

## ORIGINAL ARTICLE

# Specific cellular signal-transduction responses to *in vivo* combination therapy with ATRA, valproic acid and theophylline in acute myeloid leukemia

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**Acute myeloid leukemia (AML) frequently comprises mutations in genes that cause perturbation in intracellular signaling pathways, thereby altering normal responses to growth factors and cytokines. Such oncogenic cellular signal transduction may be therapeutic if targeted directly or through epigenetic regulation. We treated 24 selected elderly AML patients with all-trans retinoic acid for 2 days before adding theophylline and the histone deacetylase inhibitor valproic acid (ClinicalTrials.gov NCT00175812; EudraCT no. 2004-001663-22), and sampled 11 patients for peripheral blood at day 0, 2 and 7 for single-cell analysis of basal level and signal-transduction responses to relevant myeloid growth factors (granulocyte-colony-stimulating factor, granulocyte/macrophage-colony-stimulating factor, interleukin-3, Flt3L, stem cell factor, erythropoietin, CXCL-12) on 10 signaling molecules (CREB, STAT1/3/5, p38, Erk1/2, Akt, c-Cbl, ZAP70/Syk and rpS6). Pretreatment analysis by unsupervised clustering and principal component analysis divided the patients into three distinguishable signaling clusters (non-potentiated, potentiated basal and potentiated signaling). Signal-transduction pathways were modulated during therapy and patients moved between the clusters. Patients with multiple leukemic clones demonstrated distinct stimulation responses and therapy-induced modulation. Individual signaling profiles together with clinical and hematological information may be used to early identify AML patients in whom epigenetic and signal-transduction targeted therapy is beneficial.**

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**Keywords:** acute myeloid leukemia; ATRA; valproic acid; theophylline; phosphorylation; cell signaling

## Introduction

Acute myelogenous leukemia (AML) is an aggressive malignant disease characterized by blocked differentiation of the myeloid cell lineage.<sup>1</sup> Extensive studies on AML cytology, cytogenetics and molecular analyses have confirmed a disease heterogeneity that is reflected in the clinical outcome of these patients.<sup>2</sup> Elderly AML patients and patients with serious comorbidities are frequently ineligible for intensive chemotherapy because of unacceptable high risk of treatment-related mortality. We and others have demonstrated that non-genotoxic therapy, including all-trans retinoic acid (ATRA), valproic acid (VPA) and theophylline, has clinical effects in a subset of patients.<sup>3–6</sup>

Responders are mainly characterized by stabilization of their disease with partial restoration of peripheral platelet counts.

Retinoic acids receptors have a key role in myeloid differentiation through the modulation of expression of target genes. ATRA has limited clinical effect in non-acute promyelocytic leukemia AML when used alone, but may enhance the effect of chemotherapy if combined with granulocyte colony-stimulating factor (G-CSF).<sup>7</sup> Along with others, we have demonstrated distinct biological effects in AML cells of patients treated with ATRA only<sup>8,9</sup> (for review see Stapnes *et al.*<sup>10</sup>). To enhance the differentiation effect of ATRA, we combined treatment with the histone deacetylase inhibitor VPA.<sup>10,11</sup> Inhibition of histone deacetylase activity results in increased protein acetylation and acetylation of histones may cause epigenetic gene modulation<sup>4</sup> that induce differentiation<sup>12</sup> and apoptosis in AML cells. The third agent used was theophylline, a phosphodiesterase inhibitor that increases intracellular cyclic AMP, and which is reported to be of benefit in chronic lymphatic leukemia and acute promyelocytic leukemia.<sup>13</sup>

Along with others, we have recognized vigorous modulation of proteins and gene expression early after start of conventional chemotherapy,<sup>14,15</sup> but there is limited knowledge on signal transduction modulation in AML blasts in patients undergoing therapy. Particularly, with new therapy directed against signal-transduction enzymes, we would presume that monitoring signaling cascades in cancer cells may provide important information about therapy response.<sup>16</sup>

Analyzing signaling response and basal phosphorylation, our 11 patients predominantly split into three signaling clusters (SCs) before start of therapy. Patients with clinical response dominate in the cluster with low basal phosphorylation and high response to stimuli. The basal phosphorylation level of signaling molecules was lower in patients characterized as responders to the therapy compared with the group of non-responders to the therapy. Furthermore, AML patients with multiple AML blast subsets demonstrated the modulation of multiple clones during therapy. We conclude that the clinical responders of this epigenetic and signal-transduction-directed therapy demonstrate a signal-transduction signature that may help to indentify responders and sensitize non-responders.

## Materials and methods

### *Patient treatment and collection of patient cells*

Eleven patients (Table 1) were selected on the basis of white blood cell counts above  $13 \times 10^9/l$ , from the total clinical trial of 24 patients. Detailed patient protocol is referred to Rynningen *et al.*,<sup>6</sup> but in short the patients received ATRA

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(Vesanoid; Roche, Basel, Switzerland) 22.5 mg/m<sup>2</sup> twice a day for 14 days. At day 2 VPA (Orfiril, Destin GmbH, Hamburg, Germany) and theophylline (Theo-Dur; Astra Zeneca, London, UK) were administered. The doses of VPA were gradually increased until side effects appeared, and doses of theophylline were adjusted on the basis of serum levels up to a therapeutic level of 50 to 100 µM.

Leukemic peripheral blood mononuclear cells were collected and cryopreserved, as described in the study by Rynningen *et al.*<sup>6</sup> and Abrahamsen *et al.*,<sup>17</sup> before treatment (day 0), 3 days after treatment with ATRA (day 2) and 5 days after (day 7) treatment with theophylline and VPA.

The clinical effects of the treatment were evaluated with regard to (i) normal peripheral blood cell counts and (ii) number/differentiation/function of circulating AML cells.<sup>6</sup> Patients were divided into responders and non-responders (Table 1).

**Stimulation of AML blasts**

Preparation and stimulation of AML blast is previously described in the study by Irish *et al.* In short, thawed cryopreserved and rested cells were either unstimulated or stimulated with a final concentration of G-CSF (20 ng/ml), granulocyte/macrophage-CSF (20 ng/ml), interleukin-3 (20 ng/ml), Flt3L (50 ng/ml), stem

**Table 1** Clinical and biological characteristics of AML patients

Patient	Age/sex	Previous malignant disease, present disease status	FAB classification	Membrane molecule expression <sup>a</sup> (%)				Mutations karyotype <sup>b</sup>	Clinical response to treatment <sup>c</sup>	Survival in days <sup>d</sup>
				CD11b	CD15	CD71	CD34			
				Day 0/2/7						
1	68/F	MDS, 1st relapse	M1	<b>9/13/19</b>	2/3/4	<b>17/30/34</b>	5/12/6	FLT3-ITD, CEBPA	A	105
4	60/M	2nd relapse	M4	<b>32/40/53</b>	<b>10/17/21</b>	8/4/3	<b>66/67/89</b>	FLT3-ITD	—	7
5	50/F	Li-Fraumeni	M1	3/5/5	2/2/2	<b>19/18/29</b>	77/82/82	Complex karyotype	—	34
9	74/M	<i>De novo</i>	M0	9/10/10	0/0/0	41/43/42	67/74/59	None	—	112
12 <sup>e</sup>	61/F	MDS, 1st relapse	M1	82/79/77	51/60/51	2/3/3	44/43/47	Complex karyotype	A	644
13	80/F	<i>De novo</i>	M2	<b>29/25/53</b>	2/1/0	<b>41/36/66</b>	48/45/50	FLT3 ITD, NPM1	B	8
14	78/M	MDS	M1	5/9/12	3/6/5	11/8/11	<b>58/78/43</b>	ND	A+B	55
15	86/M	<i>De novo</i>	M4	ND	ND	ND	<b>42/ND/23</b>	ND	A+B	58
16	67/M	MDS, 1st relapse	ND	<b>40/14/72</b>	51/28/47	8/0/2	<b>32/48/48</b>	FLT3-ITD, CEBPA	B	23
21	70/F	Chemotherapy	M4	<b>44/41/25</b>	37/39/30	2/1/1	<b>11/16/26</b>	NPM1	B	15
23	68/M	Myelofibrosis	M1	<b>8/6/24</b>	2/6/11	23/18/17	80/84/86	FLT3-TDK	B	70

Abbreviations: AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ND, not determined.

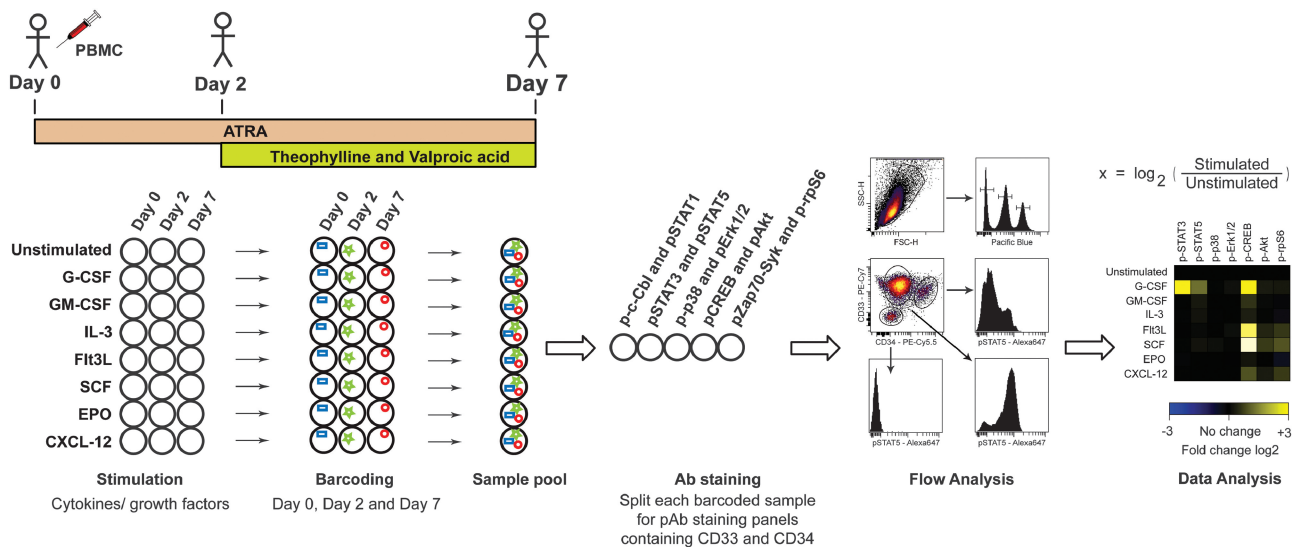
<sup>a</sup>The results are presented as the percentage of positive cells. Differences exceeding 10% after 7 days are shown in bold.

<sup>b</sup>Karyotype is normal if not other indicated.

<sup>c</sup>Responses were classified as either peripheral blood normal cell counts (A) or decreased circulating AML blasts (B).

<sup>d</sup>Survival from start of treatment.

<sup>e</sup>Patients showing hematological improvement according to the MDS criteria.



**Figure 1** Phosphoprotein profiling and bioinformatics analysis of AML cells from patients undergoing combination treatment with all-trans retinoic acid (ATRA), valproic acid and theophylline. Peripheral blood mononuclear cells were obtained from the patients at three time points: before treatment at day 0, after *all-trans* retinoic acid monotherapy at day 2 and after additional therapy with theophylline and valproic acid at day 7. At the given sample points, the cells were stimulated with growth factors as indicated, before barcoding and pooling into one sample. Each sample was then divided into five antibody staining panels before being acquired by flow cytometry and analyzed by open-source Cytobank software developed for this purpose ([www.cytobank.org](http://www.cytobank.org)).

cell factor (50 ng/ml), erythropoietin (10 U/ml) and CXCL-12 (20 ng/ml; all from PeproTech, Rocky Hill, NJ, USA).

### Intracellular staining

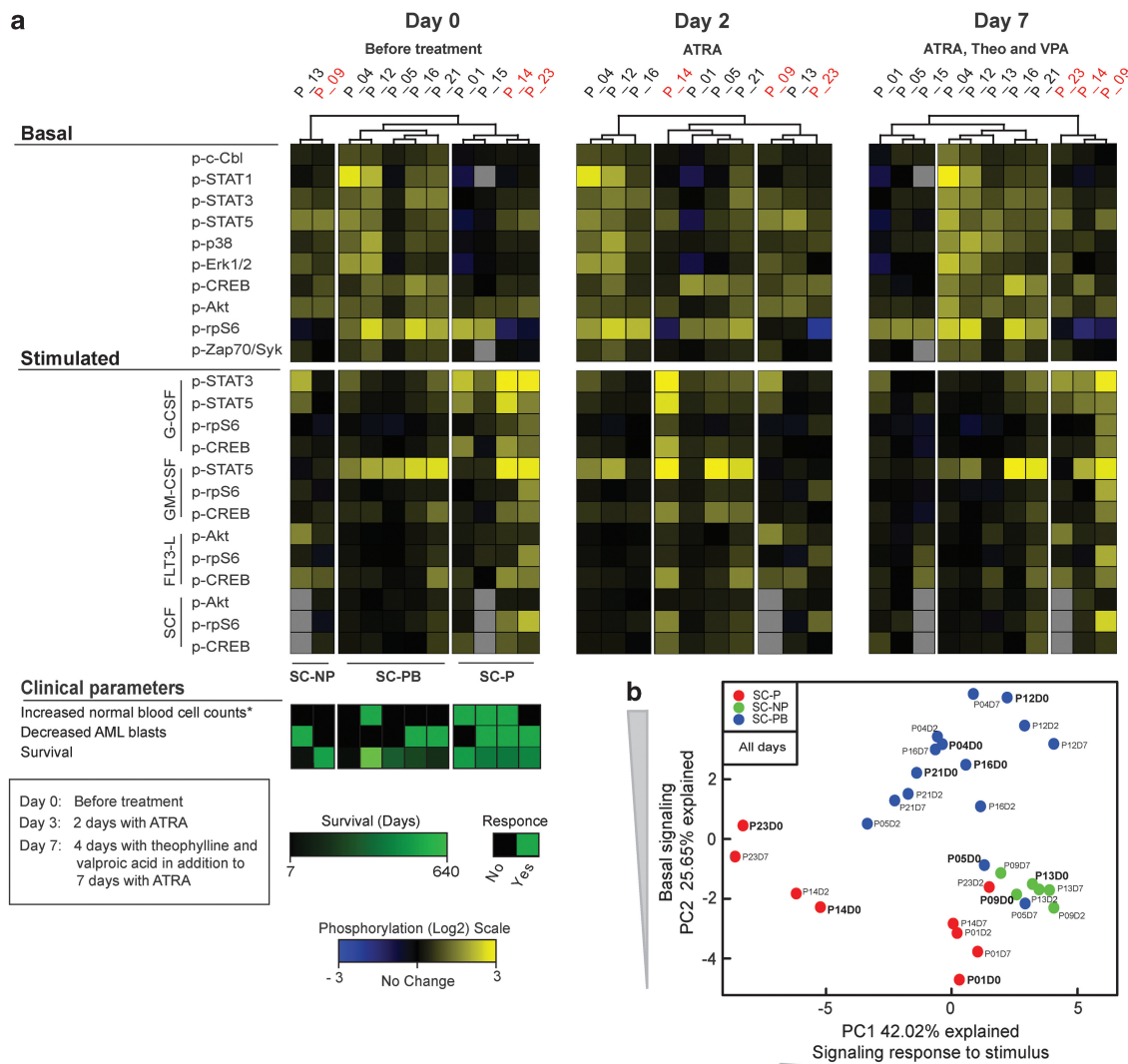
We fluorescent cell barcoded the three sample points using Pacific Blue amine-reactive fluorescent dye (Molecular Probe, Eugene, OR, USA).<sup>18</sup>

Paraformaldehyde-fixed, methanol-permeabilized and fluorescent barcoded cells were equally split and stained with five different antibody panels. All panels contained the same four surface antibodies: CD11b(ICRF44) Alexa Flour 700, CD15(HI98) PE, CD33(P67.6) PE-Cy7 and CD34(581) PE-Cy5.5 (all from BD Pharmingen, San Diego, CA, USA). Two phospho antibodies were added to each panel with the respective direct-conjugated dye Alexa Flour 488 and Alexa Flour 647; c-Cbl(pY700) and STAT1 (pY701), STAT3(pY705) and STAT5(pY694), p38(pT180/pY182) and ERK2(pT202/pY204), pCREB(pS133) and Akt/PKB(pS473),

Zap70/Syk(pY319/pY352) and ribosomal protein S6 (rpS6)(pS240/pS244) (all from BD Pharmingen, except Akt/PKB and rpS6 from Cell Signal(CS), Danvers, MA, USA). rpS6 was conjugated with Alexa Flour 647 protein labeling kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### Data collection and analysis

Phospho-flow analyses were performed on a four-laser FACSria flow cytometer (BD Biosciences). To analyze only live cells, a strict gate was set in forward- and side-scatter dot plot including the live population. A log<sub>2</sub> change from the median of measured phospho antibody median fluorescent differences between a stimulated sample and its unstimulated/basal phosphorylation state was calculated. The phosphorylation scale bar is set to maximum range for the data set analyzed. Analysis of signaling profiles and identification of leukemia cell subsets was carried out using open-source Cytobank software



**Figure 2** Cluster analyses of basal signaling state and signaling response. The biosignature is presented in a heat map as fold change (log<sub>2</sub>). Stimulated samples are calculated by dividing by the corresponding unstimulated sample. Basal phosphorylation is relative to phosphorylation in the average lymphocyte population. (a) Hierarchical clustering using a Pearson correlation-based distance gave three signaling clusters at sample time day 0 and given a signaling cluster (SC) nomenclature based on their signaling appearance P—potentiated, NP—non-potentiated and PB—potentiated basal. Positive clinical parameters represent an increase in normal cell counts or >50% reduction in peripheral blasts. \*Normal cell count response was used as response criteria of the clinical protocol. (b) PCA analysis of phospho-specific signaling data for all three sample points reflects the patients signaling profiles. The x axis represents stimuli-activated phosphorylation. Y axis represents basal phosphorylation.

developed for this purpose (www.cytobank.org), and FlowJo (TreeStar, Ashland, OR, USA). The hierarchical clustering used a Pearson correlation-based distance measure using TM4: a free, open-source system for microarray data management and analysis.<sup>19</sup> Principal component analysis (PCA) was carried out using the prcomp module in the open-source script-based statistics environment R (http://www.r-project.org/).

## Results

Heterogeneity among AML patients reflects differences in the constitutive expression of signaling proteins in leukemic cells. To find characteristic signaling profiles for each patient on the basis of phosphorylation state of signaling proteins (CREB, STAT1/3/5, p38, Erk1/2, Akt, c-Cbl, ZAP70/Syk and rpS6), a set of relevant ligands (G-CSF, GM-CSF, interleukin-3, Flt3L, stem cell factor, erythropoietin and CXCL-12) was used to stimulate the pretreated patient cells. Each individual AML patient seems to have a unique phospho protein signaling profile probably reflecting the genetic and epigenetic abnormalities in the leukemic cells (Figure 1, Supplementary Figure S1). To carry out the process as identically as possible for the three time points, we barcoded the cell samples allowing staining procedure for all three samples in the same tube and thereby avoiding variation between sample time points. Basal phosphorylation level in resting leukemic cells (90% or more of cells) was analyzed by dividing the phosphorylation value by the average basal phosphorylation in lymphocytes (10% or less of leukocyte in samples) in the same sample. The basal phosphorylation level *in vivo* are previously reported by others not to be altered because of freezing and thawing procedure.<sup>20</sup>

### Patients cluster in three groups by Pearson correlation and PCA

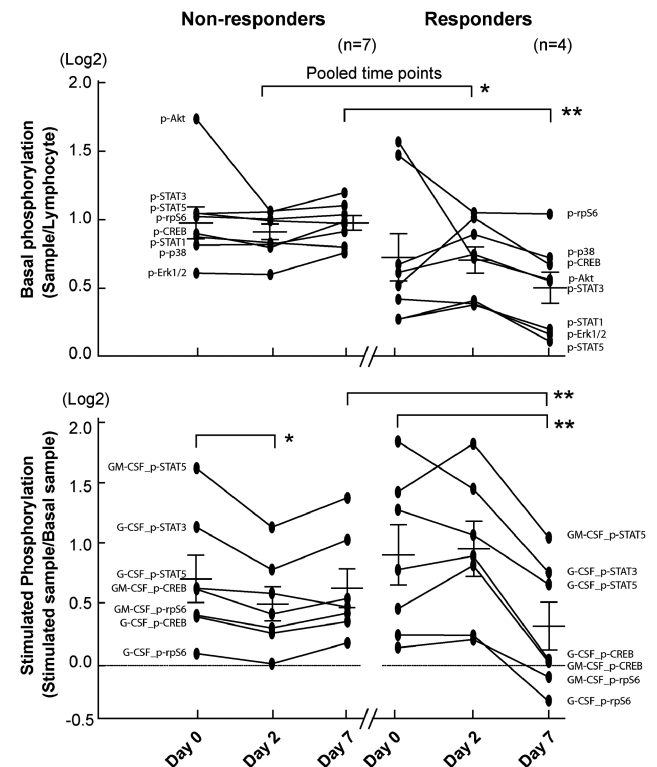
The phosphorylation data were analyzed by hierarchical clustering using Pearson correlation-based distance (Figure 2a) or a PCA (Figure 2b) to examine if patient signaling profile analysis could indicate patient treatment outcome. An extraction of all phosphorylation data was performed by variance analysis (Supplementary Figure S2A) to rule out data of no significance for hierarchical clustering. We found significant variance in nine of the basal phosphorylated data and in 21 combinations of stimulated signaling nodes having a change in variance above 0.1. From this extraction we selected all basal readouts and 12 of the most prominent response readouts representing responsive signaling nodes. Before the treatment was initiated (day 0), the hierarchical clustering analysis reveals three distinct SCs. The clusters can be divided into a non-potentiated signaling cluster (SC-NP) in which there is a relatively low basal phosphorylation and hardly any signaling response. The second falls into a potentiated basal cluster (SC-PB) that has a uniformly higher basal phosphorylation and some signaling response. The third cluster is a potentiated signaling cluster (SC-P) of four patients, all with a high signaling response to stimulation but relatively low basal activity. The time of survival was on average 2.4 months (range 55–105 days,  $n=4$ ) in SC-P, versus 4.8 months (range 7–644 days,  $n=5$ ) in cluster SC-PB and 6.7 months (range 8–392 days,  $n=2$ ).

*Change in patient clustering reflects treatment response*  
ATRA treatment, and the combination treatment, gave an altered effect on the signaling in several patients. It is either seen in

different basal phosphorylation or as a change in response to stimuli. Calculation of a hierarchical clustering using a Pearson correlation-based distance measure on the phospho-flow data after 2 days with ATRA led to a new patient clustering. At day 7, yet another re-grouping of patients formed three clusters. As one can see, several patients still group together but some patients have moved to another group.

### PCA of patient data from 0, 2 and 7 days of treatment

PCA of all signaling responses in all patients from all three time points (Figure 2b) returned similar patient subgroups as the hierarchical clustering on day 0 (Figure 2a). However, the use of all the data together in combination with the two-dimensional clustering achieved with the PCA method also reveals that patient no. 5 (SC-PB) is rather close to the SC-NP cluster. The first principal component ( $x$  axis), and hence the greatest variance in the data (42.02%), correlates the highest to lowest signaling response to stimulus. The second principal component correlates the lowest to highest signaling level in the basal state (describing a further 25.65% of the variance). It also shows that data from each patient on different days has a strong tendency to cluster close together, thus demonstrating the stability of the cancerous states of the AML blasts in each patient. With some variation among the clusters, data from the same patient also clusters more closely than the data from other patients belonging to the same cluster group, thus also demonstrating patient heterogeneity. The very different



**Figure 3** Comparing signaling response between responders and non-responders. The phosphorylation status for patients representing the two groups, responders ( $n=4$ ) and non-responders ( $n=7$ ) to the therapy, was analyzed together to calculate differences. A Student's two-tailed  $t$ -test was performed to calculate significance ( $*P<0.05$ ,  $**P<0.001$ ). Basal and stimulated phosphorylations are presented as  $\log_2$ . The standard error of the mean (s.e.m.) is given for all signals for each time point.

responses to treatment in patients belonging to the same clustering groups on day 0, causing the re-grouping seen in the hierarchical clustering patterns for days 2 and 7, indicate that the response is worthy of further studies in larger patient groups.

### Basal and stimulated phosphorylation differs in patients responding to therapy compared with the non-responding patients

We have previously proposed a significantly increased survival in patients responding with improvement in peripheral blood values (Figure 2a).<sup>6</sup> Looking at the average signaling pattern in these groups we found several differences (Figure 3). Basal phosphorylation was lower in the responding group than in the non-responding group as a whole ( $*P < 0.05$ ), and phosphorylation at day 7 ( $**P < 0.001$ ) was also significantly reduced. In response to stimuli, signaling was reduced in both groups but was significant in the non-responding group at day 2 and in the responding group at day 7 compared with day 0. Four of seven phospho-nodes were upregulated in responders to ATRA treatment, whereas the opposite response was seen in all seven in the non-responding group.

### Modulation of cellular sub-population

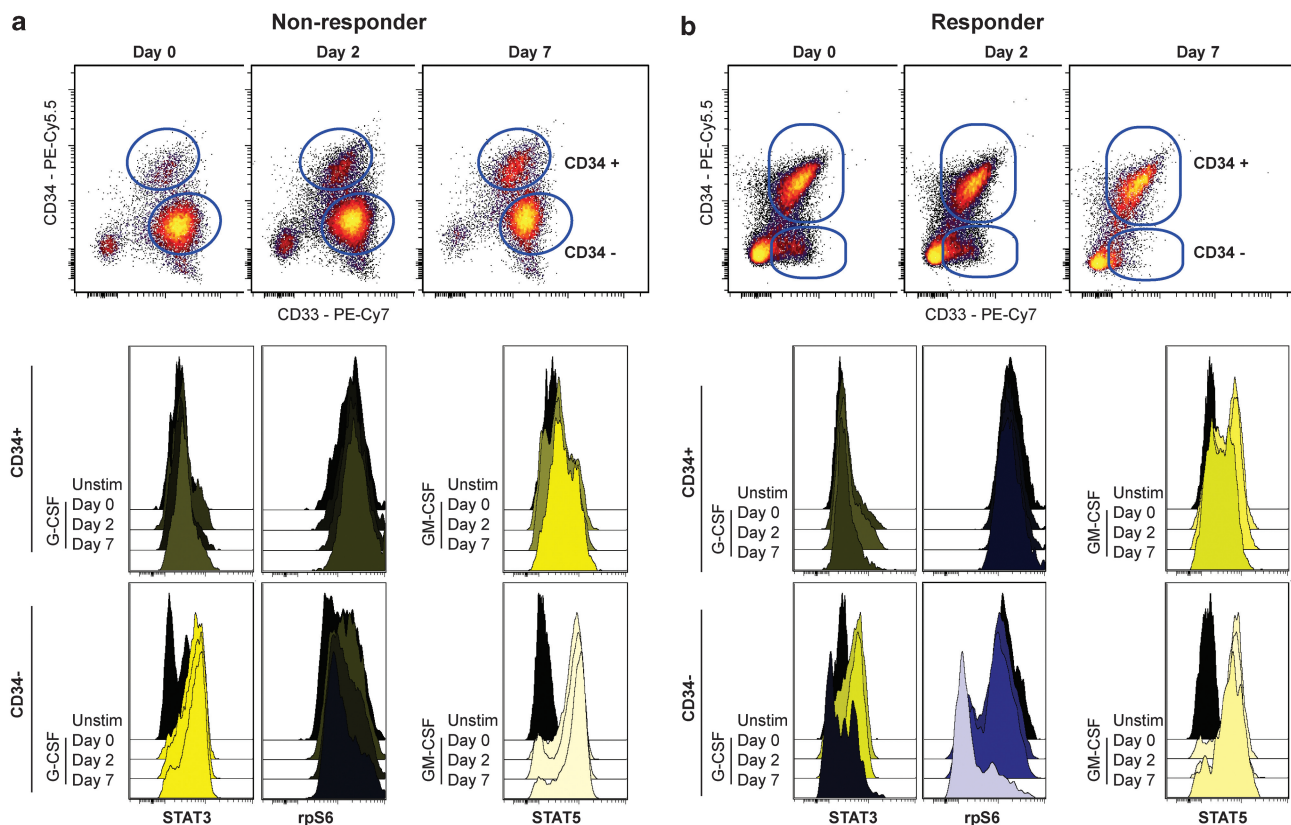
Surface markers revealed sub-populations of leukemic cells in the majority of the patients analyzed. After 2 and 7 days of treatment there are changes in population size. Figure 4 shows two patients representing a non-responder and a responder.

The patient representing the non-responding group (P-21) has an increase in the CD34+ population by 10.9, 16.2 and 25.9% for the respective sample days 0, 2 and 7. The changes in signaling for this patient are representative for the majority of the non-responding group. For the responding group all patients responded in a similar manner with regard to signaling, and represented patient (P-12; Figure 4b). A small reduction in response to stimuli was seen in the main CD34+ population at day 7. The biggest reduction in stimulus response was observed in the smaller CD34- population in which it was remarkably reduced at day 7. For this patient there were small changes in the CD34+ population (44.0, 42.7 and 47.0%).

### Discussion

Along with others, we have shown that combination therapy with ATRA and VPA is of clinical benefit in a subset of AML patients.<sup>11</sup> In this study, we analyzed signal-transduction nodes in leukemic cells from AML patients treated with ATRA, VPA and theophylline (Figure 1). Through phospho-flow cytometry combined with bioinformatics analysis, we searched for determinants for prediction of long-term responders.

Even if the major effects of ATRA and VPA epigenetic therapy develop after at least a week of therapy, immediate effects related to signal transduction have been reported for VPA, theophylline and ATRA. Within hours, VPA is shown to activate the PI3K/AKT pathway,<sup>21</sup> depolarize mitochondria with depletion of mitochondrial cytochrome *c*<sup>22,23</sup> and increase



**Figure 4** Signaling in leukemic cell sub-population. Phospho-signaling analysis of cellular sub-populations in non-responder (a) and responder (b) patients. Tight gates on sub-population or gating high and low CD marker expression was analyzed for G-CSF-stimulated p-STAT3 and p-rpS6, and GM-CSF-stimulated p-STAT5. There was no change in unstimulated samples for days 0, 2 and 7, except for a small decreased p-rpS6 in CD34- for the non-responder (a).

cellular redox stress.<sup>24</sup> This indicates that several pathways and mechanisms influence the signaling profiles that we observe in the patient's cells analyzed in this study (Figure 2, Supplementary Figure S1), probably resulting in a new steady state signaling response reflecting the combined therapy as depicted at day 2 and 7 (Figures 2a and b).

Both PCA and non-supervised hierarchical clustering analysis of basal and stimulated AML cells revealed three groups of patients with different signaling patterns (Figure 2a, Supplementary Figure S2B). The three clusters were similar to response clusters previously reported in a set of 30 AML patients,<sup>25</sup> in which the patients in the SC-P were overrepresented with poor prognostic markers and the worst outcome. Surprisingly, the opposite was seen in the cluster (SC-P; Figure 2) of the combination therapy, where all four patients indicated beneficial response in clinical parameters. Both our previous study and the data shown in this study are limited with respect to the number of patients. In our previous study, prognosis was related to conventional chemotherapy. The patients in this study were treated with compounds that predominantly altered signal transduction, and we may speculate whether such a non-genotoxic therapeutic approach may be more feasible in this SC-P group of advanced AML.

The SC-P cluster signal response indicated a high signaling response to G-CSF and GM-CSF, and a relatively low basal phosphorylation. It is reported that G-CSF treatment in combination with ATRA can sensitize myeloid cells to differentiation,<sup>26,27</sup> and may represent a possible mechanism of therapy response in the SC-P group in our study. For some patients dramatic changes were observed either in basal phosphorylation or in stimulation response after 3 days of monotherapy with ATRA, and resulted in reorganization of the cluster pattern (Figure 2a), indicating that ATRA monotherapy has altered signaling profile at least in some patients. We observed altered signaling response after 3 days of ATRA monotherapy (Figures 2a and 3). This change in clustering is consistent with biological response in AML cells of all patients,<sup>6</sup> further emphasized in analysis of average signal modulation (Figure 3).

On the basis of improvement of peripheral blood counts, we may discriminate between responder and non-responder patients,<sup>6</sup> and non-responders demonstrated limited therapy-induced changes in signaling and basal phosphorylation (Figure 2). High basal phosphorylation may reflect a high cellular activation state with limited possibility to enhance the phosphorylation by stimulation. In contrast, responder patients demonstrated a lower basal phosphorylation corresponding to a stronger response to stimulation with growth factors. The responding patients demonstrated attenuation of basal phosphorylation level after ATRA monotherapy, which is further attenuated by combination therapy for 7 days (Figure 3).

Furthermore, we examined the leukemic subpopulations by CD34/CD33 surface markers, and found that in patients with multiple leukemic cell populations (8 of 11 patients) therapy altered distribution of population and changed the signaling response (Figure 4). For patient P-12, the CD34<sup>+</sup> population was decreasing, and the cell population demonstrated weaker phosphorylation of rpS6 and STAT3 in response to G-CSF. This may explain the actual signal modulation that is related to therapy response, and that AML patients with multiple leukemic cell populations demonstrate heterogeneous signaling responses.

This study indicates the feasibility of single-cell analysis in early therapy response evaluation of cancer cells in combination therapy, and should be followed by more vigorous testing in larger clinical trials.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

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