Implementing a functional precision medicine tumor board for acute myeloid leukemia

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CONFLICTS OF INTEREST

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ABSTRACT

We generated ex vivo drug response and multi-omics profiling data for a prospective series of 252 samples from 186 acute myeloid leukemia (AML) patients. Functional precision medicine tumor board (FPMTB) integrated clinical, molecular and functional data for application in clinical treatment decisions. Actionable drugs were found for 97% of AML patients and the recommendations were clinically implemented in 37 relapsed or refractory patients. We report a 59% objective response rate for the individually tailored therapies, including 13 complete responses, as well as bridging five AML patients to allogeneic hematopoietic stem cell transplantation. Data integration across all cases enabled identification of drug response biomarkers, such as the association of IL15 overexpression with resistance to FLT3-inhibitors. Integration of molecular profiling and large-scale drug response data across many patients will enable continuous improvement of the FPMTB recommendations, providing a paradigm for individualized implementation of functional precision cancer medicine.

STATEMENT OF SIGNIFICANCE

Oncogenomics data can guide clinical treatment decisions, but often such data are not actionable nor predictive. Functional ex vivo drug testing contributes significant additional, clinically actionable therapeutic insights for individual AML patients. Such data can be generated in 4 days, enabling rapid translation via a functional precision medicine tumor board.
INTRODUCTION

High-dose chemotherapy and allogeneic hematopoietic stem cell transplantation (alloHSCT) can cure up to 60% of younger patients with adult acute myeloid leukemia (AML), but many patients relapse and suffer from life-long toxicities of treatment (1). Refractory or relapsed (R/R) AML patients, particularly older ones, have limited treatment options, and survival has remained poor (1,2). Genomic profiling has helped to deconvolute the biological basis, heterogeneity and clonal evolution of AML and highlighted novel therapeutic targets and subgroups (3-6). FLT3 inhibitors midostaurin (7) and gilteritinib (8), IDH1 mutant inhibitor ivosidenib (9), IDH2 mutant inhibitor enasidenib (10) offer new genetically guided treatment options for AML patients. However, only a fraction of patients harbor these mutations and even fewer respond to the treatments assigned by genetics (11,12). Furthermore, often no actionable mutations are seen to guide therapy decisions and many therapies do not even have any confirmed (genomic) biomarkers(13,14). For example, the BCL-2 inhibitor venetoclax can provide significant clinical benefits in AML therapy, but we lack effective biomarkers to identify patients likely to benefit (15-17).

We and others have utilized high-throughput ex vivo testing of AML cells to functionally identify drug response patterns(18-24). The Beat AML study reported on the functional testing of 122 small molecule inhibitors combined with genomic profiling in a cohort of 562 biobanked AML patient samples(23). Snijder et al. applied an image-based drug testing assay (pharmacoscopy) to demonstrate that the assay predicted clinical response to chemotherapy (21). Flow-cytometry has also been used to quantify responses in distinct cell subpopulations (19,25-27). Nevertheless, many of these studies are retrospective and lack the integration of functional and molecular data. Prospective implementation of these assays in the clinical decision-making process is needed.

Here, we performed ex vivo drug sensitivity and resistance testing (DSRT) of up to 347 emerging and 168 approved cancer drugs in AML patient cells. Molecular and functional data were interpreted and integrated for individual patients to consider novel therapy options for R/R AML patients. To implement the results in real-time for clinical translation, we designed a multidisciplinary functional precision medicine tumor board (FPMTB). We report here that, i) ex vivo DSRT is informative in highlighting the cancer-specific efficacy of drugs and that combined with molecular data, ii) such information is actionable in the treatment of refractory and recurrent (R/R) AML, and that iii) therapies tailored based on FPMTB recommendations are effective and provide clinically meaningful responses. The three-day DSRT assay provided actionable data faster than genomics and transcriptomics profiling, which was advantageous in rapidly...
recommending treatments for patients for whom standard therapy alternatives had been exhausted and for whom alternative therapy options were urgently needed.
RESULTS

Functional precision medicine tumor board (FPMTB) workflow and criteria

To quickly identify and implement selective treatment options for R/R AML with the help of *ex vivo* drug testing, we set up a multidisciplinary functional precision medicine tumor board (FPMTB) in a large single-center academic hospital setting. The FPMTB prospectively reviewed 61 R/R AML patients utilizing clinical parameters, *ex vivo* drug testing from each individual patient, as well as genomic and transcriptomic profiling data when these were available on time. To uncover novel associations of genotypes and drug response phenotypes, we also performed multi-omic profiling of all newly diagnosed AML patients (Figure 1).

The FPMTB composition, aims and treatment selection algorithms are presented in Supplementary Table 1 and the FTMB patient and sample flow is depicted as a CONSORT diagram in Supplementary Figure 1 and the meeting scheduling in Supplementary Figure 2. Data available at the FPMTB meetings included: i) full clinical patient history, ii) diagnostic workup (laboratory values, cytogenetics, clinical mutation data), iii) *ex vivo* drug sensitivity testing with 515 anti-cancer drugs, iv) whole exome (WES) and v) transcriptomics sequencing data.

Treatments for R/R AML need to be determined quickly, and therefore, genomic and transcriptomic profiling data were often not available in time for the clinical decision making. In contrast, *ex vivo* drug testing has a turnaround time of four days (three-days assay, one-day for data analysis). Thus, the treatment recommendations by the FPMTB were primarily based on drug-sensitivity testing, complemented with clinical disease history, routine molecular diagnostics (e.g. flow cytometry, cytogenetics, FLT3-ITD, NPM1, IDH1/2, WT1 mutation status), with support from genomics and transcriptomics when such data were available. If the BM blast count was low (<20%), as was observed in five relapsed patients, blast-specific drug sensitivity was assayed using flow cytometry.

**FPMTB guided therapy led to successful responses in 59% of R/R AML patients**

The FPMTB recommendations were implemented for 37 R/R patients, of whom 29 were eligible for objective response evaluation. The criteria for the selection of individual drugs and combinations are shown in Supplementary Table 1. The most frequently used targeted drugs for the clinical implementation of the FPMTB data were venetoclax (BCL-2i), dasatinib (inhibitor of ABL1 and other kinases), sunitinib (VEGFR- and FLT3-kinase) and temsirolimus (mTORi). The
drugs were administered as a customized combination of 2-3 drugs based on patient-specific sensitivity to single drugs and molecular data (Supplementary Table 2). Treatment was given to individual consenting patients as prospective n=1 case studies, not as a formal clinical drug study with case and control groups.

Implementing the FPMTB recommended therapy resulted in clinically meaningful responses (complete and partial responses, morphologic leukemia-free state, evaluated per ELN2017 criteria(28)) in 17 of 29 patients (59% objective response rate), including 13 (45%) patients achieving CR/CRi after treatment (Figure 2a). The remaining 12 patients progressed with resistant disease. The median time from the beginning of therapy to CR was 36 days (range 23-110 days).

In five R/R patients with no other conventional treatment options, the targeted treatments given enabled the patient to be bridged to alloHSCT, which then resulted in a long-term remission and survival. The time from the beginning of therapy to transplantation ranged from 1.3 to 37.7 months (mean 10.7). The median overall survival for all 37 patients was 7.5 months (range 14 days to 4.7+ years), with a median follow-up for surviving patients of 23 months (range 13 to 66 months) (Figure 2b). Comparing the drugs applied in clinical translation, the best predictive value of the DSRT was observed for venetoclax: 11/15 patients who showed ex vivo sensitivity had a CR/CRi response to venetoclax therapy (positive predictive value 73%) (Supplementary Figure 3a). We further explored expression of BCL-2 family members found that the expression of BFL1 may be associated with venetoclax resistance while expression of MCL1 does not associate with ex vivo response to venetoclax (Supplementary Figure 3b-e).

Novel drug combinations, such as those used in this study, may result in novel, unexpected toxicities. However, as most of the drugs used in clinical translation were molecularly targeted agents, with limited toxicity profiles as single agent drugs, we did not observe any grade 3-4 adverse effects attributable to the drug combinations used.

Overview of drug response and molecular profiling data

In order to reveal molecular patterns underlying drug responses, we generated comprehensive functional, genomic and transcriptomic data from 252 consecutive samples from 186 individual AML patients. By assembling a large database where molecular and functional data can be mined, we think we can eventually improve the FPMTB rules and clinical implementation. The dataset includes, i) disease status information for 252 samples, ii) DSRT assay details for 164 patient and 17 healthy control samples, iii) drug sensitivity data for each sample, along with full
annotated data from the 515 chemical compounds, iv) exome-seq genomic data for 226 samples, v) RNA-seq gene expression and fusion gene data for 163 patient samples and vi) DSRT and RNA-seq data for 4 healthy control samples. The AML patient cohort and sample information are given in Supplementary Table 3,4. The overview of mutation and gene expression patterns across all samples are given in Supplementary Figure 4 and 5a-c.

**Ex vivo drug responses providing functional insights in AML**

*Ex vivo* drug sensitivity and resistance profiles were determined for 164 consecutive AML patient samples by a high-throughput testing of a library of 515 chemical compounds (Supplementary Table 5,6). This analysis revealed selective efficacy profiles for individual drugs in individual patients, hence enabling us to determine the proportion of samples sensitive to each of the drugs in the library (Supplementary Table 7,8). The DSRT was performed in MCM or CM medium which provide different response levels for the FLT3, BET and JAK inhibitors as shown previously(29). The data for the two media types were therefore analyzed separately. The drug responses were quantified as an area under curve (calculated as described previously(30)) and then normalized using data from healthy control and expressed as selective drug sensitivity scores (sDSS). The distribution of blast cell percent and cell viability without drug treatment is given in Supplementary Figure 6a. These two variables were not significantly associated with drug responses in the 164 samples (Supplementary Figure 6b). The cut-off for a significant drug response was defined by a sDSS value 8.7 that represents the 95% percentile of the sDSS distribution of all drugs in all cases (Supplementary Figure 7a). The most often effective 50 drugs were further categorized in sub-classes based on their known mechanisms of action, such as BCL-2i, PI3Ki, HSP90i, JAKi, MEKi, CDKi, and BETi (Supplementary Figure 7b). We observed that about half of all the chemical compounds (n=272) in the drug library were effective in 3 or more samples (Supplementary Figure 7c). Of these *ex vivo* effective drugs, 15% (n=77) were drugs that were already approved for some oncology indications, and hence could readily repurposed for AML. The effective drugs included chemotherapeutics such as topoisomerase inhibitors but also targeted drugs e.g. tyrosine kinase inhibitors, immunomodulators, mTORi, JAKi, MEKi, BCL-2i, CDKi, PI3Ki (Supplementary Figure 7d).

Drugs with the same mode of action or with the same molecular targets often clustered together as expected (Supplementary Figure 8a). Interestingly, drugs representing different molecular mechanisms also sometimes showed clustering. For example, we found a strong association of
responses to BCL-2i venetoclax and the MDM2 antagonist idasanutlin. These two drugs clustered in their own branch of the dendrogram with a significant correlation (Supplementary Figure 8b). Another example of drugs with different molecular mechanisms showing similar response patterns was the co-clustering of BETi, MEKi and HDACi (Supplementary Figure 8c), with correlation coefficients between the individual drugs ranging from 0.64 to 0.71 (p<0.001) (Supplementary Figure 8d).

We then analyzed drug responses in paired diagnostic and relapsed samples from the same patients, tested in identical conditions. We found that the average responses to BCL-2 and PI3K/mTOR inhibitors were higher at the time of diagnosis. In contrast, responses to MEK inhibitors and dexamethasone responses were stronger in the relapsed samples (Supplementary Figure 9).

**Drug sensitivities associated with mutations**

We first analyzed *ex vivo* drug response profiles of all AML patient samples in distinct molecular groups defined by the common AML mutations (*FLT3, NPM1, IDH1* or *IDH2* and *NRAS* or *KRAS*) to obtain an overview of genomic subset-specific drug response (Figure 3). For this analysis, 146 drugs were selected based on sample-wise average sDSS values (>5) and variance (>10) (Supplementary Figure 10a). A total of 121 significant associations were observed between mutations (Supplementary Figure 10b) and responses of individual drugs. Many of these associations were between a mutation and several drugs of the same class, (Figure 4a, Supplementary Table 9). Previously known findings included the association of *FLT3* mutations (point mutations and ITD) with response to several FLT3 and tyrosine kinase inhibitors(23), or between *RAS* mutations with different MEKi(31). (Supplementary Figure 11a,b). The *FLT3* mutated samples, including ITD and point mutations, clustered functionally in two distinct subgroups based on the response patterns to FLT3i and other multi-kinase inhibitors. When we compared the average sDSS values for all drugs in *FLT3* mutant and *RAS*-mutant samples, a clear pattern emerged where all MEKi were more effective in *RAS*-mutant and all FLT3i in *FLT3* mutant AML samples (Supplementary Figure 11c). Similar segregation between key genetic lesions causing constitutive activation of cytokine receptors STAT5 and RAS-MEK signaling pathways was found in B cell acute lymphoblastic leukemia (B-ALL) (32). *NPM1*-mutated samples showed *ex vivo* sensitivity to JAKi. This was observed for drugs targeting JAK1i, JAK2i and pan-JAKi, particularly in the CM medium (Figure 4b,c). *NPM1* mutated cases that also harbored *IDH1*...
or IDH2 mutations were even more strongly sensitive to all JAKi (Figure 4d) including the clinically approved JAK1/2i ruxolitinib (Figure 4e). This observation was also validated in the Beat AML dataset (Figure 4f). We then analyzed combinations of two mutations predicting stronger sensitivity to individual drugs using the LOBICO method (33). For example, navitoclax sensitivity was strongly associated with IDH1 and IDH2 mutations (Supplementary Figure 12).

**Actionability of mutation data in AML**

Samples were divided into those with an actionable driver mutation (52%) and those without (48%) (Figure 5a). The mutations considered “potentially actionable” included FLT3-ITD and FLT3 (point mutation), IDH1/2, NPM1 and KRAS/NRAS. The efficacy of FLT3i in FLT3 mutant AML, venetoclax in IDH1/2 mutant AML patients as well as increased efficacy of JAKi in NPM1 mutated and that of MEKi in KRAS/NRAS mutated samples represent significant associations of mutations with ex vivo drug efficacies in our study. However, not all samples carrying these mutations were sensitive to the corresponding drugs (Figure 5b). Furthermore, efficacies of these drugs were also observed in cases where no mutations in the corresponding genes were found. For example, 16% of the FLT3 wild type cases responded to FLT3i midostaurin and 35% of the RAS wild type cases also responded to MEKi trametinib.

**Integration of drug response with mutations and pathway data: A basis for continuous development and refinement of FPMTB rules.**

In 97% of the samples (119/122), drug response data alone provided potential clinically applicable information for approved drugs (Fig 5c, drug response panel). We found that activity of key pathways measured by gene expression (34) was associated with drug sensitivities in cases where mutations were not informative. Thus, by incorporation of ex vivo DSRT, mutations and gene expression data, we were able to define groups of patients responding to drugs based on three lines of evidence. For example, such associations were defined for midostaurin with FLT3mut, ruxolitinib with NPM1mut-JAK-STAT pathway, venetoclax with IDH1/2mut-apoptosis pathway, trametinib with RASmut-MAPK pathway, dasatinib with KIT pathway (as was previously observed in a smaller sample set (35)) and temsirolimus with mTOR pathway activation (Figure 5c, Supplementary Figure 13a-c). We believe that the combination of DSRT, genomic and transcriptomic data will provide a means to further improve the reliability of the FPMTB.
recommendations for specific therapeutic alternatives. To demonstrate this, we quantified the overlap among drug efficacies and corresponding mutation and pathway activities. For example, expression of the BCL-2 pathway genes (n=23) was more concordant with *ex vivo* efficacy of venetoclax as compared to *IDH1/IDH2* mutation (n=4). The combination of mutation and deregulated pathways together gave complementary data in support of *ex vivo* drug responses (Figure 5d,e). The results suggested that a systematic data-driven strategy combining all the profiling data, will enable further refinement of drug response predictions.

**IL15 overexpression as a functional biomarker for resistance to FLT3 inhibitors**

This integrated data resource on AML provides insights on biomarkers of drug response and potential mechanistic insights that could help to understand the sensitivity, resistance and development of combinatorial therapies. We analyzed here the value of transcriptomic data in predicting the response to FLT3i in the subgroup of *FLT3*-mutant AML cases. Analogous to the clinical situation, *FLT3*-ITD mutant AML samples clustered in two distinct groups based on DSRT data indicating that about half of these patients are responsive (Figure 6a). Analysis of differential gene expression between FLT3i-sensitive vs FLT3i-resistant AML patient samples resulted in 57 genes with FDR<0.1 and a log-fold change>2 (Supplementary Table 10). We discovered interleukin 15 (*IL15*) as one of the most significantly overexpressed (average 4-fold upregulated) genes in FLT3i resistant samples (Figure 6b). This was validated in FLT3i resistant samples by RT-qPCR (Supplementary Figure 14a). Furthermore, data mining of the Beat AML dataset confirmed this association (Figure 6c, Supplementary Figure 14b). In line with Mathew *et al.* (36), we observed that the addition of recombinant IL15 protein reduced the sensitivity of *FLT3*-ITD mutated AML cell lines to FLT3i, sorafenib in particular (Supplementary Figure 14c). The MAPK pathway was the most upregulated (FDR<0.001) pathway in the FLT3i-resistant cases (Figure 6d). We then tested ERK phosphorylation after IL15 stimulation in AML patient samples using phospho-flow cytometry analysis. The treatment of human recombinant IL15 markedly increased phosphorylation of ERK and reduced phosphorylation of AKT in FLT3i-sensitive samples compared to FLT3i-resistant samples (Figure 6e, Supplementary Figure 14d). The results suggest that IL15 may not only be a biomarker but that the overexpression and production of IL15 protein may activate the ERK-MAPK pathway and hence contribute to FLT3i resistance. Finally, our *ex vivo* DSRT data from the same samples indicated a higher efficacy of MEKi in the FLT3i-resistant samples compared to the sensitive samples, suggesting activation of this pathway leads to subsequent MEKi sensitivity, which could provide novel treatment options to be tested in clinical
trials (Supplementary Figure 14e, f). We retrospectively assessed whether IL15 expression could predict the clinical response to FLT3i. We validated the high expression of IL15 in the patients who did not respond to the FLT3i-based treatments (Supplementary Table 11). Also, higher expression of IL15 along with the development of resistance to FLT3i was seen in serial samples from patient AML_129, thus further validating a potential functional link between IL15 and FLT3i resistance (Figure 6f).

To explore the origin of the IL15 and the nature of the signalling in patient samples in vivo, we explored both the original RNAseq data as well as a new dataset of scRNAseq data from 8 AML patient samples in a previously published study (37). ScRNAseq data indicated that both IL15 and IL15 receptor are expressed in many cell types including the monocytic lineage as expected, but also in the CD34-positive AML blast cells (Figure 6g, Supplementary Figure 15a-c). The bulk RNA sequencing data indicated that the FLT3 inhibitor resistant FLT3-ITD-mutant AML cells have a higher expression of not just IL15, but also monocyte-markers CD14 and CD300E (Figure 6b, Supplement table 10). Taken together, these findings suggest that in the FLT3-inhibitor resistant cases, production of IL15 takes place in the blast cells and/or within the monocytic lineage and that these cells also harbor the IL15 receptor. These findings are compatible with an autocrine signaling hypothesis, although paracrine signaling from other cell types or the stroma cannot be excluded.
DISCUSSION

Multidisciplinary molecular tumor boards increasingly interpret cancer genomic data in order to match cancer patients to clinical trials with targeted agents or to allocate novel clinical treatments to cancer patients (38,39). However, for many patients, genomic analyses often fail to provide clues on clinically actionable therapies (13,14). Furthermore, across all cancer types, less than half of the patients receiving genetically assigned approved therapies successfully respond as predicted (40,41).

Here, we developed and implemented a functional precision medicine tumor board (FPMTB) to integrate functional drug testing with genomics, transcriptomics and clinical laboratory data to define patient treatments. The FPMTB processed consecutive AML patients during 2011-2019 and recommended therapeutic options for individual relapsed or refractory patients. The outcome of FPMTB-recommended individualized treatments in 37 patients with multi-refractory, often end-stage AML patients was encouraging, with an overall response rate of 59%. Five patients could be bridged to curative hematopoietic stem cell transplantation therapy. In many of these patients, the FPMTB-guided therapy was started at a low disease burden (MRD). Persistent MRD is a major cause for treatment failure in AML and may be the ideal setting for implementing personalized targeted therapies.

The response and survival rates of these patients warrant a randomized, controlled clinical trial to be launched to formally validate the benefit of FPMTB-based therapeutic recommendations. The approach has become more and more informative over the years, as the number of clinically approved, better tolerated drugs has increased (13,42). We found that the clinical efficacy of venetoclax could be predicted by ex vivo testing in AML. This is of particular importance as this drug is a major advance in AML, and also had a significant impact on the positive clinical responses in the present study. Particularly in the relapsed/refractory AML setting, where many patients do not respond well to venetoclax (or experience short responses), an ex vivo drug sensitivity assay may prove valuable for selecting patients most likely to respond and directing non-responding patients to alternative therapies.

Compared to genomics-based precision medicine, ex vivo testing provides informative results in a substantially higher fraction of patients as well as for more drugs. One or more clinically applicable drugs were considered selectively effective in 97% of the evaluated AML cases. Furthermore, ex vivo drug testing assay provided results in a clinically applicable time frame (median of 4 days), and with comparative efficacy estimates across all the 515 tested drugs in...
each sample. This time-scale is particularly relevant for aggressive, rapidly progressing cancers such as AML.

There is a need to further standardize the approaches used for functional laboratory testing and the molecular analyses used to characterize each patient sample. For example, we have documented the effect of different media types (regular and stromal cell conditioned media) (29) and readouts (cell viability and flow cytometric assays) on drug testing results (26). Further refinement and standardization will improve our ability to predict drug responses in the clinic as well as to understand the driver signals and vulnerabilities of each AML patient.

The large integrated data set described here enables continuous improvement of the FPMTB rules as well as exploration of the data to identify potential biological insights and biomarkers of drug efficacy in subsets of patients. The continuous improvement of FPMTB could in the future include machine learning-based decision trees as key components of a learning health care infrastructure. Our analysis of these data already revealed insights on mechanisms-of-action that could be clinically applied. For example, our data suggested that IL15 may act both as a biomarker as well as functionally contribute to FLT3i resistance in FLT3-ITD mutant AML. This observation was validated in an independent gene expression data from the BeatAML dataset(23). The administration of recombinant IL15 protein efficacy of FLT3i in FLT3-ITD-positive AML cells in vitro. The mechanistic link between IL15 and FLT3 was previously proposed by Mathew et al.(36).

We showed how IL15 increased phosphorylation of ERK in FLT3i-resistant, but not in FLT3i-sensitive samples, pointing to the ERK-MAPK pathway as a possible escape route for FLT3-inhibition(43,44). FLT3i-resistant FLT3-ITD mutant AML cells showed ex vivo sensitivity to MEK inhibition suggesting potential combinatorial strategies for future clinical studies. Data integration also revealed insights to the role of AML blasts and monocytes in producing IL-15, including a potential signaling loop involving the IL15-receptor.

A major hurdle in implementing precision medicine is the limited access to potentially effective drugs for patients. Many drugs showing ex vivo efficacy are neither available for off-label indications, nor approved at all or not even in clinical trials. There are also financial, legislative and policy-related implications that make the design of clinical drug studies for individually tailored (combinatorial) therapies challenging. However, n=1 proof of concept studies as described here should be highly encouraging and informative for the design of formal clinical studies. The FPMTB approach and the specific findings on ex vivo drug response described here should be explored to set up international multicenter collaborations between private and public stakeholders to solve issues that currently hinder the application of individually tailored functional precision medicine.
In conclusion, ex vivo drug testing is a powerful approach for understanding AML biology and drug sensitivity as well as for facilitating repositioning known and emerging drugs for AML therapy. Systematic data integration prioritizes the most promising drugs and biomarkers for drug development and clinical trials. While further research is warranted, the combination of molecular and functional assays is warranted in a clinical cancer drug trial setting.

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METHODS

AML patient cohort and samples

Samples (n=252) from 186 adult AML patients and 17 healthy donors were collected with signed informed consent with protocols in accordance with the Declaration of Helsinki (study acronym HRUHLAB2, HUS Ethical Committee Statement 303/13/03/01/2011 (original), latest amendment 7 dated June 15, 2016. Latest HUS study permit HUS/395/2018 dated February 13, 2018). Mononuclear cells (MNCs) were isolated by Ficoll-Paque centrifugation from freshly collected bone marrow and peripheral blood specimens of 133 diagnosis, 78 relapsed, 41 chemorefractory stage patients. For 42 patients we profiled two or more consecutive samples. Skin biopsies were collected from all patients for germline DNA analysis. The median age at diagnosis of the patient cohort was 62 years. Other clinical details of the AML patients are given in Supplementary Table 1. A summary of the cohort of patients including demographic information, clinical and treatment data are given in Supplementary Table 1.

Functional molecular precision medicine tumor boards (FPMTB)

The FPMTB consisted of the AML tumor group chair and clinicians managing the patients, clinical laboratory specialists, translational scientists familiar with the functional assays and multi-omics data, bioinformaticians, study nurses and by referral a genetic counsellor for actionable germline variants (Supplementary Table 1). The meetings were scheduled every week (Wednesdays) and also ad hoc if necessary, when a patient case was submitted (meeting <1 week of sampling). The task of the FPMTB was to overview and analyze clinical, molecular and functional characterization of all consecutive patients with newly diagnosed or R/R AML patients, assign risk groups, evaluate standard of care options and open clinical trials. In addition, in case of R/R AML, candidate drugs were evaluated for on- or off-label treatment to make rational therapy recommendations based on DSRT and other profiling data. The board also analyzed treatment follow-up and responses for eligible patients, recommended bridging to alloHSCT (Supplementary Figure 1). More detailed criteria for patient and treatment selection are shown in Supplementary Table 1.

Drug sensitivity and resistance testing (DSRT)

A library of 515 commercially available chemotherapeutic and targeted oncology compounds consisted of 168 approved drugs, 261 investigational compounds and 86 probes (Supplementary
Table 2). The chemical compounds, DMSO (negative control) and benzethonium chloride (positive controls) were added to 384-well plates using an acoustic liquid dispensing system Echo 500/550 (Labcyte). Freshly isolated MNCs were counted and resuspended in MCM (Mononuclear Cell Medium, PromoCell) with 0.5μg/mL gentamicin and 2.5μg/mL amphotericin or in CM (conditioned medium) constituted of 77.5% RPMI 1640, 10% FCS, 12.5% human HS-5 bone marrow stromal cell line derived conditioned medium and 1% penicillin and streptomycin. A 5μL cell free medium was added to dissolve compounds followed by 20μL cell suspension containing 5,000-10,000 cells to each well using multidrop (ThermoFisher). The plates were incubated at 37°C in 5% CO2 for 72 h. Subsequently, CellTiter-Glo (Promega) reagent was added to all wells and cell viability as luminescence generated by total cellular ATP was measured using a PHERAstar (BMG Labtech).

The drug responses passing the data quality assessment were included in further analysis (45). Drug sensitivity scores (DSS) were calculated as shown previously (30) and selective DSS (sDSS) were calculated by normalizing drug responses against 17 healthy controls.

Exome and RNA sequencing

Exome and RNA sequence analysis were performed in real-time under individualized systems medicine program using DNA and RNA materials isolated from MNCs as described previously (20,46). The skin biopsies from the same patient were used as germline control for exome-seq data analysis. The detailed methods are given in the Supplementary text.

Mutation and drug response association analysis

Cancer and AML-specific genes were selected for the drug-mutation association analysis. The AML genes were collected from published studies, TCGA (n=23), InToGen (n=32), Papeammanuil et al. (6) (n=111) and other cancer-associated genes (n=616) were obtained from Census database (47). Out of altogether 667 genes, 340 genes were found mutated in our dataset. Furthermore, the genes with mutation in at least two samples and tumor variant allele frequency (VAF) >25% were selected for the analysis. The VAF below 25% was disregarded for the analysis considering no significant impact on drug responses. The drugs were selected based on effectiveness across all samples. The analysis was done independently on MCM (n=61) and CM (n=82) subsets to avoid impact of media on efficacy of key AML drugs and misleading the
biological signal. To test significant differences in drug responses, Wilcoxon signed rank test was applied using R package "exactRankTests" (version 0.8-29) between mutated and wild type samples for each gene. For adjusting drug-wise multiple comparisons, FDR was calculated using the Benjamini and Hochberg method. FDR < 0.1 and mean sDSS difference <= 5 or =>5 between wild type and mutated samples was considered significant.

Gene set variance analysis (GSVA)

GSVA(48) (Gene-set Variation Analysis) was used to calculate pathway activation scores (R package version 1.22.4). As an input log2 CPM of protein-coding genes from all the AML patient samples and four healthy controls (CD34+ sorted cells) were used. GSVA calculates relative enrichment of a gene set for each sample across the sample space, allowing for sample-wise comparison of gene set enrichment within a dataset. A positive enrichment value for a sample indicates overall higher expression of the genes in the pathway in the sample, compared to the other samples analyzed. Pathway definitions were taken for canonical pathways (CP) that had 1329 gene sets (MSigDB database v6.2). The gene sets used were i) CP:BIOCARTA (n=217), ii) CP:KEGG (n=186), iii) CP:REACTOME(n=674). To consider a pathway to be active, we used a robust, four-step methodology. First, to get the highly significant active pathways (P values) in a sample, we applied 1000 bootstrap iterations on GSVA scores. Pathways wise P values were corrected by applying Benjamini and Hochberg (BH) method and FDR < 0.01 were considered significant. Second, we chose highly variable pathways which had a GSVA score > 0.2 (cutoff based on overall distribution). Third, these significant pathways were further normalized to four healthy controls (CD34+). A given pathway was only considered active when it had a GSVA score more than the average GSVA score of healthy controls. The final step involved choosing only those active pathways which passed the above three criteria and were also active in at least two databases. For example, apoptosis pathway was considered active if found to be deregulated in at least two of the databases; BIOCARTA_TCAPOPTOSIS_PATHWAY, KEGG_APOPTOSIS, REACTOME_APOPTOSIS.

Differential gene expression and pathway enrichment analysis

Differential gene expression analysis was performed using the R package DESeq2(49). The analysis was performed using raw read counts from FLT3i sensitive and resistant samples. To
remove any batch effects in the data, we corrected for RNA-seq library preparation protocols and
gender by modifying the design formula (~batch + condition) and then applied a likelihood ratio
test (LRT) to get the differentially expressed genes. The Benjamini Hochberg (BH) method was
used to control the false discovery rate (FDR). A cutoff value of absolute log2 fold-change of
greater than or equal to 2 and FDR < 0.1 were used as additional filters to select differentially
expressed genes (DEGs) for the downstream analysis. Pathway analysis was performed using
the genes upregulated in FLT3i resistant samples. Enrichr web-tool was used for pathway
enrichment analysis. Outputs from KEGG 2016 and Wiki pathways were considered for further
analysis.

Phospho-flow cytometry and data analysis

Viably frozen mononuclear cells from AML patients were thawed and resuspended in RPMI 1640
medium supplemented with 10% FBS and Pen/Strep. Cells were treated with 50μL of DNase
(Promega) for 2 h at 37°C to dissolve dead cell clumps. Cells were centrifuged and resuspended
in RPMI 1640 medium with Pen/Strep without serum. Cells were stained with Zombie violet cell
viability dye (423113, BioLegend) stimulated with 100 ng/mL human recombinant IL15
(PeproTech) for 20 min at 37°C. Subsequently, cells were washed with ice cold PBS, centrifuged
at 1000 x g, fixed with 500μL of 4% formaldehyde and incubated at 37°C for 10 min. PBS was
directly added to the fixed cells and centrifuged at 1000 x g and the supernatant was discarded.
Ice cold methanol was added dropwise to the cell pellets and incubated on ice for 30 min to
permeabilize the cell membrane. Cells were washed with PBS, counted and added to 96 well V
bottom plates. The surface IgG was blocked using human IgG Fc receptor inhibitor (Invitrogen) in
staining buffer (0.5% bovine serum albumin in PBS) for 15 min on shaker at room temperature
and washed with PBS. The antibodies for CD45 (563716, BD Biosciences), pERK (612566, BD
Biosciences), pAKT S473 (4075S, Cell Signaling Technologies) and isotype controls were added
to the respective wells and incubated for 30 mins on a plate shaker. The cells were washed with
staining buffer and PBS before flow cytometry analysis using iQue Screener Plus (Intellicyte).
Antibody-stained UltraComp beads (01-2222-41, Invitrogen) and cells without viability staining
were used for compensation. The data was analyzed using Cytobank cellmass software.

Testing of FLT3 inhibitors in FLT3-ITD mutated AML cell lines
FLT3-ITD mutated AML cell lines MOLM-13 and MV4-11 were purchased from DSMZ and were cultured in recommended media. Sorafenib was dispensed in 9 different doses in 384 well plates. MOLM-13 and MV4-11 cells stimulated with 100ng/ml human recombinant IL15 (PeproTech) at 37°C for 1 hour. AML cell lines were resuspended in their respective medium with CellTox Green reagent (Promega). 3000 cells per well were dispensed in pre-drugged plates and incubated at 37°C for 72 hours. Fluorescence was detected using a Phearastar plate reader (BMG LabTech) and dose-response was generated using four parameters logistic regression.

Data access

Basic demographics, clinical laboratory values, drug therapies, treatment responses, ex vivo drug testing and sequencing (exome and RNA sequencing) data will be available at the publication-specific analysis environment at the Helsinki University Hospital datalake. This is an EU GDPR-compliant (General Data Protection Regulation, https://gdpr.eu/), secure, cloud-based data environment accessible by a virtual machine (IP-restricted, 2-level authentication), including all key analytical tooling. Datalake onboarding commences by sending an email request to tietopalvelu@hus.fi.
REFERENCES


**FIGURE LEGENDS**

**Figure 1. Schematic of the design for functional precision systems medicine study.** The diagram illustrates how functional molecular precision systems medicine integrates high-throughput drug response assays and molecular profiling, aiming at individualized clinical translation of data for AML patients. The n=1 cycle on the left illustrates prospective real-time clinical translation through a functional precision medicine tumor board approach. The drug response and sequencing data are analyzed and integrated within a patient with a goal to tailor therapies in a realistic time frame. The n=many cycle on the right illustrates the data integration across a larger large sample set. The main goal here is to find possible biomarkers of drug responses, which eventually could also help to refine the rules of the FPMTB.

**Figure 2. The outcome of patients treated with FPMTB-guided personalized therapies.** a, the overall survival estimated by the Kaplan-Meier method of all patients (grey area denotes 95% confidence interval). b, swimmer plot illustrates survival and therapy responses in 37 relapsed and refractory AML patients upon FPMTB guided therapies, where asterisk represents patients who received allogeneic hematopoietic stem cell transplantation after the treatment and arrows represents the patients who are alive. The zero month represents the starting time point of the FPMTB recommended therapy. The therapy responses, CR-MRDneg - complete response with minimal residual disease negative, CR-MRDpos-complete response with minimal residual disease positive, CR - complete remission, CRi - complete remission with incomplete hematological recovery, PR- progressive disease, MLFS – bone marrow blasts <5%, RD – resistant disease, were defined by ELN-2017 criteria.

**Figure 3. Drug response patterns in molecular subsets of AML.** AML patient samples were categorized in molecular sub-classes according to mutation status in common AML driver genes. Hierarchical clustering of samples using Euclidean distance and ward linkage for sDSS of 146 drugs in individual molecular subsets. The drugs were selected considering variance >10 and data points available in at least 20% of the samples. Grey bars in the drug response heatmap indicate missing data. Fourteen recurrent AML driver genes, with at least 3 samples recurrently mutated and VAF>25%, were displayed to indicate the mutation patterns in the molecular subsets. The diseased status, age, medium used for drug testing and cytogenetics information for each patient are displayed in the last panel.

**Figure 4. Somatic mutations as a molecular denominator of drug sensitivities.** a, an overview of the mutation-drug response association analysis results. The upper part of the table lists the total number of positive (drug sensitivity) and negative (drug resistance) gene-drug
associations identified separately in the MCM and CM sample sets. The associations at FDR<0.1 were considered significant. The lower part shows the number of drugs significantly associated with each selected gene mutation. For example, the FLT3-ITD mutation is positively associated with six drug responses in the MCM sample set and with three drugs in the CM sample set. b, The volcano plot illustrates the mean difference of sDSS values on X-axis and adjusted P values on Y-axis for each drug-gene pair in the CM sample set. The significant (FDR <0.1, mean difference >5 or <-5) drug-gene pairs were highlighted in blue (negative associations) or red (positive associations) where dark red dots show significant positive associations with NPM1 gene. c, The P values for NPM1 mutation associated JAKi including approved drugs ruxolitinib, baricitinib and tofacitinib in CM sample set. d, Hierarchical clustering of NPM1 mutant samples and sDSS of nine JAKi divide samples in two distinct subsets based on the presence of IDH1 or IDH2. e, The co-existing IDH1 or IDH2 in NPM1 mutated samples were significantly associated with strong JAKi sensitivity f, The same association was significantly observed for ruxolitinib in the BeatAML dataset(23).

**Figure 5. Genomic and transcriptomics-based prediction of ex vivo drug efficacies.** a, The division of 143 AML patient samples in actionable and non-actionable subsets. b, Ex vivo drug sensitivity of FLT3i in FLT3-ITD and point mutation positive samples and of MEKi in KRAS/NRAS mutation positive samples. c, The samples with complete molecular profiling and drug response data ordered as per actionable driver mutations and subsequently non-actionable mutations. Selective drug responses for FDA/EMA approved 77 drugs were depicted on Y-axis and individual patient samples on X-axis, where ineffective drugs below sDSS 8.7 were marked with grey rings. The common effective drugs were highlighted for integration with mutation and pathway activation. The lower panel illustrates integrated ex vivo efficacy and the presence of respective mutations and pathways for each sample. d, The statistics of patient samples showing evidences of drug sensitivity, the presence of mutation and pathway activation for key targeted drugs in AML including BCL-2i venetoclax, FLT3i midostaurin, TKi dasatinib, JAKi ruxolitinib, MEKi trametinib and JAKi ruxolitinib. e, The drug-wise percent of samples showing any evidence and no evidence from effective drug response, mutation and/or pathway up-regulation.

**Figure 6. IL15 overexpression is associated with resistance to FLT3i in FLT3-ITD+ cells.** a, Hierarchical clustering of FLT3-ITD positive AML patient samples and six FLT3i resulted in two groups of the samples with high (sensitive group) and low (resistant group) efficacy to FLT3 inhibitors (FLT3i). b, Differential gene expression of FLT3i sensitive versus resistant samples depicts up-regulation of the IL15, CD14 and CD300E genes in the FLT3i resistant group. c, The
overexpression of IL15 was significant in FLT3-ITD mutant FLT3i resistant samples in our data and in Beat_AML dataset. d, Gene set enrichment analysis of the genes upregulates in FLT3i resistant samples depict MAPK pathway as top enriched pathway e, AML patient cells stimulated with human recombinant IL15 had increased phosphorylation of ERK compared to unstimulated control cells using phospho-flow cytometry. The color bar displays phosphorylation ratio to the control cells. f, FLT3i sensitivity and expression of IL15 in serial samples from the patient AML_129. g, The UMAP plots demonstrate expression of IL15 and IL15 receptor (IL15RA) in eight AML patient samples from published study (Dufva et al. Cancer Cell 2020(37)).
Ex-vivo drug sensitivity and resistance testing (DSRT) 

Clinical translation of 515 drugs

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Recommendation for each patient

FPM

Data integration

Subgroups

Biomarkers

Reference data

Feedback learning to improve FPMTB

Therapy recommendation

FPM tumor board

Data integration for each patient

Data integration across all patients

Translational research

Individualized clinical translation

Mononuclear cells from bone marrow and blood

Ex-vivo drug sensitivity and resistance testing (DSRT)

Exome and RNA sequencing

515 drugs

Investigational

Approved

252 patient samples
17 healthy controls

Clinical data

Gene expression

Chromosomal alterations

Drug responses

Somatic mutations

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Figure 2

Therapy response

CR−MRDneg
CR−MRDpos
CRi
PR
MLFS
RD
Not evaluable

a

Survival (months)

Proportion

1.00
0.75
0.50
0.25
0.00

0 6 12 18 24 30 36 42 48 54 60

b

Patient number

Therapy response
    CR−MRDneg
    CR−MRDpos
    CR
    CRi
    PR
    MLFS
    RD
    Not evaluable

⁕ Allogeneic hematopoietic stem cell transplantation after FPMTB based therapy

Survival (months)

0 6 12 18 24 30 36 42 48 54 60

0.00
0.25
0.50
0.75
1.00

0 6 12 18 24 30 36 42 48 54 60

Proportion

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37

0 6 12 18 24 30 36 42 48 54 60

Survival (months)
Figure 4

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<td>8</td>
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</tr>
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**Flt3** refers to the **Flt3**-ITD mutation, which is a common genetic alteration in acute myeloid leukemia (AML) that results in constitutive activation of the JAK/STAT pathway.

**FLT3** refers to the **Flt3** receptor tyrosine kinase, which is involved in hematopoietic cell proliferation.

**IDH1** and **IDH2** are isoenzymes that are mutated in a subset of AML patients, leading to altered metabolism and tumorigenesis.

**NPM1** refers to the **NPM1** gene, which is mutated in a significant proportion of AML patients, influencing disease progression and response to therapy.

**Gender** refers to the sex of the patient, which may influence the outcome of AML treatment.

**Disease status** includes **Relapse**, **Diagnosis**, and **Refractory** stages, indicating different phases of the disease.

**JAK inhibitors** are a class of drugs that inhibit the Janus kinase (JAK) family of kinases, which are integral to the JAK/STAT signaling pathway.

**Mean difference (sDSS)** refers to the mean difference in survival rates between different groups, with **Non significant** indicating no significant difference.

**Area under curve** is a measure of the cumulative hazard over time, used to assess the effectiveness of treatments.

**Research.** on December 21, 2021. © 2021 American Association for Cancer Research.
Figure 5

**a** No actionable driver mutations (n=68) 48%
Actionable driver mutations (n=75) 52%

**b** Mutations Deregulated pathway
- FLT3-ITD/FLT3
- IDH1/IDH2
- NRAS/KRAS

**c** Drug response profiles
- FLT3
- FLT3-ITD
- IDH1/IDH2
- NPM1
- NRAS/KRAS

**d** Drug recommendation system prioritized on ex-vivo drug efficacies

**e** Mutations Pathways
- IDH1/2
- FLT3-ITD + PM
- NA
- NA
- NPM1
- KRAS/NRAS
- NA
- mTOR

Drug
- Venetoclax
- Midostaurin
- Dasatinib
- Ruxolitinib
- Trametinib
- Temsirolimus

Any evidence
- 61/92 (66.3%)
- 50/116 (43.1%)
- 53/122 (43.4%)
- 81/122 (66.3%)
- 74/122 (60.6%)
- 64/120 (53.3%)

No evidence
- 31/92 (33.7%)
- 66/116 (56.9%)
- 69/122 (56.5%)
- 41/122 (33.6%)
- 48/122 (39.3%)
- 56/120 (46.6%)
**Figure 6**

**a**

A heat map showing expression levels of various genes in different samples. The heatmap includes columns for gender, disease status, and FLT3 ligand response status.

**b**

A scatter plot comparing the log2 fold change of IL-15 expression between FLT3i sensitive and resistant samples. The plot includes data points for different compounds and pathways.

**c**

A box plot showing the distribution of IL-15 expression in FLT3i sensitive and resistant samples. The p-value for the difference is 0.0077.

**d**

A chart comparing the log2 fold change of IL-15 expression across different pathways. The chart includes KEGG pathways and Wiki pathways.

**e**

A set of histograms showing the expression levels of pERK and pAkt in FLT3i sensitive and resistant samples. The patient IDs are AML_120_01, AML_046_01, AML_040_01, and AML_062_01.

**f**

A graph showing the relationship between sDSS and log2 CPM for AML_129_02 and AML_129_03.

**g**

A visualization of UMAP for IL5 and IL15RA with CD34+ blasts and Monocytes highlighted.
Implementing a functional precision medicine tumor board for acute myeloid leukemia

Disha Malani, Ashwini Kumar, Oscar Bruck, et al.

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