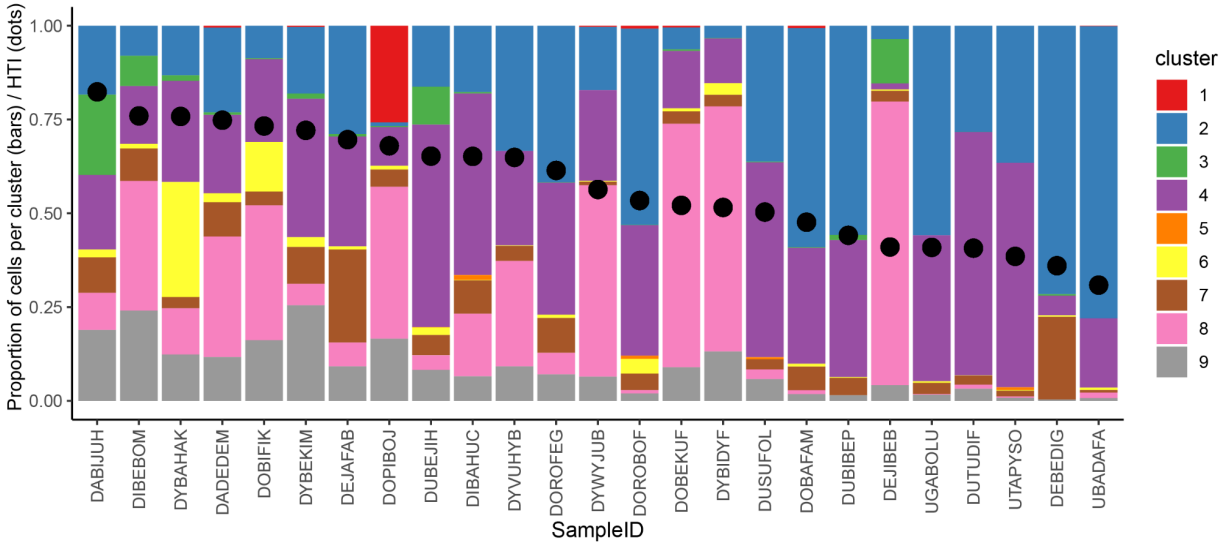
We agree that it would be exciting to extend the single-cell multi-omics profiling presented here to a larger cohort, based on the successful implementation shown in this feasibility trial. This will be the focus of future trials. However, with the current set of samples analyzed, we do not go into more detail with regards to prior therapies and treatment resistance mechanisms because our cohort is simply too small and heterogeneous to do such analyses, with the number of prior therapies received ranging from 1 to 7 (see **Table S1**).

One of the realities that has become very apparent clinically in patients with AML is the tremendous clonal heterogeneity and hierarchy at the time of diagnosis which changes based on the treatment paradigm and which inevitably gives rise to resistant subclones that form the basis for relapsed disease. The authors allude to this within their introduction. However in reviewing the techniques implemented here and the single cell data provided in these relapsed patients, there is less focus on this heterogeneity and clonal hierarchy at the time of relapse, including clonal dominance, or potential drivers of future resistance. It may be interesting to include this information if it can be derived from the available data.

Clonal heterogeneity and evolution:
Despite the single-cell resolution of the data, we were not able to systematically infer clonal herarchy. The main reason for this is that the single-cell DNA sequencing approach employed here (10X Genomics Chromium scDNA, https://www.10xgenomics.com/products/single-cell-cnv) can only detect large scale copy number variations, which turned out to be rare in AML. We also tried to infer clonality from FoundationOne Heme variant allele frequencies, however, this was not trivial since this measurement is done on bulk samples, and some mutations can be present in nonmalignant cell populations as well.

Single-cell heterogeneity:

The clustering analysis shown in **Supplementary Figure 4** highlights that most samples contain cells that fall into multiple clusters, indicating a high degree of intra-sample heterogeneity. We further quantified this using a heterogeneity index (HTI (Levy-Jurgenson et al. 2020), **Reviewer Figure 3**). The HTI in essence captures how distributed the cells from each samples are across a given set of clusters, where a value of 1 = completely evenly distributed, and a value of 0 = completely contained within one cluster. Thus, the higher the HTI, the more heterogeneous the sample. However, we found no association between the HTI and any clinical variable or drug response, therefore we did not analyze this further.



***Reviewer Figure 3**: Proportion of cells per cluster and sample (colored bars). Clusters are derived from scRNA-seq gene expression profiles of 46930 AML cells (see **Figure S4**). Samples are ordered by the heterogeneity index (HTI, value indicated by black dots).*

In addition to the above comments, below are some comments by section from the results:

1. Single cell molecular and functional profiling of relapsed refractory AML samples.

A. In terms of feasibility it appears that only 10 visits or 32% were able to be analyzed by all of the technologies listed. Is this is something that can be optimized in the clinical setting, to make sure a complete characterization is facilitated? How much sample would be needed in a prospective manner to be able to perform all of the testing demonstrated.

Yes, we think this could be optimized further. While the majority of patient visits (28/31) were analyzed by at least five technologies, there were some technology-specific limitations that led to a lower fraction of samples analyzed by all technologies (e.g. cutoffs for blast content or limited capacity to run samples). To perform all of the tests presented here, the minimum cell number is 2 million, and the ideal cell number is around 7 million. However, there are additional technology-

specific requirements such as minimal blast content or cell viability (see Table below, we also added this information to **Table S1**).

Technology	Minimal cell number in million	Optimal cell number in million	Additional requirements
CyTOF	0.4	0.6	none
Pharmacoscopy	0.5	2.5	Ideally > 5% blasts
scRNA and scDNA	0.2	0.5	> 50% viability
4i Drug Response Profiling	0.5	2.5	<= 4 samples / week
Proteotyping	0.2	0.5	Ideally > 20% blasts
bkRNA	0.2	0.5	Ideally > 20% blasts
FFPE material			
Technology	Number of tissue sections		
FoundationOne Heme	11		none
Digital Pathology	16		none

B. Figure 1D is not well explained within the text, nor the figure Legend. The order in which each figures explained also seems to be backwards. Please consider rewording sentences to describe this figure and reordering the subfigures so it is easier for the reader to understand what you are trying to show.

We thank the reviewer for this comment. Following this remark and point 4 raised by reviewer 1, we have restructured **Figure 1d** (see below). Specifically, we removed the middle panel and instead added example correlation plots to better explain what is compared. We also reordered the x-axis of the box plot such that it matches the description the main text (**lines 158-162**):

“Blood and bone marrow samples taken at the same visit showed highly similar protein levels and *ex vivo* drug responses. This similarity slightly decreased when comparing samples from the same patient across different time points, likely reflecting the effect of treatment between visits. Nevertheless it remained greater than that of unmatched samples.”

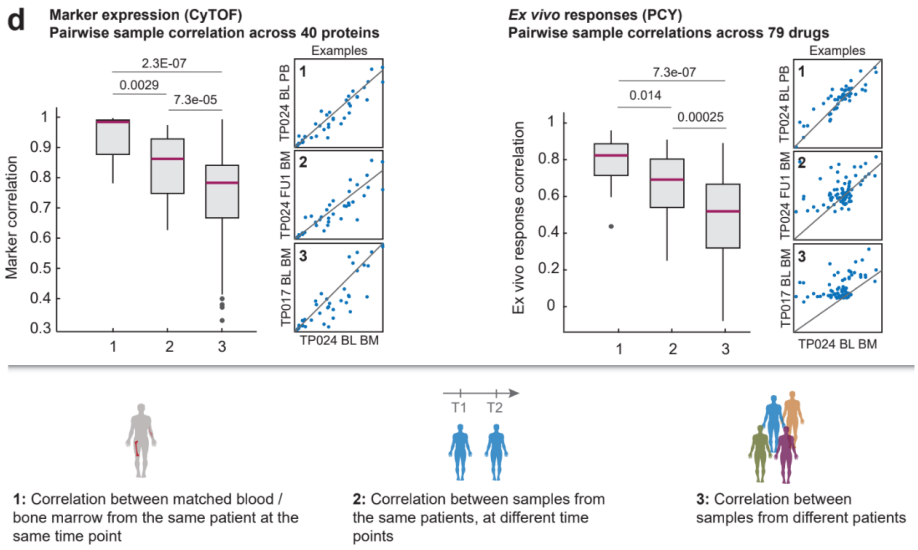


***Figure 1d**: Correlation (Spearman) of molecular (CyTOF marker expression across 40 proteins, left) and functional (PCY ex vivo responses across 79 drugs, right) profiles between 1) pairs of matched blood / bone marrow samples from the same patient taken at the same visit (CyTOF n=26; PCY n=21), 2) pairs of samples from the same patient taken at different visits (CyTOF n=44; PCY n=40), 3) pairs of samples from different patients (CyTOF n=1526; PCY n=1067). Small scatterplots show an example pairwise comparison for each category. P values from two-sided, two-sample Wilcoxon test. Box plots indicate the median (horizontal line) and 25% and 75% ranges (box) and whiskers indicate the 1.5x interquartile range above or below the box. Outliers beyond this range are shown as individual data points.*

2. Prior venetoclax treatment shapes the ex vivo drug response landscape.

A. 79 drug and drug combinations tested ex vivo on the samples is quite impressive.

We thank the reviewer for highlighting this.

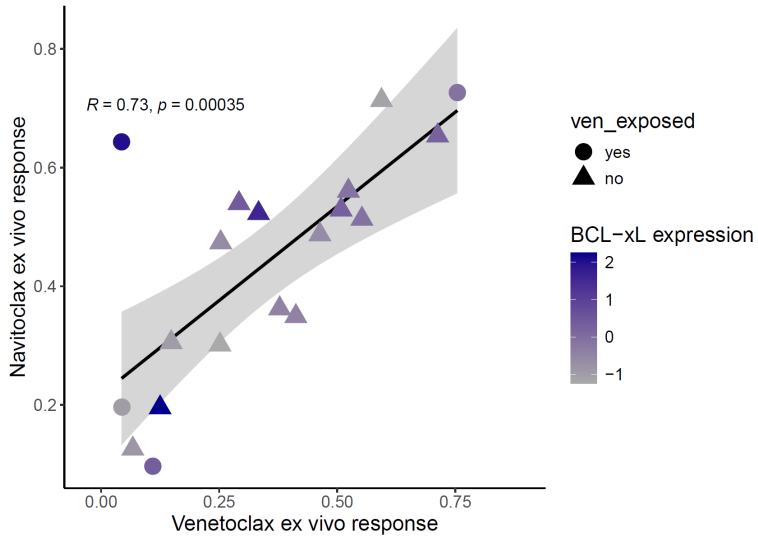
3. Multimodal recovery of innate and acquired venetoclax resistance mechanisms

A. Observing acquired venetoclax resistance in patients treated with prior venetoclax is intuitive and clinically very apparent. The question is the mechanism of this resistance which has been previously reported to by several of the authors cited, including altered dependency to BH 3 family of proteins, as well as modulation of expression of various effectors of intrinsic apoptosis. Much of this is confirmatory findings from prior studies, with additional observation of CD36 up regulation correlating strongly with venetoclax resistance. Any new findings within this data to explain this dynamic resistance.

Any followup ex vivo testing of the resistant samples treated with Venetoclax based combinations with other partners found in the screen (ie. Bcl-2i + bcl-xli, etc.)

We thank the reviewer for this suggestion. While upregulation of Bcl-xL (encoded by *BCL2L1*) is a well described mechanism of venetoclax resistance, we found that in this cohort, expression of

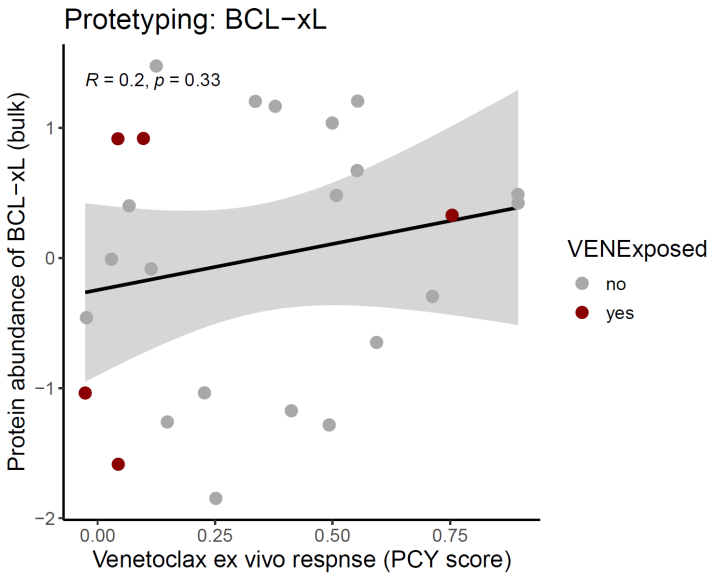
BCL2L1 was only mildly associated with venetoclax response (**Figure S5a, previously Figure S6a**). Interestingly, we also observed that response to venetoclax (specifically targeting Bcl-2) was strongly correlated with response to navitoclax (targeting Bcl-2, Bcl-xL and Bcl-w), suggesting that targeting Bcl-xL is not enough to overcome resistance in the majority of cases (**Reviewer Figure 4**). However, there was one outlier sample with very high Bcl-xL expression, which responded well to navitoclax but not venetoclax. For this one case, it is therefore likely that Bcl-xL upregulation was indeed the primary mechanism of VEN resistance.



Reviewer Figure 4: *Correlation of venetoclax and navitoclax ex vivo response (PCY score). Dots are colored by expression of BCL2L1 (BCL-xL) in AML cells (scRNA-seq).*

It would be interesting to see here further characterization of BH 3 family proteins including MCL–1, and BCL–XL as drivers of resistance in these patients and whether he is or present as intrinsic resistance among those patients who are naïve to venetoclax.

We analyzed the association between gene expression of all detected BCL- and BH3-family members and venetoclax response (**Figure 3d**), and provide the corresponding correlation plots in **Figure S5a** (previously Figure S6a). These proteins were not measured as part of the CyTOF panel and we therefore cannot make any claims about the protein levels specifically on AML blasts. Furthermore, bulk proteomics did not detect MCL-1, while BCL-xL protein levels showed no association with venetoclax PCY score (**Figure S6c,** previously S7c) and **Reviewer Figure 5**), consistent with its weak association on the RNA level (**Figure S5a**).



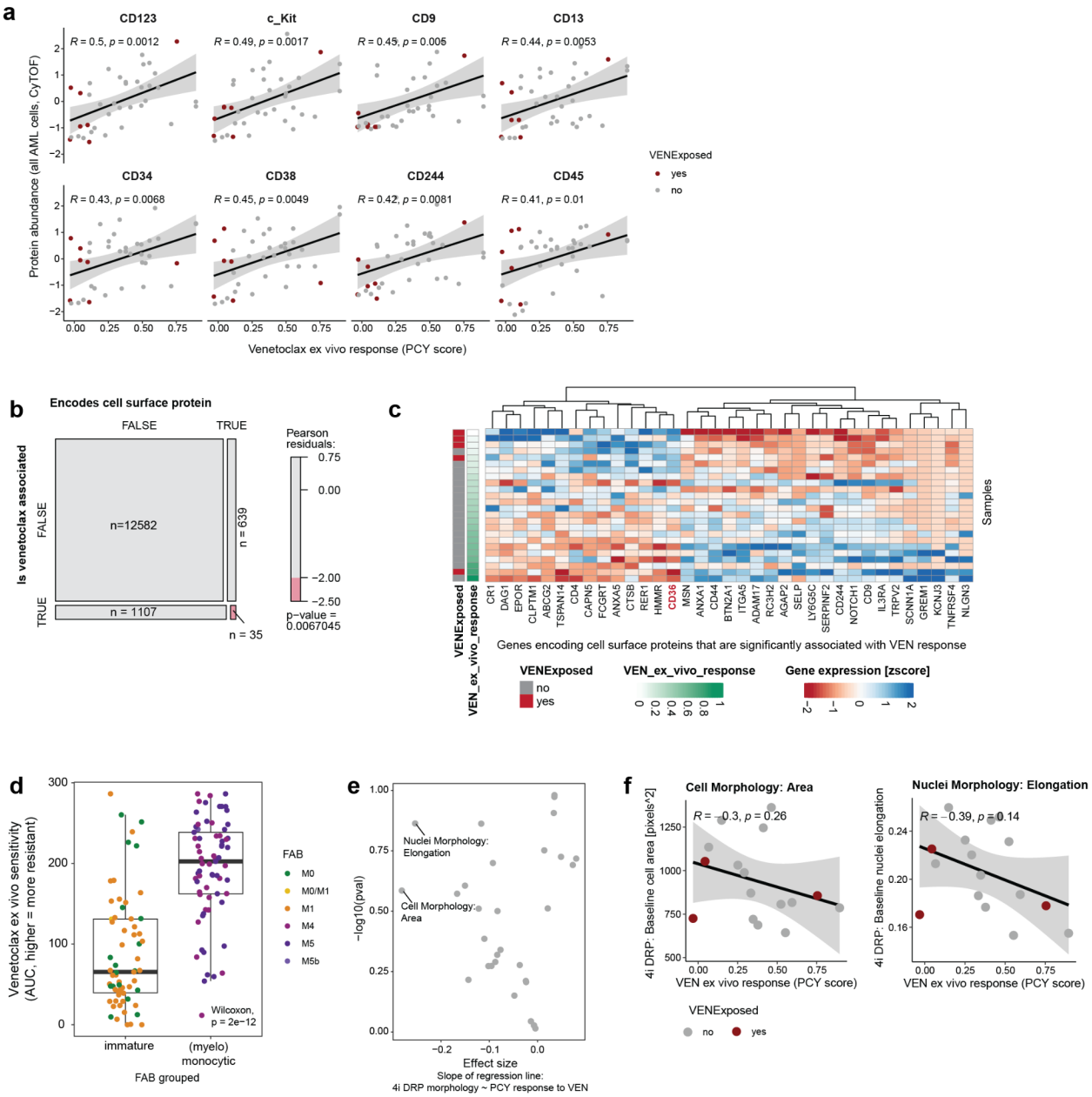
Reviewer Figure 5*: BCL-xL protein abundance by bulk proteotyping correlated with venetoclax ex vivo response (PCY score).*

Any analysis to correlate surface markers (besides CD36) and morphology with Ven resistance?

We analyzed all markers measured by CyTOF (see **Figure S6, previously S7**). There are additional surface markers that are associated with both innate and treatment-related venetoclax response (positive association: CD38, CD34; negative association: CD90), although these associations are weaker than with CD36. When considering all samples together, in addition to Bcl-2 and CD36, we find significant positive associations (FDR < 0.05) between venetoclax *ex vivo* response and the protein levels of CD123, CD117 (c-Kit), CD9, CD13, CD34, CD38, CD244 and CD45 (**Reviewer Figure 6a**). In addition, we checked which of the 1142 genes that were significantly associated with venetoclax resistance in the scRNA-seq measurements were localized on the cell surface (GO:0009986, cellular compartment: cell surface). Surface proteins were slightly underrepresented in the venetoclax associated genes (**Reviewer Figure 6b**), with 35 venetoclax-associated genes encoding predicted surface proteins. These genes are shown in **Reviewer Figure 6c**.

Regarding blast morphology: The samples in this cohort were not classified according to the FAB classification system, however, we do see a clear association between FAB blast morphology class and venetoclax sensitivity in the BEAT-AML cohort (**Reviewer Figure 6d**). To link blast morphology to venetoclax sensitivity in the TuPro cohort, we have analyzed the relationship of baseline morphology features from 4i DRP and venetoclax sensitivity. While none of the associations were significant, we found that the most strongly correlated features were blast size (cell area) and nucleus elongation (**Reviewer Figure 6e-f**), both of which were negatively correlated with venetoclax response. This is consistent with the FAB associations, where smaller, more rounded immature blasts were more sensitive to venetoclax.

Reviewer Figure 6

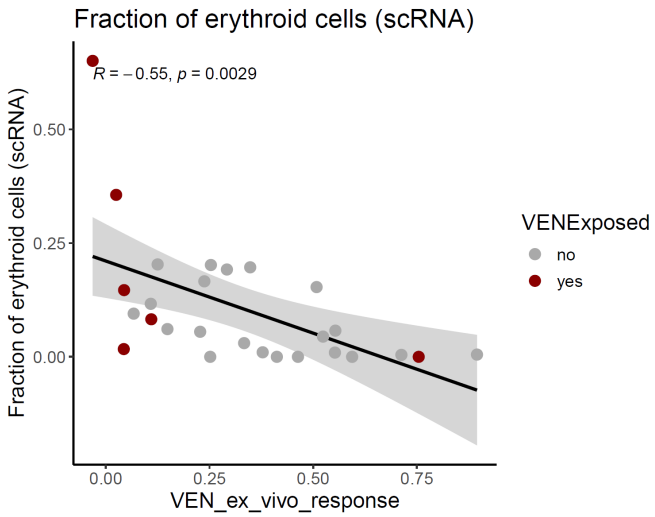


Reviewer Figure 6: *Analysis of surface markers and blast morphologies associated with VEN response.* ***a****: Surface markers in the CyTOF panel that are significantly associated with VEN ex vivo response (PCY score).* ***b****: Mosaic plot splitting all genes measured by scRNA-seq by whether their expression is associated with VEN ex vivo response (horizontal split) and whether they are cell surface proteins (GO:0009986 - cellular compartment cell surface, vertical split). Areas are colored by Pearson residuals, p-value from Chi-squared test.* ***c****: Expression of the VEN-associated genes encoding cell surface proteins.* ***d****: Association between VEN ex vivo response (AUC) and FAB blast morphology classification in the BEAT-AML cohort.* ***e****: Volcano plot for the association between VEN ex vivo response (PCY score) and morphology features measured by 4i DRP at baseline (control condition).* ***f****: Selected 4i DRP morphology features correlated with VEN ex vivo response (PCY score).*

There is recent data suggesting that erythroid AML is resistant to Ven. Did this show up in your screen? Correlation between an erythroid gene signature (RNA seq) and ven resistance?

We did not systematically classify our samples based on the FAB classification system, however, since erythroid AML is very rare, we would not expect more than one or two patients to fall into this category. We checked the expression of *CD36* for different FAB-subtypes in the BEAT-AML cohort, and now also added M6 (erythroid AML) to **Figure 5k**. Indeed, CD36 expression was very high in erythroid AML, suggesting resistance to venetoclax. Unfortunately, venetoclax sensitivity was not measured on any of the M6 samples in BEAT-AML, therefore we cannot perform a direct comparison with venetoclax sensitivity.

In our scRNA-seq data, erythroid cells were classified as a separate cell type (not AML blasts), therefore we do not generally see an erythroid gene signature expressed in AML cells. However, the fraction of erythroid cells per sample was negatively correlated with venetoclax *ex vivo* response (**Reviewer Figure 7**).



***Reviewer Figure 7**: Fraction of erythroid cells measured by scRNA-seq correlated with venetoclax ex vivo response (PCY score)*

B. Furthermore the data regarding Bcl-2 expression by Cytof among patients who were exposed are naïve to venetoclax seems to show very little difference, whereas the single cell RNA shows a more significant difference. How would you explain this?

We thank the reviewer for pointing this out. Following this comment made by both reviewer 1 and 3, we performed an in-depth analysis of the CyTOF data. We realized that a group of cells labeled "putative AML" likely corresponds to nonmalignant myeloid progenitors, but also more mature, CD34 negative AML blasts that are particularly abundant in venetoclax exposed samples. We therefore decided to re-do all analyses presented in **Figures 1d and 2-4**, with the following changes:

- We now exclude samples with low blast content (<5%) by pathology

- For the bulkified CyTOF marker profiles, we now include both the CD34 positive AML and the putative AML cell fractions

With these changes, we now get much better consistency between RNA and protein levels for Bcl-2 (**Figure 3b**) as well as other markers included in the CyTOF panel (**Figure S6a-b, previously S7a-b**).

4. Global transcriptional analysis limits oxidative phosphorylation, proliferation, and mitochondrial metabolism to venetoclax resistance.

A. This is a very interesting section demonstrating the potential importance of oxidative phosphorylation related genes as a mechanism or resistance to venetoclax. There were also comments on enrichment for proteins involvement mitochondrial complex assembly which may have been increased as well. From the paper in the description, it is not clear what is the hypothesis behind this. How does the increase in oxidative Phosphorylation genes and mitochondrial protein genes promote resistance to venetoclax?

Besides inhibiting Bcl-2, venetoclax has also been shown to affect cellular metabolism. Specifically, the drug inhibits mitochondrial respiration and the TCA cycle even in Bcl-2 knockout cell lines (Roca-Portoles et al. 2020). In this study, higher Bcl-2 expression was linked to higher respiration rates. In our cohort, we observe the opposite, potentially indicating that in venetoclax resistant cells, cellular respiration is decoupled from Bcl-2 expression and potentially no longer affected by venetoclax treatment. This hypothesis is consistent with another study demonstrating that a CLL cell line with acquired resistance to venetoclax showed higher respiration rates compared to the parental cell line, and respiration was no longer decreased in response to venetoclax (Guièze et al. 2019). This was linked to increased activity of AMPK (*PRKAA1*), which we also find to be associated with venetoclax resistance in this cohort at the level of gene expression. Decreased respiration in response to venetoclax treatment specifically in venetoclax sensitive samples was also observed in AML patient samples (Stevens et al. 2020). In this study, the authors find that this might be due to a switch from amino acids fueling the TCA cycle in leukemic stem cells towards fatty acid oxidation (FAO) in more mature and / or resistant cells. This switch was accompanied by upregulation of fatty acid transporters (CD36, CPT1A and CPT1C), and knocking down any of those genes restored sensitivity to venetoclax. In this cohort, CD36 upregulation is strongly associated with VEN resistance, however CPT1A shows an opposite association and CPT1C was not detected. Based on these prior studies and our data, we hypothesize that increased OXPHOS might be linked to increased availability of fatty acids (through CD36) to fuel the TCA cycle. In turn, high metabolic activity might enable increased proliferation rates, which we also observe in venetoclax resistant samples. However, with our current data, we cannot be fully sure whether these effects are causative of venetoclax resistance.

We have added a discussion of this link between OXPHOS, CD36 and VEN resistance (**lines 345-353**):

"In this cohort, increased expression of genes involved in OXPHOS was associated with VEN resistance, in addition to elevated levels of the fatty acid receptor and transporter CD36. Switching from amino acids to fatty acids to fuel the TCA cycle and OXPHOS has previously been identified as a mechanism by which AML cells circumvent the metabolic effects of Bcl-2 inhibition [44], thereby leading to venetoclax resistance [42]. Based on these prior studies and our data, we hypothesize that increased OXPHOS might be linked to increased availability of fatty acids (through CD36) to fuel the TCA cycle. In turn, high metabolic activity might enable increased proliferation rates, which we also observe in VEN-resistant samples."

B. The polo-like kinase inhibitor volasertib seem to provide significant activity in samples that were venetoclax resistance. How was this chosen, or was it just part of the panel? Any rationale behind why PLK1 inhibitors would be particularly active here? Any other classes of drugs that could provide activity in this population based on your analysis?

Our drug panel was based on prior studies performed on AML and other hematological malignancies (Snijder et al. 2017; Kornauth et al. 2022; Schmid et al. 2024). The panel was set up such that it included standard of care drugs for AML as well as promising candidates in clinical trials. Volasertib was part of this panel because it initially showed activity against AML in a clinical trials (Platzbecker et al. 2022; Döhner et al. 2014), but we had no prior expectation that it would be effective specifically in VEN-resistant samples. We hypothesize that the increased sensitivity to volasertib might be linked to higher expression of PLK1 in venetoclax resistant samples (**Figure 4e**), or potentially the more proliferative signature observed in these samples (**Figure 4b**). Other drugs that showed ex vivo activity in venetoclax resistant samples include Flt-3 inhibitors (crenolanib, quizartinib), afatinib, and omacetaxine mepesuccinate (**Figure S6g, previously S7g**). However, while volasertib response seems to be specifically increased in venetoclax resistant samples, the other compounds showed ex vivo efficacy on all samples independent of venetoclax sensitivity.

Reviewer #4

Expertise in AML functional precision medicine (Remarks to the Author):

In this manuscript Wegmann et al report on single cell genomics and ex vivo drug testing of rrAML patient samples. Overall this is a well written manuscript that leverages 21 rrAML patient samples to compare bulk and single cell DNA, RNA, and protein level data with ex vivo drug testing. The team focused on venetoclax exposure and resistance mechanisms in this cohort but this approach could be used for any promising novel agent and demonstrates a comprehensive approach to better understanding how to best utilize some of the novel therapeutics that have recently entered the armamentarium for AML therapy. Importantly the team demonstrated that a subset of venetoclax naïve patients were intrinsically venetoclax resistant and also outlined potential mechanisms by which ven naïve and ven expsosed patients acquired resistance.
The expansion of technology allowing for single cell resolution of data at the DNA, RNA, and protein levels has the potential to provide a wealth of information to guide investigations into chemotherapy resistance. Here, Wegmann et al have provided a useful repository of key data

exploring potential mechanisms by which AML blasts are surviving upfront chemotherapy and the use of targeted agents. This data is well annotated and novel for the field and appropriate conclusions are drawn from the extensive genomic data and ex vivo response testing that was done.

Major Critiques:
A particular strength of this manuscript is that 7 of the 21 rrAML patients included in this cohort had their leukemia blast burden quantified and profiled at multiple points during treatment and that both blood and bone marrow were collected. I was disappointed to see that there was not more data presented on clonal evolution over time for those patients with serial samples. Figure 1D shows high correlation over time points for CyTOF data but the manuscript would be improved if differences in ex vivo responses and in single cell profiling was elaborated on. In addition, information on clinical events between paired sample collections should be provided.

We thank the reviewer for this suggestion. We have performed an in-depth analysis of 6 patients with longitudinal samples (excluding one patient, TP037, for whom the second visit was only profiled by one technology). These are now provided in the **Supplementary Case Studies**.

Figure 2 – would strongly advise changing the appearance of the blast cell in Fig 2 panel A to have the appearance of a blast rather than that of a band cell.

We thank the reviewer for their attention to detail. We have updated the schema in **Figure 2a.**

Figure 2C, 2D and throughout the remainder of the manuscript would change to VEN exposed rather than experienced.

As mentioned in our reply to reviewer 1, point 1, we have re-defined our definition of venetoclax exposure, and we now call the group of samples from patients who were treated with venetoclax right before or at the time of sampling “VEN exposed” throughout the manuscript.

For Figure 5G data from patient TP031 is not clearly called out in the text.

We have amended this in the main text (**lines 291-293**), which now reads as follows:

“This effect was most pronounced in one sample from patient TP038 taken at the first visit and was dose-dependent, while CD36 blocking had no effect even at the highest dose in CD36 low samples such as TP031 baseline (Figure 5g)”

Lastly, it would have been nice to see more work testing CD36 blockade in AML beyond the automated microscopy to better delineate how this extensive testing can potentially translate to clinically useful therapeutics. Ideally, PDX models would be tested with a CD36 blocking antibody to improve the pre-clinical evaluation of these potentially promising results. If further preclinical evaluations are planned as a part of future directions it would be useful to the reader to explicitly state this.

We have added a sentence to the discussion to clarify this point (**lines 376-379**):

"CD36 blockage showed promising activity in vivo in a mouse model of human oral carcinoma (Pascual et al. 2017). In the future, our findings will need to be validated in an AML patient-derived xenograft model treated with an anti-CD36 blocking antibody in order to improve the pre-clinical evaluation of this approach."

Minor Critiques
Line 287 - towards rather than to wards

Fixed.

Figure 5G legend refers to left and right though these panels are oriented up and down.

We thank the reviewer for spotting this mistake. This has been corrected.

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Previously, I wrote:

SUMMARY
Overall, this manuscript describes a tour-de-force in primary patient AML sample profiling, integrating bulk and single cell high-parameter technologies with 2 powerful and functional precision medicine techniques. Applying this many analyses to 21 relapsed and refractory patients is powerful and represents a highly valuable resource.

Overall impact:
The analyses they perform, primarily to understand molecular markers and mechanisms of resistance to venetoclax, are interesting and impactful, despite the large body of literature devoted to this question, this large scale, highly integrated dataset significantly advances our understanding of this frequently-addressed question. The authors leverage their unique dataset to investigate this question and provide two possible solutions/treatment strategies to overcome venetoclax resistance. Given the very widespread use of venetoclax in AML patients, mechanisms of resistance represent a critical area of research. Developing resistance is nearly inevitable for a vast majority of patients. Strategies to overcome that resistance represent an important research priority.

Methods: the technologies that are integrated in this trial (TUPRO) are highly innovative and impactful. This is a stellar line-up of assays.

In this revision:
The authors have been fantastically responsive to earlier comments and concerns. I enjoyed reading this manuscript and I congratulate the authors on putting together a wonderful manuscript and highlighting interesting biological and clinical features from this very complex dataset. The detailed investigation of resistance mechanisms, cell types, potentially effective drugs/targets, and the role of CD36 and CD36 blocking is interesting and impactful.

I have noticed a few spots in the manuscript that could benefit from some minor additions, which I have suggested here, but these are just suggestions. I feel the manuscript is ready for publication.

Figure 1B: what is “Not Applicable” in references for allo-HSCT or Relapsed/Refractory? Is it “unknown”? A more specific designation would be helpful here.

In the description leading up to Figure 1d “we assessed the correlation of results obtained from matched blood and bone marrow…” might be confusing and might be more accurate to say, “we assess the correlation of features of matched blood and bone marrow..”

In main article body description of Figure 2, it might be useful to explicitly state that “on-target” effects refer to the specific killing of blast cells and sparing of non-malignant

elements. As written, I assumed you meant effects on the predicted molecular target (and I am left to wonder how you assessed that across all these conditions). I recognize that this is defined in the figure legend, but it would be helpful in the body of the article as well.

The data in this manuscript regarding the relationship of ox-phos/mitochondria/CD36 with ven-resistance is very interesting and contributes to the field. However, some of the work describing this relationship has not been referenced and may be mentioned (see work by Craig T Jordan, Daniel Pollyea, Eleni Lagadinou, and colleagues).

In the description leading up to Figure 5j, it might be useful to clarify the sentence "The majority of clusters observed consisted of.." by explaining what is meant by cluster (at this point in the manuscript, I assume the authors are referring to some sort of UMAP/tSNE/computational cluster). I might clarify "The majority of cell-interaction clusters observed consisted of…"

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed my comments. Thank you.

Reviewer #4 (Remarks to the Author):

The authors have done an outanding job of responding to reviewer comments and have produced a stronger manuscript as a result. All revisions are excellent and provide additional clarity for this valuable approach to comprehensive testing for patients with AML. The supplemental case series section is very well done and provides some additional information on how this data could potential inform clinical care and some of the limitations that will invariably remain with some cases demonstrating lack of correlation between ex vivo responses and patient responses (ie TP031).

- A typo was noted in the supplemental case studies section for TP025 (blat rather than blast).

Single-cell landscape of innate and acquired drug resistance in acute myeloid leukemia (NCOMMS-24-13462A)

Response to reviewers

We would like to thank the reviewers for their timely and constructive feedback throughout the review process.

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We specified both “allo-HSCT before TuPro” and “Relapsed/Refractory” as “not applicable” for follow-up samples, since this information was collected at the time of the first sample only. We clarified this meaning in the legend of Figure 1 now.

In the description leading up to Figure 1d “we assessed the correlation of results obtained from matched blood and bone marrow…” might be confusing and might be more accurate to say, “we assess the correlation of features of matched blood and bone marrow..”

We thank the reviewer for this suggestion. We have adapted this sentence in the main text.

In main article body description of Figure 2, it might be useful to explicitly state that “on-target” effects refer to the specific killing of blast cells and sparing of non-malignant elements. As written, I assumed you meant effects on the predicted molecular target (and I am left to wonder how you assessed that across all these conditions). I recognize that this is defined in the figure legend, but it would be helpful in the body of the article as well.

We have added a definition of “cellular on-target” response to the description of Figure 2 in the main text: “Thus, the PCY score measures a “cellular on-target effect”, i.e. a specific reduction in the target AML blast cell population”.

The data in this manuscript regarding the relationship of ox-phos/mitochondria/CD36 with ven-resistance is very interesting and contributes to the field. However, some of the work describing this relationship has not been referenced and may be mentioned (see work by Craig T Jordan, Daniel Pollyea, Eleni Lagadinou, and colleagues).

We have added additional citations referencing this work to the discussion.

In the description leading up to Figure 5j, it might be useful to clarify the sentence “The majority of clusters observed consisted of..” by explaining what is meant by cluster (at this point in the manuscript, I assume the authors are referring to some sort of UMAP/tSNE/computational cluster). I might clarify “The majority of cell-interaction clusters observed consisted of…”

Thank you for the suggestion, we have clarified this.

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Corrected.