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ABSTRACT

Background: A fundamental hallmark of cancer cells is their ability to sustain proliferative signaling and cell survival, reflected in a cellular chemotherapy response that is poorly understood. We questioned whether chemotherapy modulated phospho-signaling at 4 and 24 h *in vivo* could provide information about long-term survival in acute myeloid leukemia (AML), and if the signaling response to therapy was more informative than analysis at time of diagnosis.

Methods: Peripheral blood was collected from 32 younger AML patients (age 16-74 years), before, 4- and 24 hours after start of induction chemotherapy. Samples were analyzed by 36-dimensional mass cytometry for assessment of alterations in immunophenotypes and intracellular signaling using unsupervised and supervised machine learning approaches. Results were validated by RNA sequencing and mass spectrometry proteomics (Super SILAC). Targeted sequencing was used to characterize patient samples for recurrent AML mutations. Drug sensitivity and resistance testing *ex vivo* was compared to activation of relevant signal transduction pathways and mutational profile.

Findings: 5-year patient survival was accurately predicted in the leukemic cell population at 24 hours after therapy onset by phospho-proteins p-ERK1/2 (T202/Y204) and p-p38 (T180/Y182). RNA sequencing showed induction of MAPK target gene expression and the AP-1 transcription complex in patients with high p-ERK1/2. Super-SILAC proteomics confirmed an increase in the abundance of p38 prime target MAPKAPK2(MK2) 24 hours after start of induction therapy. *Ex vivo* drug sensitivity testing demonstrated high sensitivity to MEK inhibitors in the patient cells with high p-ERK1/2 measured at diagnosis or 24 hours after start of chemotherapy.

Interpretation: Early single cell signaling response to chemotherapy provided precise prognostic information independent of stratification by genetics. We propose that early functional measurement of chemotherapy-potentiated MAPK pathway signaling could identify non-responders to intensive chemotherapy allowing precise treatment adjustment.

Graphical abstract

Graphical abstract a, Peripheral blood samples were collected from 32 AML patients treated with conventional induction therapy ("7+3" cytarabine + daunorubicin). Samples were collected before start of treatment, at 4- and 24- hours after start of treatment and immediately fixed to preserve *in vivo* signaling. **b**, Conventional therapy response assessment by bone marrow aspiration at day 17 post-treatment or before cycle two of induction therapy and ELN 2017 Risk classification for the 32 patients in this study. 5-year survival are shown in Kaplan-Meier curves. **c**, Early therapy response assessment by mass cytometry at 4- and 24-hours post-treatment by investigation of intracellular signaling response to chemotherapy. Machine learning approaches identified p-ERK1/2 in the blast cell population to be predictive of patient 5-year survival at 24 hours after start of induction therapy.

INTRODUCTION

Acute myeloid leukemia (AML) is a hematopoietic stem cell-derived myeloid malignancy characterized by manifold genetic aberrations and overall poor overall survival, where more than half of the patients display aberrated signal transduction.¹⁻³ Standard treatment for newly diagnosed AML patients fit for intensive chemotherapy includes a combination of an anthracycline (daunorubicin 60-90mg/m² or idarubicin 10-12 mg/m²) for three days, and cytarabine (Ara-C) (200mg/ m²) for seven days with an initial remission rate of 60-80%.² However, relapse is a challenge in more than 40% of AML patients. In AML, like in most cancers, response to therapy is evaluated weeks to months after start of therapy. Early detection tools of responders and non-responders will be essential to improve cancer patient survival, providing physicians with patient specific information to change treatment strategy early and avoid unnecessary adverse effects.⁴⁻⁸

Aberrant signaling in cancer is known to regulate cancer cell proliferation, protect against cell death and modulate interactions with the micro-environment.⁹ Approximately 60% of AML patients harbor mutations in signal transduction pathways,¹⁰ including mutations resulting in abnormal activation of signaling that is associated with prognosis.² Conventional chemotherapeutics affect signaling directly or through induction of cell stress. In AML, this is demonstrated within hours after start of chemotherapy, involving gene expression of proteins central in regulation of cell death and survival.^{11,12}

The intratumor heterogeneity of AML underscores the need for methods that provide single cell resolution. Single cell immune and signaling profiling by mass cytometry provides a high dimensional method that deciphers the clonal- and functional heterogeneity in cancer by simultaneous analysis of multiple parameters in single cells.¹³ The application of >40 antibodies permits simultaneous analysis of both intracellular signaling networks and phenotypic characterization of cells,¹⁴ and thus allows for assessment of the intratumor heterogeneity of AML. Furthermore, the possibility of sample multiplexing allows for direct comparison of sequentially acquired patient samples, making this an ideal tool for assessment of therapy response in a clinical setting.¹⁵

The current risk stratification for predicting long-term response in AML is based on genetic analyses in cancer cells sampled at the time of diagnosis.² However, risk stratifications that included clinical and biological information, and are under discussion.¹⁶ Functional analyses of drug sensitivity and signaling responses are suggested,^{14,17-19} but based on our previous

observations of protein and gene expression hours after start of chemotherapy,^{11,12} we hypothesize that early chemotherapy-induced alterations in intracellular signaling networks may be a more accurate predictor of long-term therapy response. Here, we have employed mass cytometry to investigate intracellular signaling networks in peripheral blood (PB) samples from 32 newly diagnosed AML patients during the first 24 hours of standardized induction chemotherapy. By correlating initial intracellular signaling response to 5 years overall survival, we demonstrate that early response evaluation by mass cytometry represent a valuable tool for early identification of patients with suboptimal treatment response to standard induction therapy.

RESULTS

Characterization of major immunophenotypic clusters in AML by FlowSOM

Based on experience with proteomics-based functional diagnostics in leukemic patients^{12,20,21}, we collected peripheral blood (PB) samples for single cell signaling profiling from 32 AML patients immediately before, at 4 h and 24 h after start of standard “7+3” induction chemotherapy (Supplementary Fig.1, 2 and Supplementary table 1) The antibody panel of 21 extracellular and 15 intracellular markers (Supplementary table 2) was designed to identify the major immunophenotypic cell subsets in the samples, including both healthy immune cells and leukemic blasts, and to investigate the major intracellular signaling pathways regulating myeloid cell proliferation and survival.^{20,22}

To avoid user bias and enable robust and efficient analysis in our dataset of more than 35 million cells from 52 individuals (45 AML/MDS/B-ALL patients and seven healthy donors), we applied the unsupervised clustering algorithm FlowSOM²³ to identify the major phenotypes (Fig. 1a). Manual analysis of increasing resolutions from 1 to 20 metaclusters (MCs) demonstrated that a total of 10 MC was sufficient to differentiate the major healthy cell populations in peripheral blood from the AML blast cells (Fig. 1b-c). No significant association between MC size and patient survival or ELN 2017 risk class were found, neither at the pre-treatment time point nor over the time course.

The 10 MCs were annotated manually based on surface marker expression (Fig. 1c). Healthy cell types (MC3-8) had a homogeneous surface marker expression among patients and healthy donors (Supplementary Fig.3). These also formed well defined, islands in the t-SNE plots (Fig. 1a). The AML blast clusters (MC1 and MC2), the myeloid cluster (MC9) and the hematopoietic stem cell cluster (MC10) were, as expected, more heterogeneous. MC1 and MC2 had a high expression of CD34 and CD117 and were therefore classified as CD34⁺ blast cells (Fig. 1c and Supplementary Fig. 3). Most patients in this cohort had a CD34⁺CD117⁺ blast population present in MC1, whilst MC2 was nearly entirely composed of patient 9 and 22 having mutated FLT3-ITD and mutated WT1 (Fig. 1b and Supplementary Fig. 2). MC2 could primarily be distinguished from MC1 by higher expression of HLA-DR, CD33 and CD123. MC9 included most cells with 43.2% of the cells in the entire cohort and was represented in all patient samples and healthy donors. MC9 had a myeloid phenotype (CD64 dim, CD33 dim and HLA-DR dim) and did not express lymphoid markers. This cluster had heterogeneous CD34 expression among patients and was negative for CD117 (Supplementary Fig. 3). MC9 was

expanded in AML patients and bone marrow (BM) of healthy donors but not in PB of healthy donors. (Fig. 1b and Supplementary Fig. 4)

The healthy donors had a similar distribution of different cell populations that resembled a normal differential blood count with approximately 60% granulocytes, 30% lymphocytes and 3% monocytes. The AML patients showed abnormal distribution of cell populations with expanded myeloid lineage and CD34+ cells, while expansion of these cells was not detected in healthy donors (MC1/MC2) (Fig. 1b).

Overall, the size of the MCs was quite consistent between the different time points (Supplementary Fig. 5). However, there was a tendency among several patients towards a more differentiated phenotype at 24 hours, with a decrease in the myeloid cluster MC9 and an increase in granulocytes (MC7) and monocytes MC6 (Supplementary Fig. 5). No significant association between MC size and patient survival or ELN 2017 risk class were found, neither at the pre-treatment time point nor over the time course.

Figure 1. Characterization of immunophenotype in 32 AML patients by FlowSOM

a, t-SNE maps of the 10 FlowSOM identified metaclusters based on surface marker expression across the 7 healthy donors and the 32 AML patients at pre-treatment, 4 hours and 24 hours (20,000 cells per plot for visualization). t-SNE maps are annotated with color-coded overlay shown in c. b, Stacked bar chart showing the metacluster relative distribution in each patient and healthy donor, as percent of total population in the pre-treatment sample. Patient numbers are colored by patient 5-year survival (blue=alive, orange=deceased). c, Heatmap showing the median marker intensity of the 19 surface markers used for clustering in the pre-treatment sample of the 32 AML patients. Total metacluster size among the 32 AML patients are shown as percent of total.

Chemotherapy-induced changes in intracellular signaling during the first 24 hours of treatment predicts long-term survival in AML.

Chemotherapy modulates intracellular phospho-protein including p53 and ribosomal P2 in bulk samples of tumor cells.^{12,21} Therefore, we examined the treatment-induced phospho-signaling in each MC in the patient samples. We employed a machine learning approach using a supervised LASSO Cox regression model with automated feature selection and nested cross-

validation to evaluate which features were predictive of patient survival. Features evaluated in the model included age, sex, three timepoints (pre-treatment, 4- and 24 hours), the change from pre-treatment to 24 hours (delta 24 h), the 10 MC sizes and in each MC the 90th percentile dual count measured for all the functional markers (cCaspase3, CyclinB1, p-4E-BP1(T37/T46), p-AKT(S473), p-AXL(Y779), p-CREB(S133), p-ERK1/2(T202/Y204), p-Histone3(S28), p-NF-kB p65(S529), p-p38(T180/Y182), p-Rb(S807/S811), p-S6(S235/S236), p-STAT1(Y701), p-STAT3(Y750) and p-STAT5(Y684))(Fig. 2a-b). The analysis identified p-ERK1/2 ($p=2.057e-4$, Hazard ratio (HR) 2.41) at 24 hours in MC9 as the most significant predictor for patient two-year survival. Furthermore, we repeated the LASSO analysis with five-year survival which revealed that p-ERK1/2 ($p=9.59e-5$, HR 2.51) at 24 h in MC9 still was the most significant feature (Fig. 2c). When the cohort was split by median p-ERK1/2 at 24 h, resulting in 16 patients in each arm (Fig.2d), we found that the patients with a high level of p-ERK1/2 in MC9 at 24 hours after start of induction chemotherapy had significantly inferior survival ($p=0.0049$ 2-y OS, $p=0.0015$, 5-y OS) compared to patients with p-ERK1/2 levels below median at 24 h (Fig. 2c). These groups will further be referred to as the high and the low 24h-p-ERK1/2 group. p-ERK1/2 levels at pre-treatment or 4 hours after start of induction therapy were not significant in predicting patient 5y- OS. The 24 h sample gave a better separation of deceased and alive patients (Supplementary Fig. 6a) and the levels of p-ERK1/2 in high group at 24 h were significantly higher than the levels in PB and BM from healthy donors (Supplementary Fig. 6b).

By further inspection of the longitudinal signaling patterns from pre-treatment to 24 h, a substantial drop in p-ERK 1/2 were observed in the low 24h-p-ERK1/2 group and vice versa in the high 24h-p-ERK1/2 group (Fig. 2d). We therefore calculated the ratio and delta between pre-treatment and 24 h for p-ERK 1/2 and performed a univariate survival analysis by dividing the patients into two groups by the median. Both p-ERK1/2 ratio and delta were significantly discriminating 5-y overall survival ($p=0.0333$)(Supplementary Fig. 6c,d). This confirms 24 h of chemotherapy induced drop in p-ERK 1/2 which provide a better prediction of survival compared to the pre-treatment basal p-ERK1/2 value. We speculate in the need for bringing p-ERK1/2 under a certain threshold to obtain optimal therapy response (e.g., P30, P5 and P31 in low 24h-p-ERK1/2 group and P11, P7 and P8 in high 24h-p-ERK1/2 group) (Fig. 2b).

Figure 2. p-ERK1/2 signaling at 24 hours after start of induction therapy predicts patient two- and five-year survival.

a, t-SNE plot highlighting the position and distribution of MC9 (red) in the t-SNE plot of all 32 patients at pre-treatment, the other metaclusters are shown in grey. b, Heatmap of the arcsinh transformed 90th percentile p-ERK1/2 in MC9 at all timepoints sorted by the 24 h value, divided by median into high and low 24h-p-ERK1/2 group. Patient numbers are color coded by 5-year survival (blue=alive, orange= deceased). Complete remission by bone marrow aspiration at day 17 or before cycle two of induction therapy, ELN 2017 risk classification and transplantation status are shown in color coded bars to the right. c, Cox LASSO regression analysis identified p-ERK1/2 at 24 hours in MC 9, as significantly associated with patient 2-year and 5-year survival. The 32 AML patients were divided into two groups based on the 24 h median value as shown in b; high and low 24h-p-ERK1/2 group. Kaplan-Meier survival curve of the 5-year overall survival (OS) in the two groups (Log-rank (Mantel-Cox) test $p=0.0015$, Hazard Ratio (logrank) low/high group 0.2307 and 95% CI of ratio 0.0945 to 0.5625) show the poor survival of the high group. d, Line-graph of the arcsinh transformed 90th percentile p-ERK1/2 value at all timepoint in the two groups.

In MC9, the level of p-p38 at 24 hours was the second most significant feature predicting patient two- and five-year survival ($p=3.42e-4$, HR 3.39 2-y OS and $p=2.969e-4$, HR 3.50 5-y OS) (Supplementary Fig.7a-c). In addition, p-Rb ($p=0.003$, HR 1.72) at 24 hours in MC9 and the change in Cyclin B1 from pre-treatment to 24 h ($p=0.003$, HR 9.3) in MC8 (NK-cells) were

significant in predicting five-year survival (data not shown). ERK1/2 and p38 are both members of the mitogen activated protein kinase (MAPK) family, and play important roles in cell proliferation, differentiation and apoptosis.²⁴ CREB is a downstream target multiple kinases, including both p-ERK1/2 and p-p38, and is also directly phosphorylated at S133 by MSK1, MSK2 or MAPKAPK2 (MK2) immediately downstream of both ERK1/2 (MSK1/2) and p38 (MSK1/2 and MK2).^{25,26,27} Our data confirmed that p-CREB (S133) was significantly higher in the patients with high p-ERK1/2 and p-p38 in MC9 at 24h (Supplementary Fig. 7d). Activation of the ERK1/2 signaling pathway has been shown to protect cancer cells specifically against apoptosis induced by chemotherapeutic cytotoxic drugs.²⁸ Previous studies in relapsed/refractory AML have shown that as many as 50-80% of AML patients have elevated p-ERK1/2 at the time of diagnosis.^{29,30} The ERK pathway has been shown to drive selection of resistant clones during induction therapy for AML *in vitro* and elevated ERK1/2 activity has also been observed in AML patients who have developed resistance to FLT3-targeted inhibitors.^{31,32} Expression of p38 has also been shown to drive chemotherapy resistance in different cancers.^{33,34} Similarly, the high ERK 1/2 and p38 activation level following chemotherapy treatment could indicate an enhanced pro-survival signaling response in the peripheral blood leukemic blasts.

Clinical parameters and signaling in MC9

To determine the prognostic impact of the high and the low 24h-p-ERK1/2 group, we investigated the distribution of complete remission (before the second cycle of induction therapy), ELN 2017 risk classification and transplantation status between the two groups (Fig. 2b). More patients achieved complete remission after induction therapy in the low 24h-p-ERK1/2 compared to the high (ten in low group vs five in high group). 6/16 patients in high 24h-p-ERK1/2 and 3/16 patients in low 24h-p-ERK1/2 group had remaining blasts (>6%) at day 17 or before cycle two. However, P18 in low 24h-p-ERK1/2 had 15% bone marrow blasts at day 17, but when reevaluated on day 26, the number had been reduced < 5%. P25 in the low group had prolonged aplasia but 30% blasts at day 29 measured by flow cytometry. The ELN 2017 risk classification was quite equally distributed between the high and low 24h-p-ERK1/2 groups. Eight patients in low 24h-p-ERK1/2 vs six patients in high 24h-p-ERK1/2 received allogeneic bone marrow transplantation. Likely due to the low number of patients in this study, there was no significant correlation between response to chemotherapy, ELN risk or

transplantation status and the high and low 24h-p-ERK1/2 groups. However, the ELN risk adapted therapy could to a certain explain survival of high-risk outliers. For example, P20, the only survivor in high 24h-p-ERK1/2 group received allo-HSCT due to adverse risk disease (ELN2017) and was most likely thereby rescued by the transplantation.

Mutations and signaling patterns in MC9

To investigate whether activation of signaling pathways was associated with specific mutations, we associated the signaling in MC9 (Myeloid cells) and MC1 (CD34+ blasts) to next generation sequencing data (TruSight myeloid panel) and cytogenetics (Supplementary Fig. 8a-f). The two patients with highest p-ERK1/2 levels in MC9 at 4 and 24 hours were the only patients in the cohort with NPM1 mutations and wild-type FLT3. Furthermore, the two patients with the highest level of p-STAT5 in MC9 at all time points had FLT3-ITD mutations. STAT5 is known to be activated in FLT3 mutated AML.³⁵ There were several examples where patients with the same mutations seemed to have a similar signaling pattern at 4- or 24 hours; p-STAT5 at 24 h in patients with IDH1/2 mutations (MC9), p-p38 at 4 hours in patients with BCOR or BCORL1 mutations (MC9) and p-NF-kB at 24 h in patients with SRSF2 mutations (MC1) (Supplementary Fig. 8b,d,f). Patients with mutations affecting DNA methylation (DNMT3A, IDH1/2 and TET2) seemed to have a strong induction of p-Histone3 in the CD34+ MC1 from 4 - to 24 h (Supplementary Fig. 8e). Due to the complexity of the mutational landscape of these patients, with several cooccurring mutations, establishing a significant coupling of mutations and signaling would demand a larger patient cohort. Nevertheless, there appear to be signaling patterns that reflect the mutation status of the patients that could be worth further investigation.

Signaling in CD34+ MC1 AML blast cluster

MC1 had high expression of the stem cell markers CD34 and CD117 and was therefore a particularly interesting cluster (Supplementary Fig.3). All patients had cells present in this metacluster at pre-treatment, but there was large variation in cluster size between patients, ranging from 0.04% to 35% (Supplementary Fig. 9a). The MC1 cluster size or the change in cluster size during the first 24 hours was not significantly correlated to survival. Furthermore, we did a univariate survival analysis of p-ERK1/2 and p-p38 by dividing the patients into two groups by median at 24h (16 patients in each group). The difference in survival between the

two groups was not significant for p-ERK 1/2, but significant for p-p38 ($p=0.014$) (Supplementary Fig.9c). We considered whether the p-ERK 1/2 and p-p38 signaling in MC9 could originate from CD34+ cells. We found that the level of both p-ERK 1/2 and p-p38 was significantly higher in MC1 than in MC9 (paired t-test $p<0.0001$) (Supplementary Fig. 9d and e). p-ERK1/2 has previously been shown to be upregulated in CD34+ cells compared CD34- cells.³¹ 10 patients had CD34+ cells in MC9, most of them (7/10) were in the high 24h-p-ERK1/2 group (Supplementary Fig. 9b). However, several patients in the high 24h-p-ERK1/2 signaling group did not have CD34 expression in MC9 and very small MC1 populations. This suggests that for some of the patients with prognostic p-ERK1/2 and p-p38 signaling this could originate from the CD34+ cells, but for others the signaling could originate from CD34- myeloid cells in MC9.

Computational validation by manual gating of mass cytometry data

Our unsupervised cell clustering was followed by an analysis with a machine learning algorithm that identified treatment-induced alterations in intracellular signaling. We evaluated whether a comparable result could be achieved by simple analysis of manual bi-axial gating of the data. Successful manual analysis may suggest that routine flow cytometry can be used for functional response assessment, e.g., by crude gating of leukemic blasts by using CD45 and side scatter of light. Mass cytometry data does not provide scatter parameters; therefore, we focused our manual gating on CD45 expression, indicative of hematological cell maturity. Cells were gated by CD45 and CD66b, which allowed identification of the majority of immature cells (CD45 low/CD66b low) and exclusion of the more mature cell types (CD45 high), including the granulocytes (CD45 low/CD66b high). The gating strategy is visualized in Fig. 3a. Healthy donor samples included in the respective barcodes were used to guide blast cell gating. After gating, we evaluated the measured dual counts (90th percentile) of p-ERK1/2, p-p38 in time-to-event analyses, where patients were stratified by median marker expression (above/below median), as described above (Fig. 3b, c). The pre-treatment and the 4 h samples were not predictive of patient survival, while the negative prognostic value of high p-pERK1/2 ($p=0.0011$), p-p38 ($p=0.0013$) at 24 h and the ratio of p-ERK1/2 (24h/0h) ($p=0.0005$) were confirmed (Fig. 3d and Supplementary Figure 15e). Interestingly, p-ERK1/2 and p-p38 at 24 h and the ratio of p-ERK1/2 was even more significant than in the unsupervised machine learning approach described above. All in all,

these results show that a simple gating strategy, easily achievable by standard flow cytometric methods, is sufficient to detect chemotherapy-induced changes in peripheral blood leukemic blasts that could provide early information on therapy response and therapy outcome.

Figure 3

Figure 3. Validation by manual blast gating confirm p-ERK1/2 and p-p38 prediction of survival

Manual gating of mass cytometry data was performed by bi-axial gating in cytobank on all 32 patients. a, Bi-axial gating strategy of patient P11 (PB=Peripheral blood) and a healthy donor (PB) for gating guidance. AML blast cells were defined as CD66 low and CD45 low, the red arrows pinpoint the exported population. Raw 90th percentile data of p-ERK1/2 and p-p38 from the CD66low, CD45low cell population was exported from cytobank and arcsinh transformed with cofactor 5. b, Heatmap show the arcsinh transformed 90th percentile data for p-ERK 1/2 and c, p-p38, sorted by their respectively 24-hour values. Patient numbers are color coded by 5-year survival (blue=alive, orange= deceased). d, Kaplan-Meier survival curves for p-ERK1/2 and p-p38. Groups are defined by the median 24-hour value for each marker and the combination. Curve comparison p-values are calculated by Log-rank (Mantel-Cox) test (p-ERK1/2; HR low/high group:0.2252, 95%CI of ratio: 0.09215 to 0.5502, p-p38; HR low/high group: 0.2437, 95%CI of ratio: 0.09791 to 0.6064)

Target genes of ERK1/2 and p38 signaling are upregulated after start of induction therapy

We examined chemotherapy-induced changes in gene expression profiles by RNA sequencing (RNAseq) in a subset of the patients in our cohort (n=14) (Fig. 4a). Our analysis was focused on previously reported primary response genes (immediate early genes (IEGs), immediate

late genes (ILGs), delayed early genes (DEGs)) and secondary response genes (SRGs) induced by ERK (n=189) or by p38 (n=501) signaling.^{36,37} In total, this included 689 genes (Supplementary Table 3), of which 525 were identified in our dataset (Fig. 4b). Due to the small sample size (n=14), the two timepoints post chemotherapy (4 and 24h) for each patient were used as post-treatment replicates when evaluating chemotherapy-induced changes. A two sample students t-test was performed on the 24h-p-ERK1/2 low post-treatment group versus the 24h-p-ERK1/2 high post-treatment group with a threshold p-value <0.05. This resulted in 62 significant genes, of which 29 were upregulated in 24h-p-ERK1/2 high post-treatment group, including FOSL1/Fra-1 (FOS Like 1) (Fig. 4c). FOSL1 is an activator protein-1 (AP-1) transcription factor and an immediate late gene shown to decode ERK signal duration to response amplitude, hence maximal expression increases with longer signal duration.^{36,38} When we investigated FOSL1 mRNA expression in each individual patient, there was a clear separation between the patients in 24h-p-ERK1/2 high group versus the patients in 24h-p-ERK 1/2 low group (Fig. 4d). Patients in 24h-p-ERK1/2 high group had a higher induction of FOSL1, especially 4 hours post-treatment. The analysis also revealed high expression of additional genes that have been linked to poor prognosis in AML in the 24h-p-ERK1/2 high group, like heat shock protein 90 (HSP90)^{39,40} and F2R like trypsin receptor 1 (F2RL1)⁴¹

Figure 4

Figure 4. RNA sequencing show AP-1 complex induction after start of induction chemotherapy

a, Venn diagram showing that 525 p-ERK1/2 and p-p38 induced genes were identified among our RNAseq data. These genes were used for further RNA sequencing data analysis. b, Heatmap of 90th percentile arcsinh transformed p-ERK1/2 mass cytometry data in M9. The high and low groups used for RNAseq data analysis were defined by the 24-hour p-ERK1/2 median value in MC9. Patient numbers are color coded by 5-year survival (blue=alive, orange= deceased). An overview of the patient sample material and the analysis performed is shown to the right. All 32 patients in the study were analyzed by mass cytometry, 15 patients with mass cytometry and proteomics and 14 patients with mass cytometry, proteomics and RNAseq. In addition, diagnostic fresh bone marrow or peripheral blood samples from 18 of the 32 patients was screened with an ex vivo drug sensitivity screen and a selective drug sensitivity score was calculated (sDSS). c, Hierarchical clustering (Euclidean distance) of the 10 patients with post-treatment samples, based on the gene expression (DESeq normalized counts) of the 62 differentially expressed genes post-treatment identified between the 24-hour p-ERK1/2 high and low groups ($p < 0.05$, two-sided students t-test). The heatmap show z-scored DESeq2 normalized counts. The two timepoints post chemotherapy (4 and 24h) for each patient were considered post-treatment replicates. Mutations identified by NGS and diagnostic cytogenetics are shown for each patient in the top panel. d, Line plots of particularly relevant genes showing the DESeq2 normalized counts at the three different timepoints for each patient. Each line represents one patient, numbers for identification are annotated next to the lines. For patients with only pre-treatment samples the patient number is annotated to the left. Patients in 24h p-ERK1/2 low group are colored in grey, patients in 24h p-ERK1/2 high group in red.

Furthermore, we performed a paired students t-test on the 4-hour versus pre-treatment sample in the 24h-p-ERK1/2 high group on the ERK and p38 inducible genes (Supplementary Fig. 10a, b). This analysis revealed another AP-1 transcription factor; ATF3,⁴² that was strongly induced at 4 hours in all patients in the 24h-p-ERK1/2 high group and decreased in the two patients in the low group that had 4-hour samples (Supplementary Fig. 10c,d). Several of these genes, there among ATF3, are shown to be upregulated 4-hours post induction therapy in our previous work.¹¹

The MAPK pathway is an important regulator of AP-1 transcriptional activation.⁴³ AP-1 proteins consist of members of the Jun and Fos families, Jun dimerization partners and the activating transcription factors (ATF) subfamilies. Our analysis identified FOSL1 and ATF3 to be particularly induced 4 hours after induction therapy in the 24h-p-ERK1/2 high group. We therefore investigated the other components of AP-1, which appeared to have a similar pattern as FOSL1 and ATF3, thus with an increase at 4 hours in the high group and a decrease in the low group (Supplementary Fig. 11a-c). Indeed, our RNAseq results demonstrate a higher expression of AP-1 transcription complex members, like FOSL1 and ATF3, in the 24h-p-ERK1/2 high group patient samples. The induction of these primary response genes was especially high 4 hours after start of induction chemotherapy, which is consistent with the mRNA half-life of these genes.³⁶ AP-1 is a nuclear target of both ERK and p38 signaling in response to growth factors and proinflammatory cytokines and play a central role in cell proliferation and survival. In addition, recent work by Martinez et al. identified AP-1 as a pioneer transcription factor in senescence that imprints a reversible transcriptional program of senescent cells.⁴⁴ Interestingly, Duy et al. showed that induction chemotherapy in AML induced a transient senescence-like state in resilient cells that was capable of initiating AML recurrence.⁴⁵ ATF3 has been shown to be critical among the AP-1 transcription factors in reconstructing the accessibility of chromatin to promote senescence.⁴⁶ Notably, ATF3 was strongly induced in all the six patients from which we had 4 hours RNAseq data from in the 24h-p-ERK1/2 high group, whilst it was decreased in the two patients that had 4-hour samples in the 24h-p-ERK1/2 low group (Supplementary Fig. 10d).

Super-SILAC proteomics revealed the p38 target MAPKAPK2 (MK2) to be significantly induced 24 hours after start of induction therapy

Proteome profiling was conducted on the 14 patients analyzed by RNAseq, focusing our analysis on the 12 patients with pre- and post-treatment samples (Fig. 5a). We quantified 7480 proteins, of which 6363 had a quantitative value in at least two samples. A paired students t-test between the pre-treatment and 24 hours samples in the 24h-p-ERK1/2 high group (n=7) were performed, with a p-value cut off at <0.02. This resulted in 114 differentially expressed proteins; of which 64 proteins increased from pre-treatment to 24 hours and 50 proteins decreased (Fig. 5b). MAPK-activated protein kinase 2 (MAPKAPK2 or MK2), a downstream substrate that is directly phosphorylated and activated by p-38, was one of the most significantly ($p=0.0039$) upregulated protein at 24 hours in the 24h-p-ERK1/2 high group (Fig. 5b-c). To determine the effect of chemotherapy on cellular proteins we calculated the change (delta) from pre-treatment to 24 hours (24h-0h) for the top 15 most significant proteins. Delta MK2 along with ferritin light chain (FTL) had the most significant difference between 24h-p-ERK1/2 low group and 24h-p-ERK1/2 high group, with a p-value of 0.0091 and 0.0026, respectively (Fig. 5c,d). All patient samples in 24h-p-ERK1/2 high group, except for P23, were also in 24h-p-p38 high group in MC9. P23 was the patient with highest p-p38 at 24 hours in 24h-p-p38 low group. MK2 is a primary target of p38 and is considered to be the master regulator of RNA-binding proteins.⁴⁷ It facilitates stability of mRNA with distinct AU-rich elements (AREs) that are found in cell cycle regulators, cytokines, growth factors and protooncogenes like c-fos and c-jun. MK2 has been shown to sustain a robust AP-1 activity in triple negative breast cancer.⁴⁸ The increase of MK2 protein by chemotherapy supports our RNAseq results of induced AP-1 activity post-treatment in the 24h-pERK1/2 high group. The p38- MK2 signaling axis has also been shown by others to be induced in quiescent (G0) leukemic cells after exposure to cytarabine, and thereby promote chemoresistance.⁴⁹ Interestingly, MK2 is especially important in p53-deficient cells for cell-cycle arrest and survival after DNA damage.⁵⁰ This is of importance in AML as most patients have wild type TP53 gene but non-functional p53 protein.⁵¹

Figure 5

Figure 5. Proteomics reveals increased MAPKAPK2 (MK2) expression at 24 hours in patients with high p-ERK1/2 signaling
a, Heatmap of the 90th percentile arcsinh transformed p-ERK1/2 in M9 mass cytometry data. The high and low groups used for proteomics data analysis were defined by the 24-hour pERK1/2 median value in MC9. Patient numbers are color coded by 5-year survival (blue=alive, orange= deceased). An overview of the patient sample material and the analysis performed is shown to the right. Groups for proteomics paired data analysis are highlighted in red. b, Hierarchical clustering (Euclidean distance) of the 53 most differentially expressed proteins (SILAC log2 ratio, z-score) identified by a paired students t-test (p<0.01) between the pre-treatment sample (0h) and 24-hours sample of the 7 patients in 24h p-ERK1/2 high group with 24-hour samples. Patients are sorted by sampling timepoint; 0h and 24-hours. c, d, The two proteins (MK2 and FTL) among the 15 most significant differentially expressed proteins in high group (n=7) that had the most significant increase in expression from pre-treatment to 24 hours compared to the 5 patients in 24h p-ERK1/2 low group. c, SILAC log2 (Light/Heavy) ratio of MK2 and FTL in all patients analyzed by proteomics (n=15) patients in low group is colored in grey, patients in high group are colored in red. d, The change in protein expression (SILAC log2 ratio) of MK2 and FTL from pre-treatment to 24 hours was calculated and compared between the 24h p-ERK1/2 low and high groups. Patients in high group had a significantly higher increase in MK2 and FTL compared to the low group (Unpaired t-test, with two tailed p-value 0.0091 for MK2 and 0.0026 for FTL).

Ferritin heavy chain (FTH1) was the most significant protein upregulated at 24h in 24h-p-ERK1/2 high group (p=0.00015) but did not show the same significance between the low and high 24h-p-ERK1/2 groups as FTL. Iron is a prerequisite for leukemic cell response to anthracyclines⁵², and upregulation of FTH1 may be a protection mechanism that attenuate

the effect of chemotherapy. Ferritin heavy/light chain (FTH1/FTL) have previously been linked to cytarabine resistance in AML, and serum ferritin levels have been shown to increase from day 1 to day 8 of induction chemotherapy.⁵³

The BCL-2 protein family has a central role in regulation of cell death and overexpression of BCL-2 in AML is associated with poor survival and resistance to conventional chemotherapy.^{54,55} ERK1/2 and p38 can regulate several members of the BCL-2 family to control cell survival.^{56,57} The pro-apoptotic protein BCL-2 associated X protein (BAX) was among the top 15 expressed proteins that increased from pre-treatment to 24 hours (p=0.0022) in 24h-pERK1/2 high group (Fig. 5b). This finding is in consistency with our previous work, where mRNA expression of BAX increased 24 hours after start of induction chemotherapy in AML.¹¹ We therefore investigated the other proteins in the BCL-2 family (Supplementary Fig. 12). BCL-2 decreased to undetectable levels at 24 hours in four out of five patient samples in 24h-p-ERK1/2 low group. On the contrary, there were only minor changes in the BCL-2 expression in the 24h-p-ERK1/2 high group, except in P4. (Supplementary Fig. 12a) This indicates that the patients with high p-ERK1/2 signaling post-treatment had a stable high expression of BCL-2, whilst the patients more sensitive to induction therapy had a drop of BCL-2 to undetectable levels as response to chemotherapy. Furthermore, expression of the pro-apoptotic BCL-2 antagonist killer 1 (BAK) was significantly higher from pre-treatment to 24 hours in the low 24h-pERK1/2 group (p=0.0145) (Supplementary Fig. 12b). These findings reveal that the stress signaling is indeed pro-survival and confers chemoresistance.

Drug sensitivity data show sensitivity for HSP90, mTOR, BCL-2 and MEK inhibitors

Ex vivo drug sensitivity and resistance testing (DSRT) of AML patient samples has been suggested to predict therapy response *in vivo*.^{58,59} We investigated the *ex vivo* drug sensitivity of a subset of patient samples in the cohort (18/32) correlated to the intracellular signaling profiles detected by mass cytometry (Fig. 6a). Fresh bone marrow or peripheral blood from diagnostic samples were screened by a drug sensitivity assay consisting of 349 drugs, using CellTiter-Glo as readout to assess cell viability. selective drug sensitivity score (sDSS) was calculated by subtracting the mean DSS of the respective drugs of control samples from five healthy donors (four BM and one PB) from each individual DSS scores. Notably, we had ten patients with sDSS data in 24h-p-ERK1/2 low group and eight patients in 24h-p-ERK1/2 high group.

We focused on the 10 drugs with highest sDSS score for each patient (Supplementary Fig. 13). The sDSS range among the top 10 drugs was from 2.8 to 35.6, with a median of sDSS of 12.3 per patient. Among the most frequent compounds with highest efficacy were HSP90-inhibitors ($n=12$), BCL-2-inhibitors ($n=11$), mTOR-inhibitors ($n=10$) and topoisomerase-inhibitors ($n=8$). We then investigated if there was a difference in which drugs the patients were more sensitive towards in the 24h-p-ERK1/2 low and high group. The most frequent drug targets among patients in the 24h-p-ERK1/2 low group were HSP90 ($n=5$), BCL-2 ($n=5$) and microtubule ($n=5$) inhibitors (Fig. 6b). In the high group the most frequent targets were HSP90 ($n=7$), BCL-2 ($n=6$) and mTOR ($n=6$) (Fig. 6c). The eight patients in the 24h-p-ERK1/2 high group had a higher median sDSS (13.6) than the ten patients in the 24h-p-ERK1/2 low group (11.2). In the high group seven out of eight patients were responsive to HSP90 inhibitors, exemplifying similarities in sensitivity to top targeted drugs. Interestingly, four out of eight (50%) patients in high group had MEK inhibitors among their top 10 targets. Notably, the patient with the highest p-ERK1/2 at 24 hours was very sensitive to MEK inhibitors and had MEK inhibitors as the top three most efficient drugs. For the remaining five patients with the highest p-ERK1/2 level at 24 hours measured by mass cytometry we did not have sDSS data. In the 24h-p-ERK1/2 low group, two patients (20%) had MEK inhibitors among their top targets. One of them, P6, had an NRAS mutation and was as expected very sensitive to MEK inhibitors. Two additional patients that were not included in the main analysis of this study, P35 and one relapsed AML patient, P37, both had NRAS mutations and MEK inhibitors among their top 10 drugs (Supplementary Fig. 14a) Especially the relapsed patient P37, with two MEK inhibitors ranking as the most efficient drugs. RAS mutated AML patients have been shown by others to be sensitive towards MEK inhibitors, and there have been several clinical trials for MEK inhibitor treatment in RAS positive AML.^{60,61} The other patient in the 24h-p-ERK1/2 low group that had a MEK inhibitor among its top 10 drugs was P30. This patient had the highest pre-treatment p-ERK1/2 value in MC9 of all patients in our cohort. Our data suggests that not only patients with RAS mutations might benefit from MEK inhibitors, but also those with high p-ERK1/2 levels at diagnosis or 24-hours.

Figure 6

Figure 6. Drug sensitivity and resistance testing (DSRT)

a, Heatmap of the 90th percentile arcsinh transformed p-ERK1/2 in M9 mass cytometry data. The high and low groups were defined by the 24-hour p-ERK1/2 median value. Patient numbers are color coded by 5-year survival (blue=alive, orange=deceased). Remission at day 17 post -treatment or before cycle two are annotated to the right. The blue circles indicate the 18 patients that was analyzed by *ex vivo* drug sensitivity screening. Diagnostic cytogenetics of inv(16), del(7q), FLT3-ITD and mutations detected by NGS are shown to the right. Mutations are grouped by gene function, colors are shown in the top panel and color code shown to the upper right. Grey squares annotate detected mutations, white squares if the mutation was not detected. b,c *Ex vivo* drug sensitivity of 18/32 patients of fresh diagnostic BM or PB samples were screened using a drug sensitivity assay consisting of 349 drugs, using CellTiter-Glo as readout to assess cell viability. The 10 drugs with the highest selective DSS (sDSS) for each patient was selected and the most common drug targets among the top 10 drugs in low and high group was summarized. The top targets among the patients in low and high group are show in the bar graphs; for example, seven patients in high group had HSP90 inhibitors among their top 10 targets. Only targets that was present in two patients or more are shown in the graphs. The circle maps below the x-axis shown which patient that had each target among its top 10 drugs. MEK inhibitors are annotated in yellow. b, Top targets among patients in low group. c, top targets among patients in high group.

DISCUSSION

The therapy responses of functional signaling at 24 h predicted survival more precisely than risk stratification by bone marrow tumor load on day 17 and the ELN 2017 genetic risk stratification. Thus, *in vivo* functional cellular responses may provide close to real-time outcome prediction in contrast to tumor load measurements, which need longer observation time.

Cancer response evaluation is based on measurement of change in tumor load determined clinically, by advanced imaging, by cytometry or histopathology and by quantitative genetics.⁶² In AML, early evaluation of peripheral blast cell clearance by flow cytometry within the first three days of chemotherapy has been shown to predict CR, relapse risk and survival.⁶³ Assessment of tumor clearance is refined in determination of measurable residual disease (MRD), established as a robust marker for optimal response in leukemia⁶⁴ and in solid cancer through emergence of liquid biopsy and PET imaging.⁶² The time it takes to obtain a minimal tumor state may be a challenge for optimal therapy, e.g., after two month long cycles of chemotherapy in AML,⁶⁴ with risk for tumor regrowth and development of therapy resistance. Measurement of functional cellular responses to therapy may be an alternative and time saving approach to response evaluation. All cancer therapeutics target cellular proteins directly, from the more selective ATP antagonistic tyrosine kinase inhibitors (TKI) to the multi-targeting antibiotics of anthracyclines. We examined protein phospho-signaling in peripheral blood of AML before and shortly after start of therapy, using signaling pathways as sensors for cell fate early after start of therapy. Our data suggest that functional phospho-signaling profiles can save precious time through early response evaluation of aggressive tumors.

Biologically relevant intracellular signal systems are affected *in vivo* early after start of treatment.^{12,21,65,66} In this study we explored a selection of 15 intracellular targets, covering three major pathways important for myeloid leukemogenesis; the JAK/STAT, RAF/MEK/ERK (MAPK) and PI3K/AKT/mTOR pathways. These signaling systems are tightly connected to regulation of hematopoiesis and are also affected by mutations in more than 50% of the AML cases.¹⁰ AML can be stratified based on these signal responses to *in vitro* stimulation with relevant cytokines and growth factors that regulate these signaling systems, into a potentiated response cluster and a non-potentiated cluster.²⁰ Analogous lines can be drawn to our study, where the chemotherapy acts as an *in vivo* stimulus (or stressor) of the signaling networks.

667 We therefore hypothesized that temporal dynamics of signaling may be explored in therapy
668 response evaluation. Using a data driven machine learning framework, we found that the 24
669 h cross sectional analysis resulted in the highest significance in risk stratification. We
670 examined the predictive impact of the various measurements manually and confirmed that
671 24 h was the most accurate time point for risk stratification. A possible explanation for the
672 higher significance at 24 h may be in the intense cellular stress response caused by the
673 combination of anthracycline and cytarabine. The 24 h chemotherapy treatment may cause
674 an optimal state for decoding the intracellular signaling into precise information about
675 response. A wide range of cellular stress response systems are activated, and most of the
676 leukemic cell death and removal start beyond 24 h.⁶⁷ This is confirmed by our cell profiling,
677 which indicates that the peripheral blood cell populations are stationary at 4 and 24 h,
678 (Supplementary Fig.3) and optimal for examination of intracellular signaling perturbations
679 that reflect therapy induced cell fate. We hypothesize that phospho-MAPK need to decrease
680 under a threshold to secure a cell fate that translate into response.

681 Functional analyses of cancer cells may improve the genetic diagnostic approach in risk
682 stratification. AML has some of the most robust genetic risk stratification systems in cancer,
683 based on a diagnostic sample and developed over decades. However, the prognostic
684 algorithms do not cover a significant proportion of patients with normal cytogenetics and
685 non-detectable mutations by NGS in a satisfactory way. At least 30% of the “good risk” AML
686 patients experience relapse and leukemia related death.⁶⁸ An increasing number of genetic
687 alterations have been included and are incorporated in revisions of the risk stratification. This
688 approach has two possible limitations. First, the analytical process of the stratification will
689 take days to weeks and is most useful for adjustment of consolidation therapy. Second, with
690 higher sensitivity of genetic diagnostics an increasing number of private genetic aberrations
691 will be discovered: genetic alterations with currently unknown impact in risk assessment. The
692 phenotype of therapy response is defined by a plethora of genetic and epigenetic
693 constitution. Multivariate single cell signaling before and early after start of therapy reflect
694 the genetic and epigenetic script that define the therapy response more accurately. Our data
695 of drug sensitivity and resistance testing confirms that inhibitors of MAPK pathway may be
696 useful (Figure 6). To fully utilize the knowledge from genomics we need to achieve a deeper
697 biological understanding of the connection between cancer phenotype and genotype. Real-

time functional response evaluation may be fundamental in deciding the best target for therapeutic intervention among the myriad of genetic lesions.¹⁸

Clonal heterogeneity may challenge the accuracy of functional response evaluation.⁶⁹ The single cell immune and signaling profiling provided here indicate clonal heterogeneity in 29/32 patients (Fig. 1b) In the unsupervised analysis of cells from patients receiving induction therapy, the bulk of AML cells returned accurate prognostic information based on p-ERK1/2(T202/Y204) and p-p38 (T180/Y182), and validation by simple manual two-dimensional analyses returned a more significant prognostication compared to the computed stratification (Fig. 3c). By selecting a low number of metaclusters capable of distinguishing blast cells from normal cells we remove noise of immunophenotypic heterogeneity and allow the analysis to focus on intracellular signaling. The clonal heterogeneity in *de novo* AML with high blood cell counts and kilograms of tumor may comprise mutational repertoires that include resistance to most available therapeutics. Emergence of new clones in a proliferative phase of relapse is also likely. This would support a single cell diagnostic approach, also for follow up, that monitor and visualize the clonal heterogeneity in AML. However, the accuracy of the single cell signaling profiling used in this study is likely incomplete in detecting rare but devastating clones, e.g., TP53 mutated subclones that predispose to treatment failure and relapse. Specific probes that detect high risk aberrancies like mutated TP53 mutations may help to solve this problem of detecting rare clones.

The possible impact of functional signaling analysis in prediction of survival need to be examined in future studies. The number of patients included in this study is limited in contrast to the studies that have established genetics in risk stratification and MRD in response evaluation.^{68,10,64,70} However, the patients are carefully selected AML patients receiving standard “7+3” induction therapy,^{71,72} and validation by manual clustering, proteomics and gene expression was performed on the data set and confirmed the observation of high versus low p-ERK1/2 groups (Fig. 3,4,5). Thus, we suggest that functional signaling analysis early after start of therapy may be an important diagnostic tool in improving therapy precision in chemotherapy of AML patients.

MATERIAL AND METHODS

Patient samples

Patients were consecutively included in this observational study, and samples were collected between 2014-2016 at Haukeland University Hospital, Bergen, Norway, and Oslo University Hospital, Rikshospitalet, Norway. The study was performed in accordance with the Declaration of Helsinki, and all samples, including samples from healthy donors, were collected following written informed consent. All patients and healthy donors were above 16 years of age. The biobank and the clinical protocols were approved by the ethical committee at the University of Bergen (Ethical approval REK Vest 2012/2245, 2012/2247 and the Regional Committee for Medical Research Ethics South-East Norway (REK 2015/2012), the Norwegian Medicines Agency and The Data Inspectorate.

The patients are numbered consecutively after their sampling date. The healthy donors are numbered after the barcode-pool (1-7) they were included in. The cohort consist of 32 AML patients (30 *de novo* and two secondary AML) that received standard “7+3” induction therapy according to the HOVON / SAKK 102 and 132 trials,^{71,72} consisting of a 30 min infusion of anthracycline; daunorubicin (60mg/m²) or idarubicin (10-12 mg/m²) for three days in combination with 24 h infusion of cytarabine (Ara-C) for seven days. Routine diagnostic workup of flowcytometry and genetic biomarkers investigated by G-banding, RT-PCR, FISH and fragment analysis of FLT3 and NPM1 was performed on all patients. 18 of these 32 patients were included in the HOVON 132 clinical trial (EudraCT Number: 2013-002843-26) and were randomized to receive either standard “7+3” (Eight patients), or “7+3” with the addition of per-oral treatment of lenalidomide at days 1-21 in cycle I and cycle II (ten patients). Additionally, we analyzed samples from 13 patients who were not included in the data analysis, as they did not receive standard induction therapy for AML, which was the main criteria for inclusion in the data analysis. These patients included two AML patients on a dose-reduced “7+3” treatment schedule (P33 and P34), three AML patients who received amsacrine 150mg/m², cytarabine 200mg/m² and etoposide 110mg/m² for five days (MAE) (P35-P37), one AML who was a secondary AML and previously allo-SCT (P38), one patient receiving palliative treatment (P39) and six patients (P40-45) with other diagnosis; three B-ALL patients treated according to The Hammersmith and NOPHO-ALL-2008 protocols (EudraCT Number: 2008-003235-20) one myelodysplastic syndrome (MDS) and two promyelocytic leukemia patients (APL) patients An overview of the selection process for the

32 AML patients included in the Cox lasso regression analysis is shown in Supplementary Figure 1.

All patient samples in this study were peripheral blood (PB) samples, collected at time of diagnosis, and 4 - and 24 hours after start of therapy. Five patients were only sampled at two timepoints (pre-treatment and 24 hours). The two patients receiving palliative treatment (P39 and P45) were only sampled at the time of diagnosis. Furthermore, four PB and three bone marrow (BM) samples from healthy volunteers were included in our analyses as healthy reference samples. Additionally, PB from seven healthy donors were used as control for batch effect standardization.

Sample preparation for mass cytometry

To minimize potential *ex vivo* changes in intracellular signaling networks, sample processing was performed within 20 minutes of sampling. White blood cells were fixed, and erythrocytes lysed using Lyse/Fix buffer (BD Phosphoflow) following manufacturer's instructions. Fixed samples were frozen in saline at -80°C for shipping and long-term storage. Samples were barcoded using the Cell-ID™ 20 Plex- Pd Barcoding Kit (Fluidigm), following manufacturer's instructions. The 20 barcoded samples were then pooled, resulting in a total of seven barcodepools. To minimize batch effects, all sequential samples collected from the same patient were contained to the same barcode. Each barcode also included one unique healthy donor sample, either bone marrow (BM, barcodes 3-5) or PB (barcodes 1, 2, 6 and 7). Additionally, an identical reference sample composed of pooled PB from seven healthy donors was aliquoted into all barcodes. This reference sample was used for standardization of batch effects (i.e. staining variability) between the seven barcodepools, as described below.

The samples were stained with an antibody panel consisting of 21 surface markers and 15 intracellular markers (Supplementary table 2). Surface markers known to be expressed by healthy cells, and frequently expressed by AML blast cells were included.⁷³ The selection of markers was based on EuroFlow panels applied for AML diagnostics,¹ and a study performed by Levine *et al.* that identifying markers highly informative in characterizing pediatric AML patients.^{14,73} Samples were stained according to the MaxPar Phospho-Protein Staining Protocol (PRD016 v3). Acquisition of samples was done using a Helios mass cytometer (Fluidigm) at the Flow Cytometry Core Facility, University of Bergen, Norway.

Mass cytometry data pre-processing and analysis

After acquisition, the collected data was normalized to EQ bead standard⁷⁴. Cells were gated by DNA-Ir191 versus event length, followed by exclusion of cell doublets through stringent gating using the two DNA stain channels (Ir191/Ir193). Files were debarcoded using a single cell debarcode algorithm (<https://github.com/nolanlab/single-cell-debarcoder>). All data were arcsinh-transformed (cofactor 5). To limit the possibility for spillover between antibodies channels the data was compensated according to the CATALYST (Cytometry Data analysis tools) pipeline.

(<https://bioconductor.org/packages/release/bioc/vignettes/CATALYST/inst/doc/preprocessing.html>).⁷⁵ The study was performed before the compensation method was available, and we therefore used beads created in a later experiment for compensation. The beads were stained with other antibodies conjugated to the same metal isotopes. We assume the difference in metal abundance between the two experiments are negligible, and that the potential difference in metal abundance between the two experiments will be outweighed by the advantage of compensating the data.

Subsequently, based on the reference samples, all samples were standardized across the seven barcodepools using quantile normalization. Barcodepool 7 was selected as normalization standard as it had the best separation of populations and the least background staining when median intensity of all surface markers was compared between barcodes. Standardization was performed following the CytoNorm approach, but without clustering, in 101 quantiles.⁷⁶ In short, we calculated 0-100 percent quantiles for reference sample in each barcode. For each barcode, we then calculated the piecewise linear function whose value at the *i*-th quantile is the *i*-th quantile of the reference barcode. Subsequently, the piecewise linear functions were applied to all non-reference samples. The differences in survival outcome based on p-ERK1/2 at 24h and ratio (24h/0h) between unnormalized, uncompensated and normalized, compensated data are shown in Supplementary figure 15. Normalized and unnormalized data without clustering are compared in Supplementary figure 16. The entire pipeline with clustering was also performed on the normalized, uncompensated data and the result was p-ERK1/2 at 24 hours in MC9 ($p=0.00133$) at patient 2-year survival.

To identify the immuno-phenotypically defined cell subsets across samples, the single cell data was clustered using FlowSOM.²³ We used a setup with a 5x5 grid for clustering and consensus meta-clustering with 10 metalusters (MCs). Nineteen of the 21 surface markers were used for clustering (AXL, CD117 (c-Kit), CD123 (IL-3R), CD14, CD16, CD20, CD3, CD33, CD34, CD38, CD4, CD45, CD56 (NCAM), CD64, CD66b, CD7, CD8a, CD90 (Thy-1) and HLA-DR). CD25 (IL-2R) and CD11b (Mac-1) were excluded as clustering channels due to large variability in staining between barcodes, not correctable by standardization. Clustering and meta-clustering was performed on all cells in the pre-treatment patient samples (baseline) and the healthy donors. We subsequently assigned each cell on post-treatment samples to the closest cluster center. Clustering of data was performed using the FlowSOM package in R (FlowSOM version 2.0.0, R version 4.1.0 (2021-05-18)).

For survival analyses, we applied a Cox Lasso regression model with automatic feature selection and nested leave-one-out cross-validation to determine the regularization parameter. Feature input in the model included, for each MC, the measured dual count (arcsinh transformed, 90th percentile) of the intracellular markers cCaspase3, CyclinB1, p-4E-BP1(T37/T46), p-AKT(S473), p-AXL(Y779), p-CREB(S133), p-ERK1/2(T202/Y204), p-Histone3(S28), p-NF-kB p65(S529), p-p38(T180/Y182), p-Rb(S807/S811), p-S6RP(S235/S236), p-STAT1(Y701), p-STAT3(Y750) and p-STAT5(Y684). Additionally, we included the change in the level of each feature from baseline to 24 hours (delta 24h), size of the 10 MCs, age and sex as features in the model. Survival of the individual patients was followed until five years post induction chemotherapy. For each selected feature we performed time-to-event analyses in a regularized Lasso Cox proportional hazards model.⁷⁷ We split the resulting features that were significantly associated with survival at the median and plotted the survival curves for patients with high and low values of each feature. We used the R package glmnet version 4.1-2.

Our analysis was initially focused on the 32 AML patients who received standard «7+3» induction therapy. However, 45 patients were analyzed by mass cytometry in this study. The 13 patients initially excluded had either received other treatments or had another diagnosis (APL, B-ALL and MDS) (Supplementary Fig.1). Two of the initially excluded patients P33 and P34 received a dose-reduced “7+3” induction therapy. When these two patients were added to the FlowSOM clustering, with the same metric as before, no change in the clusters’ immunophenotype was found, but some minor changes in the metacluster distribution was

observed (data not shown). When we re-calculated the LASSO analysis with these two patients included, we found that p-ERK1/2 ($p=0.0019$, HR 1.25) and p-p38 ($p=0.0020$, HR 2.07) in MC 9 at 24 hours was significant at predicting two-year survival. When analyzing five-year survival with P33 and P34 included, significance was identified in p-ERK1/2 at 24h in myeloid blasts (MC9) ($p=0.0011$, HR 1.29) and p-Rb at 24 hours in the B-cells (MC5) ($p=0.003$, HR1.67).

Drug sensitivity and resistance testing (DSRT)

Cells derived from 23 of the patients included in our study were screened for sensitivity to 349 drugs (anti-cancer compound library #L3000 from Selleck Chemicals) using DSRT.¹⁷ 18 of these patients received standard “7+3” induction therapy and were included in the LASSO analysis.

The 349 compounds from anti-cancer Selleck Chemical library were pre-aliquoted at five concentrations, increasing in 10-fold steps from 1 nM to 10 μ M, in 384-well plates using an Echo Liquid Handler. Each plate also contained eight positive (benzethonium chloride, BzCl) and eight negative (DMSO only) controls. Fresh diagnostic (pre-treatment) BM or PB was lymphoprepmed (15 BM and 8 PB), and cells were seeded directly into the plates containing the drugs at a concentration of 10,000 cells/well and incubated for 72 hours. Finally, cell viability was assessed by CellTiter-Glo Luminescent viability assay. All drugs were also tested against samples from five healthy donors (four BM and one PB).

All patient samples were normalized to the median of the positive (BzCl) and negative (DMSO) controls per plate and presented as selective Drug Sensitivity Score (sDSS), calculated using the online tool Breeze (https://breeze.fimm.fi/28489_mc43oty0nzywmcaxnje3nzwgnzaw/index.php#)

Patient sample preparation for RNA sequencing, proteomics, and next generation sequencing (NGS).

PB samples at pre-treatment, 4 - and 24 hours after start of treatment for proteomics, RNA sequencing and targeted DNA-seq was sampled in BC Vacutainer CPT™ Mononuclear cell preparation tubes with sodium heparin. Mononuclear cells were prepared by density gradient separation (Lymphoprep, Axis-shield). For RNA sequencing, approximately 1×10^6 cells were dissolved in TRIzol™ Reagent (Thermo Fisher) and stored at -80°C until analysis. For proteomics, approximately 5×10^6 cells were precipitated in 7% trichloroacetic acid (TCA) and

stored at -80°C until analysis. For targeted DNA-seq, approximately 5x10⁶ cells were pelleted, supernatant removed and stored as a dry pellet at -80°C until analysis. The gradient separation and sample preparation for storage was done immediately after sampling and the same standardized method for sample preparation was used at both collection sites in Oslo and Bergen.

TruSight myeloid panel sequencing

54 frequently mutated genes in myeloid malignancies were sequenced using Illumina TruSight Myeloid Gene Panel with Miseq where v3 reagent kit was used (all from Illumina, San Diego, CA, USA).^{78,79} Amplicon sequencing library was prepared from 50 ng DNA according to the manufacturer's instructions except for normalization, which was done manually by measuring each sample on Qubit and thereafter dilute each sample to a concentration of 4nM. 16 samples were sequenced each time and the total DNA input on the flow cell was 15 picomolar. Secondary analysis was done using MiSeqReporter version 2.6.2.3 (Illumina) mapping to the human genome reference hg19 and variant calling using Somatic variant caller 3.5.2.1. Sequence alignment of selected variants was manually examined with the Integrative Genomics Viewer (IGV)⁸⁰. Annotation was done by snpSIFT and snpEFF v 4.1. Variant filtering and interpretation were done in Coremine Oncology (PubGene AS, Oslo, Norway) with >1% minor allele frequency in the 1000 genomes data were presumed to be germline and removed from further interpretations. Synonymous substitutions and intronic variants that were not at the splice site and variants that were interpreted either as benign or most likely benign were not included. The variant allele frequency (VAF) was calculated for each mutation as number of variant reads divided by total reads. Cut-off for reported variants was for VAF >8% with read depth of 100. Only variants interpreted as pathogenic, probably pathogenic and variants of unknown significance were reported. The nomenclature is according to HGSV (<http://varnomen.hgvs.org>). FLT3 and NPM1 was also investigated by fragment analysis and CEBPA by sanger sequencing.

Super-SILAC proteomics sample preparation and data analysis

TCA-precipitated samples for proteomics were washed three times in cold water-saturated ether prior to proteomics sample preparation.²¹ Samples was then lysed in 4% sodium dodecyl

922 sulphate (SDS)/0.1 M Tris-HCl (pH 7.6), heated at 95°C for 7 min under mild shaking and
923 sonicated (3 cycles at 30% amplitude for 30 seconds with 1 min rest between each cycle). Cell
924 debris was removed by centrifugation (13.000rpm/14.000xg for 10 min) and the protein
925 concentration was determined with the Pierce BCA Protein Assay kit (Thermo Fisher
926 Scientific). Samples were kept at -80°C until analysis. For labelled proteomics, 20 mg of each
927 patient sample was mixed with 10 mg of a super-SILAC mix composed of five heterogenous
928 AML cell lines labelled with Arg6 and Lys8 isotopes.⁸¹ The patient cell lysate was further
929 processed by filter-aided sample preparation (FASP) and small-scale proteome fractionation
930 as described by Hernandez-Valladares *et al.*⁸²
931 Abundance and peptide identification were conducted on an Q Exactive HF Orbitrap mass
932 spectrometer coupled to an Ultimate 3000 Rapid Separation LC system (Thermo scientific).⁸³
933 LC-MS/MS raw files were processed with MaxQuant software version 1.5.2.8.⁸⁴ The spectra
934 were searched against the concatenated forward and reversed-decoy Swiss-Prot Homo
935 sapiens database (version 2018_02), using the Andromeda search engine.⁸⁵ The Perseus
936 1.6.1.1 platform was used for data analysis and statistics. MaxQuant-normalized SILAC ratios
937 were inverted, log2 transformed and normalized again, using width adjustment.

938 939 **RNAseq data analysis**

940 36 samples were sequenced in Hiseq 4000 with 75 X2 chemistry. The demultiplexed fastq files
941 were aligned to human genome GRCh38.p13 using HISAT2 aligner.⁸⁶ The aligned bam files
942 were quantified as whole read count matrix using tool FeatureCount .⁸⁷ The generated read
943 count was normalized using DESEQ2 R package. Variance stabilizing transformation was
944 adopted. P-values below 0.05 were considered as significant The Perseus 1.6.1.1 platform was
945 used for RNAseq data analysis and statistics.

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AUTHOR CONTRIBUTIONS

The study was designed by BST and BTG, and the manuscript composed primarily by BST, MH and BTG. Patient samples were collected and processed by BST, OHEF and THD in Bergen and PAD, DST and LP in Oslo. BST, MH, OHEF and SEG performed mass cytometry experiments. NB wrote R scripts for mass cytometry data analyses. BST, MH and BTG analyzed mass cytometry data. AS and BST performed analysis of RNA sequencing data. BST performed proteomics wet lab and EB and BST analyzed data. PAD, LP and DST performed drug sensitivity and resistance testing, which was supervised by JME. RH and PB contributed with molecular diagnostics and analyses of sequencing data. YF was responsible for collection of biologic samples and patient treatment. All authors contributed to manuscript preparation.

CONFLICTS OF INTEREST

The authors declare no relevant conflicts of interest.

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