Death Receptor Expression on Blasts in AML Is Associated with Unfavorable Prognosis

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Abstract. *Background: Tumor necrosis factor (TNF) receptor family members play a key role in the regulation of biological functions such as differentiation, proliferation and apoptosis of various cell types. Materials and Methods: We studied co-expression profiles of death receptors from the TNF family [TNF-related apoptosis-inducing ligand receptor (TRAILR) 1 to 3, TNF receptor 1 (TNFR1) and FAS receptor (FAS)] on peripheral blood blasts from 46 patients with acute myeloid leukemia (AML) at first diagnosis by flow cytometry and correlated the obtained specific fluorescence indices (SFI) with morphological, cytogenetic and clinical parameters. Results: We found that the expression of TRAILR2 and R3 was significantly increased in unfavorable risk groups, according to the National Comprehensive Cancer Network. Additionally, cut-off analyses for TRAILR2 and TNFR1 showed significantly shorter overall survival, earlier disease onset, higher proportions of cases with unfavorable prognosis and higher probability of relapse when SFIs were above the established cut-off. Conclusion: We demonstrate that high co-expression of death receptors on blasts is an independent predictor of poor prognosis in AML.*

Acute myeloid leukemia (AML) is a clonal disorder of the hematopoetic system characterized by an accumulation of neoplastic myeloid cells and impaired production of normal hematopoetic cells due to dysregulated apoptosis (1).

Expression profiles of death receptors (DRs), which are normally associated with the regulation of apoptosis, might serve to allocate AML cases to prognostic risk groups, to predict cells' response to chemotherapy or to develop targeted-therapies addressing those receptors. DRs, such as members of the tumor necrosis factor (TNF) receptor family such as TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1, -2 and -3), Tumor necrosis factor receptor 1 (TNFR1), and FAS receptor (FAS) (CD95) are known to be associated with apoptosis.

Upon activation by corresponding ligands, TNFR family members trigger several signaling pathways that control and regulate biological functions such as apoptosis, differentiation, and proliferation, as well as immune regulation *e.g.* by impairing natural killer (NK) cell tumor surveillance (2).

In leukemia cells, induction of apoptosis is mediated by TRAILR1 and -2 (3). In chronic lymphocytic leukemia and mantle cell lymphoma, only TRAILR1 is known to be responsible for induction of cell death (3). TRAILR3 is a decoy receptor lacking a functional death domain. Despite this fact recent data show a strong association of TRAILR3 expression with shortened overall survival of patients with AML, probably due to impaired apoptosis of blasts in these cases (4).

TNFR1, the receptor for the immune-modulating cytokine TNF, released from monocytes/macrophages and T-lymphocytes, has been shown to influence proliferation of blasts in AML *in vitro* by modulating responses to hematopoetic growth factors such as interleukin-3, granulocyte-macrophage colony-stimulating factor, granulocyte-colony-stimulating factor, macrophage-colony-stimulating factor and stem cell factor (5-7). Prognostic relevance of DRs in patients with AML remains unclear.

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Table I. *Expression profiles and main functions of death receptors in normal hematopoiesis.*

Receptor/cluster of differentiation (CD)	Expression profile	Function
Tumor necrosis factor -related apoptosis-inducing ligand receptor 1/2 (CD261/CD262)	Constitutively expressed on lymphoid cells such as T-cells and natural killer cells.	Induction of apoptosis
Tumor necrosis factor -related apoptosis-inducing ligand receptor 3 (CD 263)	Constitutively expressed on neutrophilic granulocytes	Regulation of apoptosis; inhibition of cell death through competitive binding activity
FAS (CD95)	Constitutively expressed on activated B- and T-cells, hepatocytes, ovarian and epithelial cells.	Induction of apoptosis; induction of proliferation of T-lymphocytes and fibroblasts; generation of proinflammatory cytokines and chemokines; stimulation of maturation of dendritic cells
Tumor necrosis factor receptor 1 (CD120a)	Constitutively expressed on monocytes, lymphocytes, granulocytes.	Mediation of cytotoxicity; signaling of fibroblast growth, endothelial activation/adhesion, antiviral activity; proliferation of thymocytes and peripheral T-cells; secretion of granulocyte-macrophage colony-stimulating factor by T-cells

FAS is the best characterized DR. Expressed in activated lymphocytes, in a variety of tissues of lymphoid and non-lymphoid origin, as well as in tumor cells, it is mainly known for inducing cell death (8). Additionally alterations affecting the FAS-related pathway lead to poor clinical outcome of AML due to a lack of pro-apoptotic regulation (9, 10). The most important functions of DRs are summarized in Table I.

The aim of the present study was to evaluate the association of co-expression of TRAILR1-3, TNFR1 and FAS on AML blasts at first diagnosis with different AML sub-types and risk groups and to combine these findings with clinical data in order to evaluate their prognostic and clinical significance.

Materials and Methods

Patients' characteristics and sample collection. Peripheral blood samples from 46 patients with AML at first diagnosis were collected after patients gave their written informed consent in accordance with the Helsinki protocol and the local Ethics Committee (13/2007V). Diagnosis and classification of AML cases was based on morphology and cytochemistry of bone marrow according to the French-American-British (FAB) classification (11). All samples were obtained before treatment.

Ten patients presented with undifferentiated leukemia (M0: n=2, M1: n=8), 20 with immature granulocytic leukemia (M2: n=17, M3: n=3) and 14 with monocytic leukemia (M4: n=7, M5: n=7); one patient had erythroleukemia (M6) and one patient was not categorized by FAB.

In 40 patients primary, and in five patients secondary AML was diagnosed. One patient was not categorized. The median age was 57.4 years (range: 18-85 years). The male:female ratio was 1:0.43. Patients' characteristics are shown in Table II.

Twenty-six patients received an anthracycline-based induction therapy (idarubicin or daunarubicin), the remaining patients (n=20) received other approved therapies or supportive therapy. Response to chemotherapy was defined for patients achieving complete remission (CR) 25 to 35 days after the start of first induction chemotherapy. Patients were then further treated by second induction therapy or bone marrow transplantation. CR was defined as cases of normocellular bone marrow, containing <5% blasts, and when neutrophilic granulocytes in peripheral blood (PB) had recovered to 1,500/μl and platelets to 100,000/μl according to the Cancer and Leukemia Groups criteria (12). Relapse was diagnosed in cases with more than 5% bone marrow blasts or when leukemic infiltration occurred at any other site. The investigated samples contained on average 77.7% (range: 21%-99%) leukemia blasts in the whole mononuclear fraction of PB.

Cytogenetics. Cytogenetic analyses were performed by standard methods at the University of Ulm, Muenchner Leukaemie Labor GmbH or Dr. Eberhard & Partner Dortmund, Germany. Samples were stratified according to the European Leukemia Network [ELN; (13)] and National Comprehensive Cancer Network [NCCN; (14)] guidelines. According to the NCCN, abnormalities with favorable risk were defined by the presence of t(8;21), t(15;17), or inv/t(16); and those with adverse risk were del/t11q23 [other than t(9;11)], der5/5q, der7/7q aberrations, t(6;9), inv3, t(3;3) aberrations or a complex karyotype (three or more numerical or structural abnormalities). According to ELN criteria, a favorable abnormality was defined by the presence of t(8;21), inv(16), t(16;16), mutation of nucleolar phosphoprotein B23 (NPM1) or of CCAAT/enhancer binding protein-alpha gene (CEBPA). Adverse abnormalities were defined as inv(3), t(3;3), t(6;9), mixed lineage leukemia rearrangement (MLL), -5 or del (5q), -7 or a complex karyotype (three or more numerical or structural abnormalities).

Cell characterization by flow cytometry. Mononuclear cells were isolated from whole PB samples by density gradient centrifugation with Ficoll-Hypaque (Biochrom, Berlin, Germany), then washed and suspended in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany). Cell counts were quantified by

Table II. *Patients' characteristics*.

No.	Age (years)/ gender	AML	Blasts (%)	Risk[1]	WBC ($\times 10^6$/l)	Hb (g/dl)	PLT ($\times 10^6$/l)	Remission[2] (yes/no)	Relapse (yes/no)	SFI				
										FASR	TNFR1	TRAILR1	TRAILR2	TRAILR3
1	69/M	pM2-nm	96	Intermediate	85.5	9.5	146	No	No	n.a.	n.a.	0.9	1.6	1.1
2	68/M	pM0-nm	93	n.a.	42.5	9.8	80	n.a.	No	2.3	3.7	0.9	1.0	1.0
3	72/F	pM1-nm	80	Intermediate	25.8	7.8	50	Yes	No	n.a.	n.a.	1.0	1.4	n.a.
4	74/F	p-M2-nm	78	n.a.	50.6	8.6	29	Yes[2a]	No	n.a.	n.a.	1.0	2.0	n.a.
5	66/M	p-M2-nm	63	Intermediate	65.4	9.5	n.a.	No	No	4.2	1.7	1.0	1.2	1.1
6	74/F	p-M2-nm	83	Intermediate	5.01	6.3	95	Yes	No	n.a.	n.a.	1.1	n.a.	n.a.
7	32/M	pM5-mo	33	Favorable	105.2	6.7	71	Yes	No	n.a.	n.a.	1.1	1.5	n.a.
8	73/M	p-M2-nm	83	Intermediate	33.8	8.1	80	n.a.	No	n.a.	n.a.	1.1	2.2	n.a.
9	79/M	s-M2-nm	99	Adverse	26.0	9.7	36	n.a.	No	n.a.	n.a.	1.1	3.8	n.a.
10	69/M	p-M2-nm	93	Intermediate	110.9	8	27	n.a.	No	n.a.	2.2	1.1	2.8	2.8
11	84/M	p-M2-nm	79	n.a.	115.2	7.8	107	n.a.	No	n.a.	n.a.	1.2	n.a.	n.a.
12	33/M	p-M4-mo	94	Favorable	65.7	12	88	Yes	No	n.a.	n.a.	1.2	1.4	n.a.
13	64/M	s-nm	91	Intermediate	394.2	7.9	189	n.a.	No	n.a.	n.a.	1.2	1.5	n.a.
14	49/M	p-M4-m	82	n.a.	316	7.1	80	n.a.	No	n.a.	n.a.	1.2	2.4	n.a.
15	61/M	s-M2-nm	32	Adverse	38.9	7.9	109	n.a.	No	n.a.	n.a.	1.2	3.3	n.a.
16	86/M	p-M5-m	92	n.a.	179.3	7.4	60	n.a.	No	n.a.	n.a.	1.2	3.4	n.a.
17	64/M	p-M2-nm	82	Intermediate	338.5	8.1	19	n.a.	No	6.7	3.2	1.2	2.2	1.3
18	74/F	p-M4-m	79	n.a.	40.3	9.6	63	n.a.	No	1.5	3.0	1.5	3.0	3.4
19	51M	p-M2-nm	98	Intermediate	5.9	13.3	8	Yes	No	2.9	1.3	1.5	1.5	1.1
20	69/F	p-M0-nm	97	n.a.	22.6	8.1	66	n.a.	No	1.5	4.0	1.7	2.7	26.2
21	76/M	p-M4-m	87	n.a.	28.6	9.9	n.a.	n.a.	No	3.5	4.0	1.8	2.2	1.1
22	70/M	p-M3-nm	82	Favorable	12.8	9.1	73	Yes	No	4.8	4.0	3.0	2.8	2.3
23	47/M	p-M3-nm	51	Favorable	23.1	13.3	25	No	No	n.a.	3.0	3.1	1.7	1.5
24	50/M	p-M1-nm	98	Intermediate	19.7	10.4	55	Yes	No	2.8	2.2	3.2	2.1	1.3
25	20/M	p-M3-nm	94	Favorable	67.6	8.8	29	Yes	No	n.a.	n.a.	3.6	2.3	n.a.
26	66/F	p-M5-m	98	n.a.	140.7	11.8	42	n.a.	No	2.4	17.2	7.3	8.7	7.6
27	81/F	p-M5-m	96	n.a.	48.0	11.4	65	n.a.	No	4.0	21.5	13.7	7.9	3.1
28	64/M	p-M5-m	51	n.a.	162.8	10.2	96	n.a.	No	1.8	11.8	17.2	6.8	5.8
29	74/M	s-M2-nm	46	Favorable	25.5	8.9	23	n.a.	No	18.1	50.9	54.7	n.a.	22.3
30	48/F	p-M1-nm	98	n.a.	56.3	9.4	60	Yes[2a]	Yes	2.5	1.6	1.2	2.1	2.8
31	40/M	p-M5-m	97	Intermediate	4.4	8.3	38	No	No	4.4	1.6	1.8	3.9	1.1
32	40/M	p-M4-m	93	Intermediate	36.5	8.5	220	Yes[2a]	Yes	1.5	2.7	7.6	3.6	2.0
33	54/M	p-M2-nm	96	Intermediate	159.9	10.7	121	Yes[2a]	Yes	1.7	6.4	4.7	2.7	2.4
34	63/F	p-M2-nm	36	Intermediate	83.2	7.8	51	Yes	No	1.7	3.5	1.1	3.2	1.3
35	38/F	s-M2-nm	33	Intermediate	7.9	8.4	25	Yes	No	n.a.	n.a.	1.1	1.4	n.a.
36	66/M	p-M2-nm	85	Favorable	41.0	8.1	9	Yes	Yes	1.5	2.5	1.1	2.5	3.9
37	18M	p-M4-m	66	Intermediate	45.1	8	75	Yes[2a]	Yes	6.3	n.a.	1.7	1.4	1.3
38	51/F	p-M1-nm	n.a.	Intermediate	13.8	8.8	25	Yes[2a]	Yes	n.a.	n.a.	1.4	1.5	n.a.
39	52/M	p-M1-nm	98	Intermediate	153.3	9.2	23	Yes	No	n.a.	n.a.	1.3	2.1	n.a.
40	24/M	p-M5-m	83	Intermediate	145.1	7.6	34	Yes[2a]	Yes	n.a.	n.a.	1.3	1.5	1.1
41	42/M	p-M2-nm	43	Intermediate	91.6	10.6	235	Yes	No	n.a.	n.a.	1.3	1.6	n.a.
42	48/F	n.a.-M1-nm	91	Intermediate	27.6	10	26	Yes	Yes	n.a.	n.a.	1.4	3.2	n.a.
43	55/M	p-M6-nm	21	Adverse	11.1	7.4	84	Yes	No	n.a.	n.a.	7.2	9.2	n.a.
44	66/M	p-M4-m	72	Adverse	61.9	5.8	18	No	No	n.a.	n.a.	1.5	3.9	n.a.
45	39/F	p-M1-nm	60	Intermediate	26.0	9.6	153	No	No	n.a.	n.a.	1.1	2.0	n.a.
46	41/F	p-M1-nm	94	Intermediate	68.7	8.1	55	Yes	No	n.a.	n.a.	1.2	2.7	n.a.

f, Female; M, male; WBC, white blood cells; Hb: hemoglobin; PLT: platelets; pM2, primary acute myeloid leukemia French-American-British classification M2; mo, monocytic; nm, non-monocytic; p, primary leukemia; s, secondary leukemia; [1]according to European leukemia network/ National comprehensive cancer network classification; [2]after first induction therapy (anthracycline-based); [2a]later than after first induction chemotherapy-yes (no) means remission (not) documented; n.a., data not available; SFI, specific fluorescence indices; FASR, FAS receptor; TNFR1, Tumor necrosis factor (TNF) receptor 1; TRAILR1-3, TNF-related apoptosis-inducing ligand receptor 1-3.

Neubauer counting chambers; cells were frozen with standardized procedures and stored in liquid nitrogen until use.

The surface expression of DRs on blasts of patients with AML was determined by flow cytometry. Since fluorochrome-labeled monoclonal antibodies (mAbs) were not available for every given DR and in order to amplify potentially weak fluorescent signals, we applied sequential staining steps (indirect staining): After blocking of unspecific binding sites with human Immunoglobulin G1 (IgG1) (10

μg/ml) in order to avoid non-specific binding of antibodies to Fc receptors, mononuclear cells were incubated with mAbs specific for human TRAILR1 to -3 (Alexis, San Diego, CA, USA), TNFR1 (R&D, Minneapolis, MN, USA) and FAS (kindly provided by Professor Gundram Jung, University of Tuebingen), or their respective isotype control mAbs (10 μg/ml each, all mouse IgG1, except for FAS where mouse IgG2b was used (BD Biosciences, Franklin Lakes, NJ, USA) to control for non-specific binding, followed by incubation with species-specific phycoerythrin conjugates. After a washing step, AML cells were selected by staining with fluorochrome-conjugated mAbs specific for CD33, CD34, or CD117, or a combination of the above mentioned mAbs depending on each individual patient's blast phenotype as determined by immunophenotyping at first diagnosis.

Analyses were performed using a FC500 (Beckmann Coulter, Krefeld, Germany). Specific fluorescence indices (SFI) were calculated by dividing the median fluorescence intensity obtained with specific mAbs by the median fluorescence intensity obtained with their respective isotype control mAbs used in the initial staining step. The threshold for surface positivity was defined as SFI ≥1.5 (2).

Statistical analyses. Data are presented as mean or median±standard deviation or 25%/75% quantiles, as appropriate, and statistical comparisons were performed using the *t*-test, Mann–Whitney test or Fisher´s exact test. The analysis was carried out with JMP® 10.0 statistical software (SAS Institute, Cary, NC, USA).

Significant differences in cases with $p=0.05$-0.1 were considered as tending towards being significant, those with $p<0.05$ as being significant, and those with $p\leq0.005$ as being highly significant.

Relapse-free survival analyses were performed by the Kaplan–Meier method in combination with log-rank tests. Values evaluated by flow cytometry are median SFIs.

In order to evaluate predictive cut-off values, we sub-grouped our cases with DR expression values in ascending order and with corresponding survival times and clinical/diagnostic variables listed. Cut-offs were obvious as a distinctive level at which grouping allowed a separation of cases into those with better and those with worse prognosis, as shown *e.g.* in Kaplan–Meier-analysis. Data are presented in combination with the corresponding clinical data (*e.g.* cytogenetic values, response to therapy, age).

Results

We studied expression profiles of DRs on blasts from patients with AML taken at first diagnosis. In some patients, it was not possible to perform all DR analyses due to limited cell counts or incomplete data sets.

Expression profiles in AML sub-types. AML samples were screened for expression of TRAILR1-R3, TNFR1 and FAS on blasts. Blasts from patients with monocytic leukemia had significantly enhanced expression of TRAILR1. In detail, comparison of monocytic *vs*. non-monocytic sub-types showed a significantly higher expression of TRAILR1 and a tendency for higher expression of TRAILR2 in monocytic sub-types (SFIs of 1.6 *vs*. 1.2, $p=0.02$ and 3.2 *vs*. 2.1, $p=0.09$, respectively; Figure 1a and b). Expression of TRAILR3, TNFR1 and FAS was higher, although not significantly different between monocytic *vs*. non-monocytic sub-types (Figure 1c-e).

Variable expression of DRs in different FAB types. A detailed analysis of expression profiles in FAB sub-types showed high expression of TRAILR3 especially in undifferentiated FAB types (M0), whereas TRAILR1 and -R2 were mainly expressed in M5 and M6 sub-types (Figure 2a, right). Lowest expression for TRAILR1, TRAILR2, TNFR1 and FAS (Figure 2a and c, right) was found in undifferentiated M0 and M1 leukemia, and of TRAILR3 in M3. Sub-types known to be prognostically unfavorable such as FAB M0, M6 (15, 16) *vs*. other FAB sub-types were not significantly different in DR expression (Figure 2a and c, left).

Expression of DRs in cases with secondary AML. Findings of higher expression levels in secondary AML for TRAILR2 (2.2 *vs*. 2.4; $p=0.9$), TRAILR3 (1.5 *vs*. 22.3; $p=0.14$), TNFR1 (3.1 *vs*. 50.9; $p=0.12$) and FAS (2.5 *vs*. 18.1; $p=0.11$) were not significant, probably due to the limited number of cases in the group with secondary compared to primary AML (n=5 *vs*. 38).

Expression of DRs by patient age and sex. The level of TRAILR1 (1.2 *vs*. 1.4; $p=0.09$) tended to be higher in patients aged 60 years or more and expression of TNFR1 (3.85 *vs*. 2.2; $p=0.02$) was significantly higher in such patients compared to patients younger than 60 years. No significant differences were seen however for TRAILR2 (2.75 *vs*. 2.1; $p=0.11$), TRAILR3 (2.8 *vs*. 1.3; $p=0.17$) and FAS (2.4 *vs*. 2.8; $p=0.94$) (Figure 2b and d).

Interestingly male patients presented with significantly lower expression of TRAILR3 compared to female patients (1.3 *vs*. 3.25; $p=0.02$), whereas no significant differences were found for TRAILR1 (1.3 *vs*. 1.2; $p=0.35$), TRAILR2 (2.2 *vs*. 2.7; $p=0.72$), TNFR1 (3 *vs*. 3.75; $p=0.37$) and FAS (3.2 *vs*. 2.05; $p=0.12$).

Prognostic evaluation. Expression levels of DRs were compared by cytogenetic risk groups as scored by ELN and NCCN. TRAILR1 expression was not significantly different in the favorable compared to the adverse-risk (1.35 *vs*. 3.0; $p=0.85$) and favorable compared to intermediate-risk groups (3.0 *vs*. 1.2; $p=0.21$). Significantly higher levels of TRAILR2 were found in the group with adverse-risk compared to the favorable-risk group (3.85 *vs*. 2.0, $p=0.01$; Figure 3a). Additionally, enhanced expression in the prognostically worse group was confirmed by comparison of the adverse with the intermediate-risk group (3.85 *vs*. 2.05; $p=0.004$). Due to a lack of cases with an adverse risk profile, TRAILR3 evaluations were restricted to favorable and intermediate cases. Significantly lower levels of TRAILR3 were found in the intermediate- compared to the favorable-risk group (2.2 *vs*. 3.5, $p=0.02$, Figure 3b). Due to a similar lack of cases with an adverse risk profile, comparisons for expression of TNFR1 and FAS could only be performed between favorable- and intermediate-risk groups. Non-

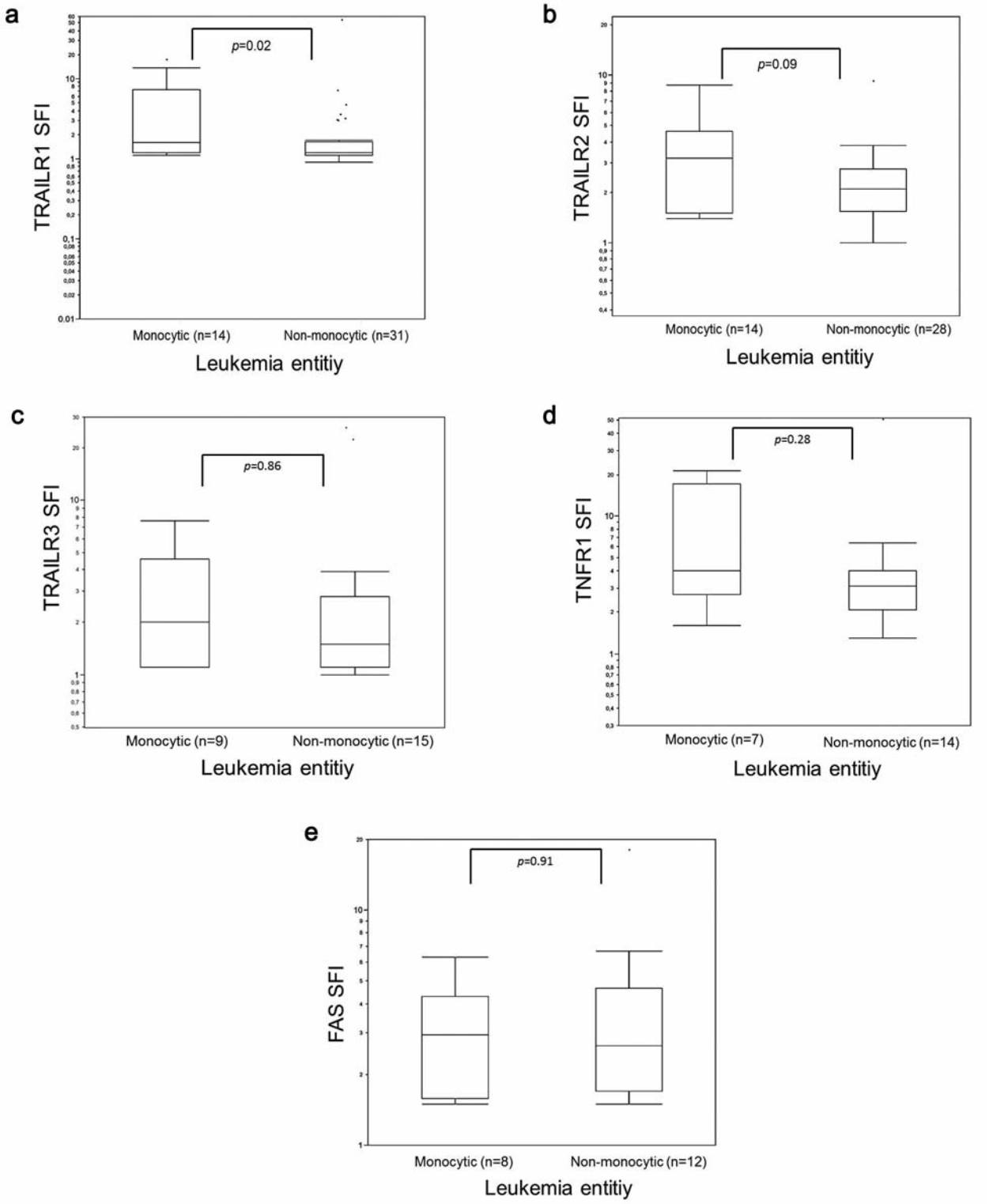

Figure 1. *Expression of death receptors on blasts from patients suffering from monocytic compared to non-monocytic leukemia. Expression of death receptors was studied by flow cytometry and the obtained specific fluorescence indices (SFIs) were calculated compared to those with isotype controls and presented in box-plots showing median, upper and lower quantiles as well as whiskers. Comparison of monocytic vs. non-monocytic sub-types showed higher expression of TNF-related apoptosis-inducing ligand receptor (TRAILR) 1 (a) and TRAILR2 (b) in monocytic sub-types. Expression levels of TRAILR3, Tumor necrosis factor receptor (TNFR) 1 and FAS receptor (FAS) were higher, although not significantly different by subtype of disease (c-e).*

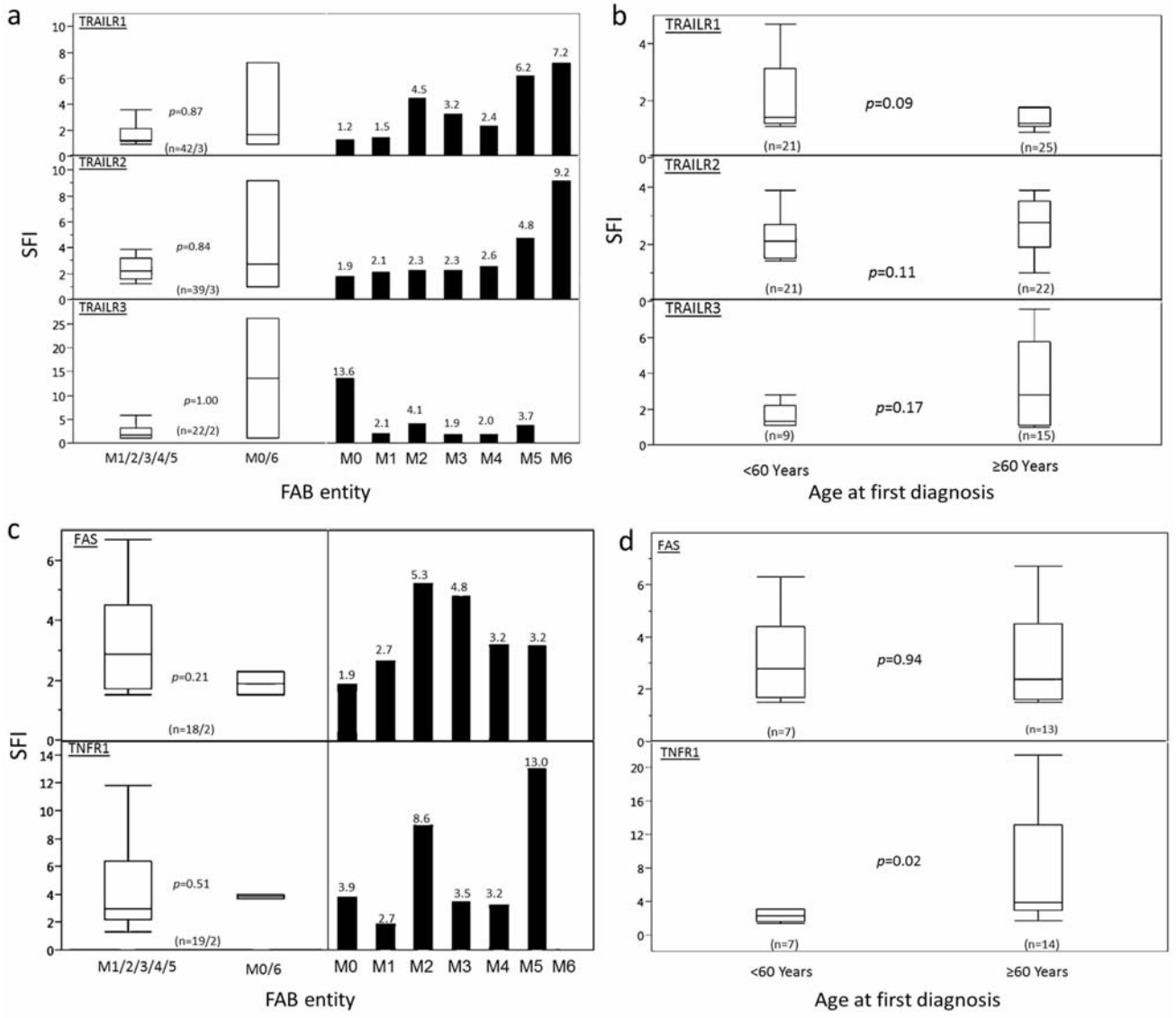

Figure 2. *Expression of death receptors on blasts from patients by French-American-British (FAB) classification (a, c) and age (b, d) groups. Expression of death receptors was studied by flow cytometry and the obtained SFIs were calculated compared to those with isotype controls and presented with box-plots. No significant differences of expressions were found in favorable compared to unfavorable FAB sub-types. DR expression profile according to FAB classification was presented as columns showing the mean distribution (right) (a, c). A tendency for enhanced expression of TRAILR1 was seen in the group of patients aged ≥60 compared to <60 years (b). Significantly enhanced expression was found for TNFR1 (d). FAS showed no significant differences within the groups (d). Data of all box-plots show median, upper and lower quantiles as well as whiskers.*

significantly lower values were found for both TNFR1 (1.3 *vs*. 3.1, p=0.14) and FAS (2.9 *vs*. 4.8; p=0.64) for the favorable- compared to the intermediate-risk group.

Further evaluation comparing response to first anthracycline-based induction chemotherapy showed no differences of DR expressions in non-responders *vs*. responders.

We related expression of DRs with patients' risk of relapse. Therefore, we grouped patients observed for at least 100 days after first diagnosis into those who did and did not experience relapse, restricting time to relapse to 260 days after first diagnosis to ensure association with the evaluated DR expression. Evaluation for TRAILR1 showed a tendency for higher values in the non-relapse group (1.25 *vs*. 1.4, p=0.5). In contrast, there was a tendency for increase in the relapse group for TRAILR2 (2.0 *vs*. 1.85, p=0.8) (one patient had to be excluded from analyses due to inconsistent results) and TNFR1 (4.45 *vs*. 2.2, p=0.3). The same tendencies were seen for FAS (3.55 *vs*. 1.5, p=0.6). Moreover, there was a tendency for lower expression of TRAILR3 (1.3 *vs*. 1.9, p=0.3) in the relapse group.

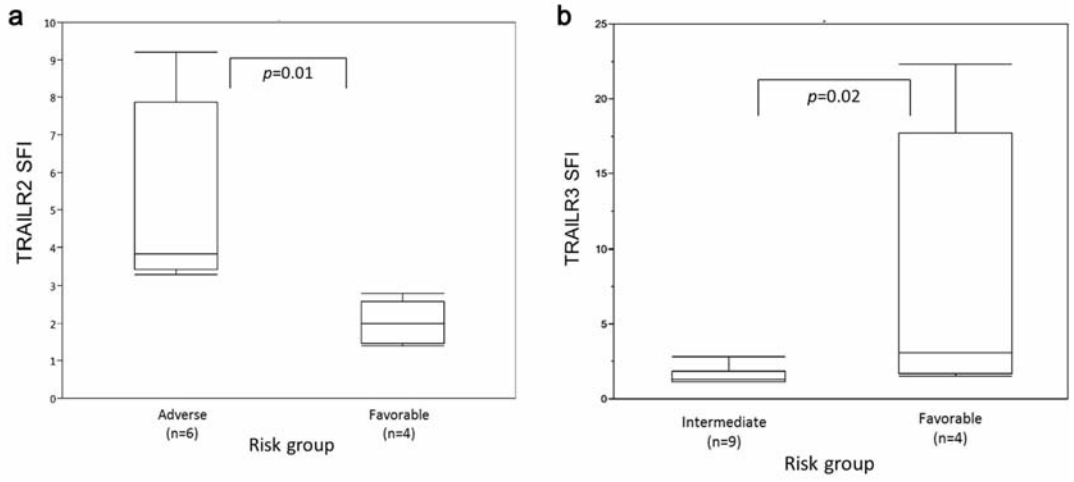

Figure 3. *Expression of death receptors on blasts from patients by National Comprehensive Cancer Network (NCCN)/European Leukemia Network (ELN) risk group. Expression of death receptors was studied by flow cytometry, obtained SFI-values were calculated compared to those with isotype controls and presented with box-plots showing median, upper and lower quantiles as well as whiskers. Significantly enhanced expression of TRAILR2 (a) was found in the adverse-risk group and significantly lower expression of TRAILR3 (b) in the intermediate-risk group vs. the favorable-risk group.*

In order to show statistical relevance of results, we performed calculations with different time periods, setting a limit of 100 days observation time and varied the maximum time to relapse between 200-260 days to ensure statistical impartiality. All results were comparable (data not shown).

Cut-off analyses to predict prognosis. We evaluated cut-off values that allowed separation of patients into those with longer and shorter overall survival and compared data with corresponding clinical and laboratory data. Clinical data and laboratory values for the groups separated by cut-off values are given in Table III, and survival analyses in Figure 4.

No cut-off value for the TRAILR1 expression in correlation with overall survival was found that allowed a separation of cases according to outcome (data not shown).

High expression of TRAILR2 on blasts was found to be associated with the unfavorable prognostic group and poor overall survival. A cut-off SFI for TRAILR2 positivity was found to be 3.2. As seen in Table III, higher proportions of patients with expression higher than this value belonged to the unfavorable AML-subtypes (e.g. higher, age, sAML, M0-M6 Fabtype, adverse NCCN-risk) and were characterized by a higher probability not to respond to anthracycline-based first induction chemotherapy. Moreover, patients with a SFI for expression of TRAILR2 higher than 3.2 on blasts, presented with significantly reduced survival after anthracycline-based first induction therapy (mean survival 24.8 *vs*. 2.0 months, p=0.013; Figure 4a). For the whole patient cohort, no significant differences in survival were apparent according to TRAILR2 expression (mean survival 24.8 *vs*. 5.2 months, p=0.28; Figure 4b).

No cut-off value for TRAILR3 expression was found that allowed for separation of cases into those with better or worse outcome (data not shown).

A cut-off value for TNFR1 positivity was at an SFI of 3.2. As seen in Table III, patients with expression higher than this value had more likelihood of belonging to unfavorable AML sub-types.

Differences in TNFR1 with regard to response rates to anthracycline-based first induction therapy and relapse rates were not significant. Patients with anthracycline-based chemotherapy and TNFR1 expression higher than 3.2 had a significantly reduced survival (mean survival 16.5 *vs*. 0.5 months, p=0.002; Figure 4c). This observation was also confirmed when including all patients regardless of type of therapy (mean survival 33.0 *vs*. 3.3 months, p=0.007; Figure 4d).

No cut-off value for FAS expression in correlation with overall survival was found that allowed separation of cases into those with a better or worse outcome (data not shown).

Discussion

The TNFR family plays significant roles in several functions of homeostasis of cells. Interactions between receptors and their ligands couple them directly to signaling pathways in cells leading to proliferation, differentiation and survival (17). Their potent effects in cellular regulation, immunological defense, apoptosis, inflammation and autoimmunity have rendered them into interesting targets to be studied in the context of AML.

The original contribution of this work is testing of the suitability of AML blast expression of DRs in a screening context for clinical purposes.

Table III. *Distribution of patient sub-types divided into groups by cut-off for tumor necrosis factor (TNF) receptor 1 (TNFR1) and TNF-related apoptosis-inducing ligand receptor 1 (TRAILR2). It was not possible to define cut-offs for the other receptors.*

		Age												
Receptor	Cut-off (SFI)	Mean (years)	Group (≥60/<60), n (%)	Gender (F/M), n (%)	AML (p/s), n (%)	FAB M0,M6 (yes/no), n (%)	mo/nm, n (%)	Blasts PB/BM (%)	WCB ($\times 10^6$/l)	Risk group (fav/adv)[1], n (%)	ABT (no/yes), n (%)	Remission (yes/no)[2], n (%)	Relapse (no/yes)[3], n (%)	
TNFR1	≤3.2	68.4	5/6 (45%/ 55%)	2/9 (18%/ 82%)	11/0 (100%/ 0%)	0/11 (0%/ 100%)	3/8 (27%/ 73%)	78.2/ 73.8	67.5	2/0 (100%/ 0%)	4/7 (36%/ 64%)	3/5 (38%/ 62%)	3/1 (75%/ 25%)	
	>3.2	55.8	9/1 (90%/ 10%)	4/6 (40%/ 60%)	9/1 (90%/ 10%)	2/8 (20%/ 80%)	4/6 (40%/ 60%)	85.2/ 76.7	72.6	2/0 (100%/ 0%)	7/3 (70%/ 30%)	2/1 (67%/ 33%)	1/1 (50%/ 50%)	
p-Value		0.01	0.06	0.36	0.48	0.21	0.7	0.45/0.72	0.88	1.0	0.2	0.55	1	
TRAILR2	≤3.2	54	15/18 (46%/ 54%)	11/22 (33%/ 67%)	30/2 (94%/ 6%)	2/30 (6%/ 94%)	7/26 (21%/ 79%)	79/74	84.1	6/0 (100%/ 0%)	12/21 (36%/ 64%)	14/9 (61%/ 39%)	4/2 (67%/ 33%)	
	>3.2	64	7/3 (70%/ 30%)	2/8 (20%/ 80%)	8/2 (80%/ 20%)	1/9 (10%/ 90%)	7/3 (70%/ 30%)	75/ 65	71.0	0/4 (0%/ 100%)	6/4 (60%/ 40%)	1/3 (25%/ 75%)	0/1 (0/ 100%)	
p-Value		0.1	0.28	0.7	0.2	1.0	0.007	0.69/0.44	0.69	0.005	0.76	0.29	0.4	

F, Female; M, male; p, primary; s, secondary; mo, monocytic; nm, non-monocytic; PB, peripheral blood; BM, bone marrow; WBC: white blood cells; fav, favorable; adv, adverse; SFI: Specific fluorescence indices; AML: acute myeloid leukemia; [1]according to European leukemia network/ National comprehensive cancer network classification; ABT, anthracycline-based therapy; [2]after first induction therapy (anthracycline-based); [3]within 260 days.

We demonstrated a significant association of TRAILR2 expression on blasts from patients of adverse risk groups by NCCN/ELN. Furthermore, we showed a negative impact on survival for cases with high TRAILR2 expression. The pro-apoptotic effects of TRAILR1 and -R2, associated with sensitivity to membrane-bound TRAIL or soluble TRAIL are an elementary step in the induction of apoptosis. No consistent data yet exist between levels of surface expression of TRAIL and sensitivity to TRAIL in leukemia. It is tempting to suppose its relevance because in multiple myeloma, expression levels of TRAILR1 and -R2 can predict sensitivity to TRAIL (18). Furthermore the expression of proteins involved in apoptosis are controlled by the TRAIL pathway. Malignancy can lead to insufficient apoptosis-related enzyme expression (19) and thus to impaired regulation or lack of apoptosis: The death-inducing signaling complex as a part of the apoptosis pathway is inhibited by proteins such as TNFR-associated factor 2 and receptor interacting protein *via* competition with caspase-8 recruitment, leading to multiple mechanisms interrupting cell death (20). These facts might explain the inconsistency in published data regarding prognostic issues (21-23) but might also show the importance of TNFR expression as the beginning of a cascade which ends with apoptosis of blasts.

For the decoy receptor TRAILR3, a significantly higher expression was found in cases with a favorable risk by NCCN/ELN. Clinical evaluations in cut-off analyses showed that the group with higher expression of TRAILR3 exhibited delayed time to relapse and enhanced overall survival. Up-regulation of decoy receptors is discussed as contributing to resistance of several tumor cell types including breast cancer (24), osteosarcoma (25) and myeloma (26), although our data for AML point to other results, as do those of other groups (4). Riccioni *et al*. found TRAILR3 overexpression on blasts from 79 patients suffering from non-promyelocytic leukemia (27), whereas Chamuleau *et al*. showed a significantly reduced TRAILR3 expression on AML blasts compared to healthy controls, suggesting a pro-apoptotic profile for myeloid blasts due to a lack of decoy receptors. In contrast, the same group presented a sub-group of patients with AML characterized by a high expression of TRAILR3 associated with significantly reduced survival compared to the group with low TRAILR3 expression (4). We can hypothesize that pro-apoptotic effects with membrane-bound or soluble TRAIL (mediated by immune effector cells) might occur and led to our observations.

Our data showed a significant negative association of a high TNFR1 expression with survival. Moreover, we found a significant association between higher expression levels and age ≥60 years. TNFR1 was shown to be frequently expressed on blasts, contributing to TNF-driven growth regulation by stimulation of interleukin-3-induced proliferation and

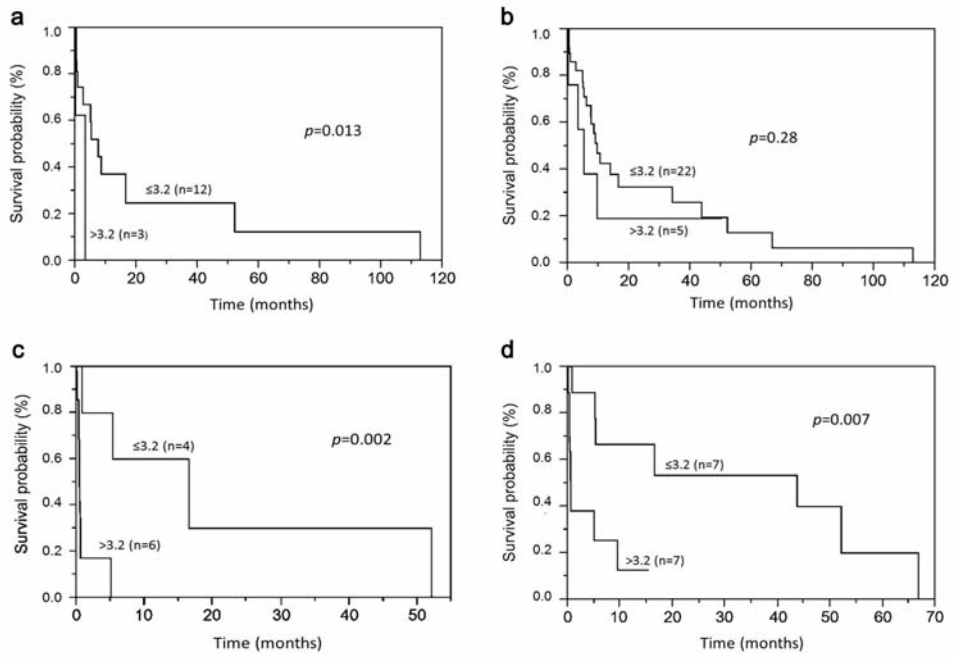

Figure 4. *Survival of patients with acute myeloid leukemia. Expression of death receptors was studied by flow cytometry, the obtained SFIs were calculated compared to those with isotype controls. Cut-off values that allowed separation of patients into those with better and those with worse prognosis were evaluated. Kaplan-Meier plots present comparative data for groups by cut-off with response of all patients to any therapy (b, d) and of patients treated by anthracycline-based therapy (a, c). Lower expression of TRAILR2 in patients treated with anthracycline-based therapy was associated with significantly prolonged survival (a). Lower expression of TNFR1 with any therapy was also associated with significantly prolonged survival (c, d).*

granulocyte-macrophage colony-stimulating factor leading to AML growth (6, 28), stimulation (29) and autocrine growth induction (28). Thus, we contribute further evidence that TNFR1 might play a role in the initiation and progression of AML blasts.

Other studies show that FAS expression is increased in myeloid leukemia (22, 30). Detailed analyses in AML, however, revealed heterogeneous expression profiles in FAB sub-types (22, 23, 31, 32). Variable expression may be present due to different maturation stages of leukemia cells (31), and our analyses are in line with this, showing no clinical relevance in prognostic parameters.

In summary with this pilot study, we showed suitability of DR expression on AML blasts in a screening context and revealed significance of TRAILR2 and TNFR1 expression on AML blasts for the clinical parameters survival and risk classification. In order to clarify the role of TRAILR3 and FAS and to determine statistical relevance for TRAILR2 and TNFR1, larger datasets are necessary.

Since TRAILR2 and TNFR1 can be easily measured by flow cytometry on a routine basis, our findings may be of value in characterizing and monitoring AML blasts in a clinical context.

Conflicts of Interest

The Authors report no conflicts of interest.

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