Function of the ABC transporters, P-glycoprotein, multidrug resistance protein and breast cancer resistance protein, in minimal residual disease in acute myeloid leukemia

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Background and Objectives. Relapse is common in acute myeloid leukemia (AML) because of persistence of minimal residual disease (MRD). ABC-transporters P-glycoprotein (Pgp) and multidrug resistance protein (MRP), are thought to contribute to treatment failure, while it is unknown whether breast cancer resistance protein (BCRP) does so. However, whether up-regulation of pump activity or selection of subpopulations with higher pump activity occurs during chemotherapy is unclear. The aim of this study was to elucidate whether ABC-transporter function changes during the course of disease.

Design and Methods. MRD cells were identified using leukemia-associated phenotypes combined with a fluorescent probe assay with substrate/modulator: Syto16/PSC833 (Pgp), calcein-AM/probenecid (MRP) and BODIPY-prazosin/Ko143 (BCRP); efflux profiles were directly compared with blasts at diagnosis and relapse from the same patient.

Results. At diagnosis BCRP activity was undetectable in AML blasts from 23/26 cases, while Pgp activity was present in 36/45 and MRP activity in 26/44 of the cases. Furthermore, no subpopulations of blasts with considerably higher drug efflux capacities were found. Overall, no consistent changes were observed at follow-up [during chemotherapy (n=20), MRD (n=37), relapse (n=26)] in forty-five patients, the mean activities (as percentages of values at diagnosis) were 97% (Pgp), 103% (MRP) and 102% (BCRP).

Interpretation and Conclusions. Emergence of MRD is thus not accompanied by either upregulation of ABC-transporter function during or after chemotherapy or by selection of pre-existing highly resistant subpopulations. The prognostic value of Pgp and MRP is, therefore, likely related to drug efflux capacity homogeneously distributed in the whole blast population, while BCRP probably has a limited function in drug efflux-related resistance in AML.

Key words: minimal residual disease, acute myeloid leukemia, drug-resistance, ABC- transporters, immunophenotyping.

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Although chemotherapy induces complete remission (<5% blasts detectable in the bone marrow with morphology) in most patients with acute myeloid leukemia (AML), many eventually relapse due to the persistence and subsequent outgrowth of minimal residual disease (MRD).[1] The question remains which mechanism(s) are responsible for the survival of leukemia cells in AML. Putative resistance mechanisms include i) resistance due to changes in apoptotic pathways[2] and ii) resistance due to the presence of ATP-dependent membrane efflux pumps, ABC-transporters,[3] such as P-glycoprotein (Pgp, ABCB1)[4] and multidrug resistance protein (MRP, ABCC1).[5] Various studies suggested that the presence of Pgp and MRP1 (henceforth referred to as MRP) at diagnosis contributed to treatment failure,[6-13] although this could not be confirmed in other studies.[14,15] Recently, a new member of the ABC transporter family was discovered to play a role in multidrug resistance (MDR), namely breast cancer resistance protein (BCRP, ABCG2).[16-18] Thus far only one study has investigated the impact of BCRP expression on outcome; using the median BCRP gene expression as the cut-off for high and low expression, it was found that children with AML who expressed high levels of BCRP had a worse overall survival.[19] In adult AML, levels of BCRP mRNA or protein expression are generally low at diagnosis,[20-23] but it is not known thus far whether BCRP plays a role in drug efflux-mediated resistance in this disease. Substrate specificities of Pgp, MRP and BCRP are distinct, but also overlapping: daunorubicin can be transported by Pgp, MRP and BCRP, etoposide by both Pgp and MRP, and mitoxantrone by BCRP and to a lesser extent by Pgp.[24] Currently available data regarding the substrate specificity of Pgp and MRP for idarubicin, the 4-demethoxy derivate of daunorubicin, are inconclusive. It is generally thought that idarubicin is probably not a direct substrate itself but that its metabolite idarubicinol might be actively involved in multidrug resistance.[25-27] The above-described drugs are commonly used in chemotherapeutic protocols in AML.

It is not only the MDR status of the blasts at diagnosis that might determine the overall response to chemotherapy but also changes in drug efflux occurring during chemotherapy. Upregulation of MDR1 gene expression has been described in human metastatic sarcoma after *in vivo* exposure to doxorubicin[28] and occurs quickly *in vitro* when AML cells are treated with an anthracycline, such as daunomycin and idarubicin, or

cytosine arabinoside,[29] or subjected to stress induction by non-cytotoxic agents.[29,30] If this phenomenon were to be observed in AML patients, it might go some way to explaining the discrepancies found between several studies addressing the prognostic significance of pump activity determined prior to treatment. Another important question is whether chemotherapy might cause a selection of subpopulation(s) with higher drug efflux ability which might in turn again refine the prognostic value of pump activity determined in the whole blast population. To elucidate whether Pgp, MRP and BCRP contribute to treatment failure in AML, the optimal approach would be to characterize the functionality of the MRD cells, since these cells have actually survived the chemotherapy and will be responsible for the occurrence of a relapse later on. By comparing the resistance profile of the MRD cells with the profile found at diagnosis and relapse in the same patient, it might be possible to establish whether chemotherapy-induced selection of resistant blasts subpopulations and/or upregulation of pump function occurs in AML. Whereas expression/functional ABC-transporter studies have been performed in diagnosed and relapsed AML, no such studies have been performed thus far in MRD situations. Some studies showed higher expression or activity of Pgp and/or MRP in blasts from refractory/relapsed patients than in blasts from untreated patients.[31-33] Direct comparison of a relapsed sample with an untreated sample from the same patient showed higher expression of MRP and unchanged expression of Pgp in the relapsed samples in one study,[34] while no consistent changes were found for Pgp and MRP function at relapse in very recent studies.[35-37] No change in BCRP protein expression or function was found in relapsed AML blasts when these were directly compared with the blasts at diagnosis in one recent study,[22] while another study showed a significant increase in BCRP mRNA levels at relapse.[37]

In order to investigate ABC transporter functions in residual leukemia cells during follow-up, MRD cells need to be identified from among many normal hematopoietic cells and, furthermore, functional studies must be combined simultaneously. While conventional morphology cannot detect MRD cells, multiparameter flow cytometry is successful in detecting MRD cells and moreover, allows the inclusion of additional parameters, e.g. expression/function of ABC transporters. This rapid and reproducible flow cytometry approach is applicable in up to 80% of AML patients, since AML blasts at diagnosis frequently display one or more leukemia associated phenotypes (LAPs).[38-40] These LAPs are combinations of cell surface markers that are usually not present, or present at very low frequencies, on normal hematopoietic cells.[41] In our own institute, in an update of a previous study,[42] one or more LAPs, useful for MRD detection, could be established in 127/156 newly diagnosed AML patients. It has been shown in several studies on AML and acute lymphoblastic leukemia that threshold numbers of MRD cells or a gradual increase of the frequency of MRD cells in follow-up samples significantly correlates with relapse rate and relapse-free survival.[43-46] A possible pitfall in using immunophenotyping to detect MRD cells is that phenotypic shifts can occur after treatment.[47-49] However, since blasts frequently display more than one LAP at diagnosis,[38,42] inclusion of all these LAPs at follow-up should minimize the occurrence of false-negative findings.[49,50] Combining functional Pgp and MRP measurements with LAP detection was shown to be feasible in a previous study[42] on both fresh and frozen-thawed diagnosed AML and MRD samples. In the present study, we used these combined investigations on leukemia cells from AML patients to determine the presence of sub-populations of blasts with different ABC transporter function at diagnosis and longitudinally at different stages of disease (during the first induction course, in different MRD situations and at relapse). Since it is possible that upregulation of pump function might only be a transient phenomenon occuring during or directly after chemotherapy, when possible, we included samples taken during the first course of induction chemotherapy and after finishing the first course. Since BCRP is a relatively newly described ABC transporter, special attention was paid to validating our detection assays for both BCRP function and expression in combination with LAP detection.

A green fluorescent probe assay with substrate/modulator combinations with proven sensitivity and specificity were used for Pgp, MRP and BCRP function: Syto16/PSC833 (for Pgp),[51,42] calcein-AM/probenecid (for MRP)[52,42] and BODIPY-prazosin/Ko143 (for BCRP).[53-55] Incubations for activity should be followed by labeling of cells with monoclonal antibodies (MoAbs) for LAP detection, particularly in situations of MRD. To our knowledge, this is the first longitudinal study presenting Pgp, MRP and BCRP activity data in leukemia cells at different stages during the disease, including minimal residual disease, thus tackling the important issues of chemotherapy-induced upregulation and subpopulation selection.

Design and Methods

Reagents

RPMI 1640 and fetal calf serum (FCS) were obtained from Life Technologies (Paisley, UK), bovine serum albumin (BSA) from ICN Biomedicals (Aurora, OH, USA). Dulbecco's minimal essential medium (DMEM) without bicarbonate and phenol red was from Flow Laboratories (Irvine, UK), phos-

phate-buffered saline (PBS) from ICN (Costa Mesa, CA, USA), Ficoll-Paque from Pharmacia Biotech (Uppsala, Sweden), 7-amino actinomycin D (7-AAD; Via-Probe) from Pharmingen (San Diego, CA, USA), dimethyl sulphoxide (DMSO) and sodium azide from Merck (Darmstadt, Germany), probenecid from Sigma (St. Louis, MO, USA), Syto16, calcein acetoxymethylester (calcein-AM) and BODIPY-prazosin from Molecular Probes (Eugene, OR, USA). Mitoxantrone (Novantrone®) was from AHP Pharma BV (Hoofddorp, The Netherlands). Ko143 (fumitremorgin C analog) was a gift from A van Loevezijn and PSC833 was a gift from Novartis (Basel, Switzerland). For sources of fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophyocyanin (APC) conjugated MoAbs see reference.[42] BXP-34 and BXP-21 monoclonal antibodies were a gift from RJ Scheper.

Cell lines

The leukemia cell line HL60 was obtained from DSMZ (German Collection of micro-organisms and cell cultures, Braunschweig, Germany), the myeloma cell line 8226, its BCRP over-expressing subline 8226/MR20,[56] the breast cancer cell line MCF7 and its BCRP over-expressing sub-line MCF7/MR[57] were obtained from RJ Scheper. All cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS at 37°C in a humid atmosphere with 5% CO_2. The 8226/MR20 and MCF7/MR were cultured in the presence of 20 and 80 nM mitoxantrone, respectively. All resistant cells were cultured for 7 days without drugs before experiments were performed.

Patients and controls

Normal bone marrow samples were obtained after informed consent from patients undergoing cardiovascular surgery. Control peripheral blood stem cells (leukapheresis material, LM) were obtained, after mobilization with granulocyte colony-stimulating factor, from healthy donors involved in an allogeneic peripheral blood stem cell transplantation protocol. After informed consent, bone marrow (BM) samples were collected from AML patients at the time of diagnosis, between December 1997 and December 2001 at the VU University Medical Center. From these patients consecutive follow-up samples were obtained at different stages: during the first course of induction chemotherapy (BM or peripheral blood 8, 25 and 53 hours after the start of treatment), in minimal residual disease, defined as a period when <5% blasts were detectable with conventional morphology (BM after 1st and 2nd course of induction chemotherapy, and when applicable, after 3rd course of consolidation chemotherapy, the autologous peripheral blood stem cell transplant and BM after autologous transplantation) and at relapse. In the present study we show data from 45 AML patients selected from a larger cohort of patients based firstly on the availability of a diagnosed sample and at least one follow-up sample with leukemia cells present and secondly on the presence of one or more LAPs enabling MRD detection. A total of 20 follow-up samples were obtained during the first course of chemotherapy (11 patients), 37 samples in minimal residual disease (24 patients) and 26 samples at relapse (26 patients). For the patients' convenience, in the studies during the first induction course of chemotherapy peripheral blood samples were taken instead of BM when blasts were present in the periphery; which was the case in eight patients. This was thought to be appropriate since in these eight cases, blasts present in BM and peripheral blood prior to treatment showed no immunophenotyping or drug efflux capacity differences. Patients were classified at presentation of disease according to the French-American British (FAB) classification. The patients were treated with intensive chemotherapy regimens according to the protocols of the Dutch-Belgian Hemato-Oncology Cooperative Group for AML. Most protocols consisted of two cycles of induction chemotherapy followed by either a third course of chemotherapy as consolidation treatment or marrow ablative chemotherapy followed by autologous or allogeneic stem cell transplantation. The patients' characteristics are shown in Table 1.

For functional studies, mononuclear cells from diagnosed and relapsed AML samples were isolated via a Ficoll gradient (1.077 g/mL). Erythrocytes were lysed afterwards by 10 min incubation on ice with an aqueous buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4. Follow-up samples were subjected only to erythrocyte lysing. Experiments were preferentially performed using fresh samples, occasionally supplemented with cryopreserved samples. We have previously shown that appropriately frozen-thawed samples can be used for detection of Pgp and MRP function combined with immunophenotyping.[42] We found no differences in BCRP expression and function between fresh and frozen-thawed samples (n=10, *data not shown*).

Flow cytometry

Flow cytometry was performed using a FACScalibur (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm argon laser with 530 nm (FL1), 585 nm (FL2) and 670 nm (FL3) band pass filters and a 635 nm red diode laser with 661 nm band pass filter (FL4). Data acquisition and analysis was performed using Cell quest software.

Establishment of leukemia-associated phenotypes and MRD detection

Leukemia-associated phenotypes (LAP) were established in diagnosed AML and MRD detected at

Table 1. Patients' characteristics.

Patient	FAB	Age[a]	Treatment cycles[b]			LAP or phenotype[c]	Follow-up samples[d]
			1st course	2nd course	3rd course		
1	M1	76	Ara-C,flu	Ara-C,flu		$CD117^+CD7^+$	t=8, t=25, after 1st, 2nd, relapse
2	Relapsed	54	Ara-C,flu			$CD45dCD34^{+*}$	t=8
3	Unknown	71	Ara-C			$CD34vCD7^+$	t=8, after 1st
4	M4	64	Ara-C,flu			$CD45dCD117^{+*}$	t=8
5	M5b	70	Ara-C			$CD45dCD56^+CD14^+$	t=8, t=25, t=53
6	M1	75	Ara-C,flu			$CD34^+CD7^+$	t=8
7	M1	74	Ara-C,flu	Ara-C,flu		$CD34^+CD45dCD4^+$	t=8, t=25, t=53, after 1st, 2nd
8	MDS	46	Ara-C			$CD34^{+*}$	t=8
9	RAEB-T	59	Ara-C			$CD34^{+*}$	t=8
10	M2	66	Ara-C,flu			$CD45dCD34^{+*}$	t=8, t=25, t=53
11	M1	52	Ara-C,ida	Ara-C,amsa	bu,cy,auto Tx	$CD34^{+*}$	relapse
12	M2	39	Ara-C,ida	Ara-C,amsa	mitox,etop	$CD45dCD117^+CD15^+$	PBSC, after 2nd (2x)
13	M2	53	Ara-C,ida	Ara-C,amsa		$CD45dCD33^{++}CD56^+$	after 1st, relapse
14	M2	67	Ara-C,dnr,p	Ara-C,dnr,p	Ara-C,dnr	CD45d*	relapse
15	M2	61	Ara-C,dnr	Ara-C,dnr		$CD34^+CD7^+$	relapse
16	M0	86	Ara-C,dnr	Ara-C,dnr		$CD34^+CD7^+$	relapse
17	M0	46	Ara-C,ida	Ara-C,amsa	bu,cy,auto Tx	$CD34^+CD7^+$	relapse
18	M4eo	41	Ara-C,ida	Ara-C,amsa		$CD34^+CD15^+$	after 2nd
19	M0	43	Ara-C,ida	Ara-C,amsa		$CD34^+CD7^+$	after 2nd
20	M2	31	Ara-C,ida	Ara-C,amsa	bu,cy,auto Tx	$CD34^+CD2^+$	PBSC, after auto Tx, relapse
21	M5b	61	Ara-C	Ara-C	Ara-C,dnr	$CD117^+CD7^+$	after 2nd, 3rd (2x)
22	M4	54	Ara-C,ida	Ara-C,amsa	bu,cy,auto Tx	$CD34^+CD7^+$	relapse
23	M5a	37	Ara-C,ida			$CD117^+CD7^+$	after 1st
24	M4eo	43	Ara-C,ida	Ara-C,amsa	mitox,etop	$CD34^+CD2^+$	relapse
25	M1	34	Ara-C,ida	Ara-C,amsa	mitox,etop	$CD34^+CD2^+$	after 1st, relapse
26	M0	75	Ara-C,dnr	Ara-C,dnr		$CD34^+CD7^+$	after 1st, after 2nd
27	M2	45	Ara-C,dnr	Ara-C,dnr	mitox,etop	$CD34^+CD7^+$	PBSC, relapse
28	M1	55	Ara-C,ida	Ara-C,amsa	mitox,etop	$CD34^+CD7^+$	PBSC, after 2nd, 3rd (2×)
29	M5	33	Ara-C,ida	Ara-C,amsa	TBI,cy,allo Tx	$CD117^+CD7^+$	relapse
30	M2	58	Ara-C,ida	Ara-C,amsa	mitox,etop	$CD34^{+*}$	relapse
31	M4eo	31	Ara-C,ida	Ara-C,amsa	TBI,cy,allo Tx	$CD34^+CD7^+$	after 2nd, relapse
32	M4	69	Ara-C,dnr	Ara-C,dnr		$CD34^+CD56^+$	after 1st, relapse
33	M4eo	53	Ara-C,ida	Ara-C,amsa	bu,cy,auto Tx	$CD117^+CD7^+$	after auto Tx, relapse
34	M2	52	Ara-C,ida	Ara-C,amsa	mitox,etop,bu,cy,auto Tx	$CD34^+CD7^+$	after auto Tx, relapse
35	M1	31	Ara-C,ida	Ara-C,amsa	bu,cy,auto Tx	$CD34^+CD7^+$	PBSC, after auto Tx, relapse
36	M5a	56	Ara-C,ida	Ara-C,amsa	bu,cy,auto Tx	$CD34^+CD2^+$	after 3rd, relapse
37	M5b	55	Ara-C,dnr			$CD34^+CD7^+$	after 1st
38	M1	52	Ara-C,ida	Ara-C,amsa		$CD34^+CD11b^+$	after 1st, relapse
39	M5a	56	Ara-C,ida	Ara-C,amsa	mitox,etop	CD45d*	relapse
40	M5a	45	Ara-C,ida	Ara-C,amsa	bu,cy,auto Tx	$CD117^+CD56^+$	after 2nd, relapse
41	M5a	68	Ara-C,dnr	Ara-C,amsa		$CD34^+CD56^+$	relapse
42	M0	66	Ara-C,dnr	Ara-C,dnr	Ara-C,mitox,etop	$CD45dCD34^{+*}$	relapse
43	M5a	69	Ara-C,dnr			$CD34^+CD7^+$	relapse
44	M4eo	20	Ara-C,ida	Ara-C,amsa	mitox,etop	$CD34^+CD2^+$	after 1st, after 3rd
45	M5a	53	Ara-C,ida			$CD117^+CD7^+$	t=8, t=25, t=53

[a]age at diagnosis; [b]Ara-C: cytosine-arabinoside; flu: fludarabine; ida: idarubicin; dnr: daunorubicin; amsa: amsacrine; etop: etoposide; mitox: mitoxantrone; bu: busulphan; cy: cyclophosphamide; p: PSC833; autoTx: autologous peripheral blood stem cell transplantation; alloTx: allogeneic peripheral blood stem cell transplantation; TBI: total body irradiation; c Leukemia associated phenotype (LAP) of blasts; d: dim; and immunophenotype of blasts (*) used in the absence of LAP for detection of leukemia cells at diagnosis, during 1st course of chemotherapy and at relapse d During chemotherapy: 8, 25 and 53 hours after start 1st course chemotherapy; minimal residual disease: after 1st, 2nd and 3rd course of chemotherapy, autologous peripheral blood stem cells (PBSC), after auto Tx; at relapse.

follow-up as described previously.[42] All LAPs found at diagnosis were used for MRD detection at follow-up and furthermore, relapse samples were characterized in detail in order to detect any changes in marker expression that might have occurred during disease progression. In the present study, 26 patients were investigated both at diagnosis and at relapse; no phenotypic shifts were detected. For the simultaneous investigation of LAP and Pgp, MRP or BCRP function (see next paragraph), two or three channels (FL2, FL4 and FL3) were available for LAP detection, since one channel (FL1) was used for the functional Pgp, MRP or BCRP assay and one channel (FL3) was used for

viability staining with 7-AAD when using frozen-thawed samples. If possible myeloid markers and CD45 labeling were excluded from the LAP combination; expression of these markers was assessed in a separate staining. If more than one LAP was present at diagnosis, we chose to use the LAP with the highest expression on the blasts and with the lowest background expression in normal control samples in the tests on follow-up samples. However, to exclude the possibility that leukemia cells with different immunophenotypes showed different drug efflux capacity, we studied all blasts populations present at diagnosis and at relapse in detail and furthermore, when applicable, we studied all MRD cells at follow-up, defined by the expression of different LAPs, for drug efflux capacity. Table 1 shows the chosen double and triplicate combinations of markers used for detection of leukemia cells in our four-color FACS assay combining assessment of LAP detection and drug efflux activity.

Combined detection of leukemia-associated phenotype and Pgp, MRP or BCRP function

LAP expression and Pgp or MRP function were measured simultaneously as described in detail in our previous study.[42] In that study it was shown that immunophenotype and Pgp or MRP function could be assessed simultaneously without interference in both diagnosed AML and in MRD samples using either fresh or appropriately frozen-thawed samples. A similar protocol was used for the functional BCRP assay which was optimized using the BCRP negative (HL60),[58,59] parental low BCRP expressing (8226/S)[59] (MCF7/S)[53,59] and BCRP over-expressing (8226/MR20),[56] (MCF7/MR),[18,53] cell lines. BODIPY-prazosin was used as substrate, since this substrate was found to be as effective as mitoxantrone in detecting BCRP-mediated efflux[53] and furthermore, it allowed detection of BCRP function in the FL1 channel of the flow cytometer. This allowed the FL2, FL3 and FL4 channels to be used for LAP detection in a similar way as done for Pgp and MRP activity assessment. We combined BODIPY-prazosin with the newly developed fumitremorgin C analog Ko143,[54] an improved version of Ko134,[55] as a sensitive, specific and non-toxic BCRP inhibitor. Cells were incubated at a concentration of 0.3×10^6 cells/mL accumulation medium (DMEM, supplemented with 10% FCS) with either accumulation medium alone, BODIPY-prazosin alone or BODIPY-prazosin combined with Ko143. A 60 min accumulation assay with 25 nM BODIPY-prazosin and 200 nM Ko143 showed maximal ratios. Activity was expressed as ratios of drug fluorescence with modulator and drug fluorescence without modulator after subtraction of the fluorescence of the control (cells in accumulation medium without drugs present). A ratio > 1.0 indicates activity. The following ratios were found for the HL60: 0.9 ± 0.1, 8226/S: 1.8 ± 0.2, 8226/MR20: 5.1 ± 0.9, MCF7/S: 1.0 ± 0.1 and MCF7/MR: 6.9 ± 1.4 (means $\pm$ s.d.; n=3-5). Figure 1A shows a representative example of the BCRP function in the tested cell lines. All above mentioned cell lines were used to control the overall performance of the BCRP activity assay and of the BCRP expression assay (*see next paragraph*).

For the patients' samples we thus used 25 nM BODIPY-prazosin in the presence of 1 μM PSC833 with or without 200 nM of the BCRP inhibitor Ko143 for a 60 min incubation period. PSC833 was included to prevent efflux of BODIPY-prazosin by functional Pgp.[24] Following accumulation with the specific substrate/modulator combinations, and ice-cold washing, cells were incubated for LAP detection with appropriate PE- and APC-conjugated MoAbs combined with either PerCP conjugated monoclonal antibody or 7-AAD (10 μL/10^6 cells) for 30 min on ice in FACS buffer (PBS with 0.1% BSA and 0.05% sodium azide). 7-AAD was always included in frozen-thawed samples to gate out dead cells in the final analysis. Cells were washed once and resuspended in 0.2 mL FACS buffer and kept in the dark on ice until acquisition.

Knowledge about the reproducibility of the functional assays is of utmost importance in order to be able to determine changes in pump function in longitudinal studies. Pump activities for Pgp and MRP were measured using three different cryopreserved diagnosis AML samples showing varying activities for Pgp and MRP (low, intermediate and high), and the assays were repeated 4-6 times on separate days. The coefficient of variation (CV) was calculated as follows: (standard deviation/mean)* 100% for each of the three different AML samples tested; the mean of the three calculated CVs was taken as the CV value belonging to the corresponding functional assay. The assay of Pgp function showed a CV of 23% and that of MRP function 13%. The reproducibility of the BCRP functional assay was determined by measuring the BCRP function 3-6 times on separate days using cryopreserved samples from HL60, 8226 and MCF7 cell lines; a CV of 13% was found.

Combined detection of leukemia-associated phenotype and BCRP expression

A consensus meeting established that the most relevant parameter for studying multidrug resistance is the function of the MDR proteins.[60] This approach was followed for Pgp and MRP in the present study, using the functional assays that we had validated in a previous study.[42] However, since this is the first study in which LAP detection and BCRP detection are combined in a flow cytometric

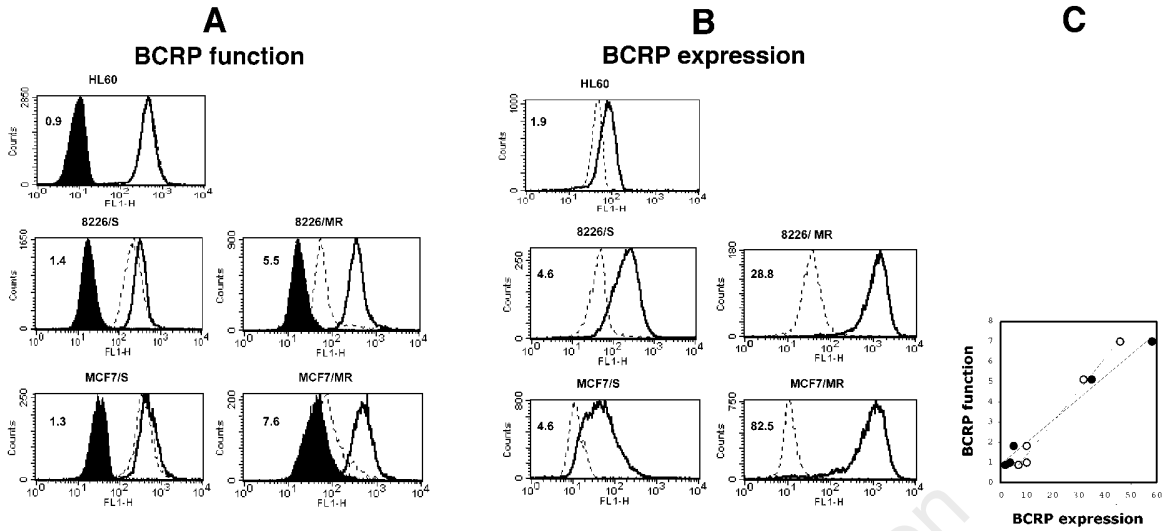

Figure 1. BCRP function and expression of cell lines. A: Representative example of BCRP function in BCRP-negative (HL60), low BCRP-expressing (8226/S, MCF7/S) and BCRP-overexpressing (8226/MR20, MCF7/MR) cell lines. The filled curve represents the autofluorescence of the cells; the dotted and solid lines represents, respectively, the fluorescence of cells incubated with BODIPY-prazosin alone and in the presence of Ko143. B: Representative example of BCRP expression in the tested cell lines. The dotted line represents the fluorescence of cells incubated with IgG1 isotype control + RAM-FITC and the solid line represents fluorescence of cells incubated with BXP-34 + RAM-FITC. C: Correlation between BCRP expression and function in the tested cell lines, depicted as the mean of 3-6 separate experiments. BXP-34 (closed circles and solid line): Rho= 1.00 (p<0.0001) and BXP-21 (open circles and dotted line): Rho=0.975 (p=0.005). No significant differences (p=0.686) and a good correlation (Rho=0.975, p=0.05) were found between BXP-34 and BXP-21 staining in these cell lines.

assay, we decided to study BCRP expression as well as BCRP function. For this purpose, we used BXP-34[58] and BXP-21[61] antibodies. For the combined detection of BCRP expression and LAP, cells were first incubated for 15 min at room temperature (RT) with 7-AAD. Next, after washing with 2 mL FACS buffer, cells were fixed with 1% paraformaldehyde (50 μL/0.3×10^6 cells) for 5 min, again at RT. After washing, cells were permeabilized with 0.1% saponine (50 μL/0.3×10^6 cells) for 15 min at RT and then washed once. All further incubation steps were performed in a total volume of 50 μL/0.3×10^6 cells. Cells were labeled with BXP-34, BXP-21 or the corresponding isotype controls (IgG1 and IgG2a, respectively) (1 μL/3×10^6 cells), for 60 min at RT. After washing, the cells were labeled with RAM-FITC (1.25 μL/0.3×10^6 cells) for 20 min, washed and then blocked with IgG1 (5 μL/0.3×10^6 cells) for 15 min at RT. Finally cells were incubated for 15 min at RT with appropriate PE- and APC-conjugated monoclonal antibodies for LAP detection. Cells were washed once and resuspended in 0.2 mL FACS buffer and kept in the dark until acquisition. To calculate the BCRP expression of blasts the mean fluorescence intensity (MFI) of LAP-positive cells for BXP-34 and BXP-21 was divided by the MFI of the corresponding isotype control.

The BCRP expression assay was optimized using the same cell lines as those for the functional assay. For BXP-34/IgG1 the following ratios (means±s.d.; n=3-6) were found for HL60: 1.9±0.1, 8226/S: 4.9±0.4, 8226/MR20: 35±8, MCF7/S: 3.6±1.6 and MCF7/MR: 58±22. For BXP-21/IgG2a the corresponding ratios were: HL60: 6.7±1.1, 8226/S: 10±1.4, 8226/MR20: 32±7, MCF7/S: 10.0±3.4 and MCF7/MR: 46±10. Figure 1B shows a representative example of BXP-34 staining in the tested cell lines. Staining with both BXP-34 and BXP-21 monoclonal antibodies showed good correlations with BCRP function in all tested cell lines (Figure 1C), indicating that the above described protocols can be used to detect BCRP function or expression accurately in combination with LAP detection in our four-color FACS assay. In order to determinine the reproducibility of this BCRP expression assay, the same cryopreserved samples were tested as with the functional BCRP assay (see preceding paragraph); a CV of 13% was found.

Statistical analysis

Wilcoxon's rank-signed test and Spearman's rank correlation test were used to determine differences and correlations. p values < 0.05 were considered statistically significant. For comparisons of the activity and expression of ABC-transporters in leukemia cells in follow-up samples with those in the corresponding diagnosis AML, a difference exceeding twice the CV of the assay (*see preceding paragraphs*) was considered to be significant.

Results

Pgp, MRP and BCRP function in diagnosed AML

At diagnosis of the 45 patients included in this longitudinal study, 80% of blasts showed Pgp activity and 59% showed MRP activity. Presence or absence of activity was defined by a cut-off of fluorescent shift ≥ 1.10, which is based on the fact that Pgp negative (human epidermoid carcinoma cell line, KB3-1) and MRP negative (human small-cell lung cancer cell line, GLC4) cell lines always display a fluorescent shift of less then 1.1.[42] These AML samples showed a median activity (and range) of 2.3 (1.1-19, n=36) for Pgp and 1.3 (1.1-1.9, n=26) for MRP. The frequency and the level of Pgp and MRP activities found in the blasts of these 45 patients were similar to those found in a larger cohort of patients (*data not shown*). Both function and expression of BCRP were determined in AML blasts; the results were similar to those found in normal CD34+ cells (Figure 2A). Low levels of BCRP expression were detectable in AML blasts (for BXP-21: mean±SD of 2.00±0.66, n=25; for BXP-34: 1.31±0.31, n=25), however, BCRP activity (mean± SD of 0.93±0.09, n=26) was absent in 23/26 samples. In the remaining three samples the activity was very low: 1.07, 1.10 and 1.12. Activity and expression of blasts from the last of these three samples are shown in Figure 2B.

Although the studied drug transporters were heterogeneously expressed among AML patients, in general the activity of all three pumps seemed to be distributed quite homogeneously in the blast population of each individual patient. It cannot be excluded, however, that blast subpopulations with higher pump activity than that in the bulk of the blasts are present at very low frequencies, and might selectively survive chemotherapy. The possible presence of subpopulations of blasts with different drug efflux capacities was studied more closely, using four different approaches which are described below. i) Since it was found, in all cases, that cells incubated in the presence of the relevant modulator showed an uniform peak of probe fluorescence, we studied in detail the fluorescence distribution patterns of the pump substrates in the absence of modulator for the existence of small subpopulations. Since at least 50 clustered events are necessary for adequate detection of a separate population in flow cytometry, for a detection limit of 1% at least 5,000 acquired events are needed. We acquired a total number of 3,500-600,000 events, which thus allowed the detection of subpopulations with frequencies ranging from 0.01% to 1.42%. There were no indications of the presence of sub-populations for Pgp (43/45), MRP (44/44) or BCRP (26/26). We detected different blast populations with different Pgp activities only in two patients; each patient displayed one blast population with Pgp activity (2.23 and 2.27, respectively) and one population without Pgp activity. In both patients the blast populations with and without Pgp activity had similar immunophenotypes.

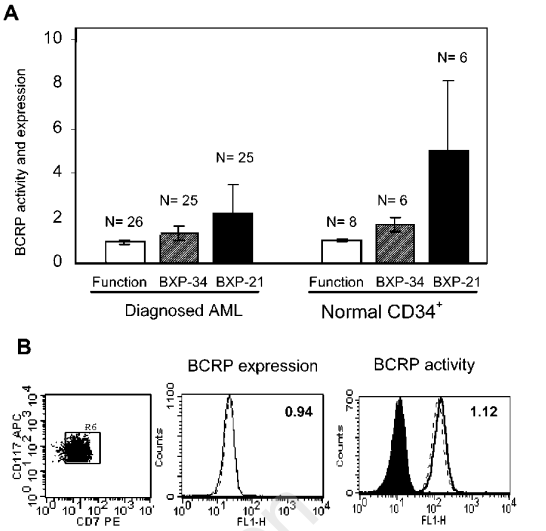

Figure 2. BCRP function and expression in AML blasts at diagnosis and in normal CD34+ cells. A: BCRP function (open bars) and BCRP expression (BXP-34: striped bars, BXP-21: black bars) in AML blasts (left side of figure) and in normal CD34+ bone marrow cells (right side of figure). B: Example of BCRP function and expression in blasts of AML patient 1. At diagnosis 68% of blasts were CD117+ of which 97% was CD7+ (left picture). BCRP expression in the CD117+ CD7+ cells was 0.94 (middle picture, dotted line: IgG1 + RAM-FITC, solid line: BXP-34 + RAM-FITC) and BCRP function was 1.12 (right picture, filled curve: autofluorescence of the cells, dotted line: BODIPY-prazosin + PSC833, solid line: BODIPY-prazosin + PSC833 + Ko143). BXP-21 staining (*not shown*) was 1.78.

ii) Five patients showed clearly different LAP expressions (range 2-3 LAPs) on the blasts at diagnosis: all these blast subpopulations were examined for Pgp and MRP function. No significant differences (differences > 2× CV of the assay, see materials and methods) in Pgp and MRP function were detected in the different LAP-positive blast cells. In addition, in ten other patients only some CD34+ blasts at diagnosis showed LAP expression. An examination of these blast subpopulations revealed no significant differences in Pgp and MRP function (p=0.445 and 0.312, respectively) between the LAP-positive and LAP-negative CD34+ blasts.

iii) Since CD34 may be a marker for primitive AML blasts[62,63] subpopulation analysis can be applied in a more focused although restrictive way by comparing CD34+ and CD34− CD45dim/SSClow AML blasts, especially when either of the popula-

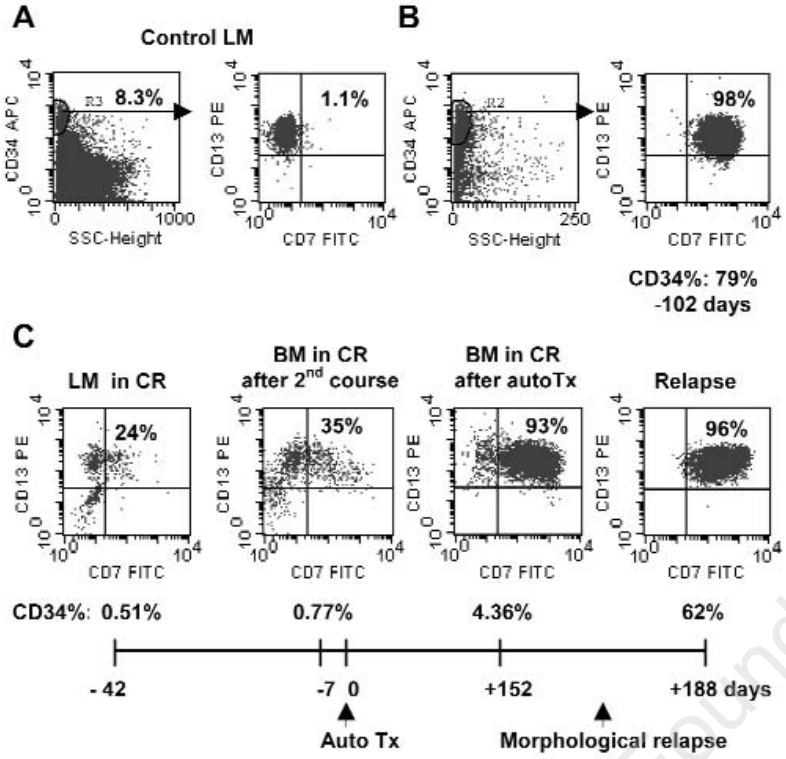

Figure 3. Detection of malignant CD34⁺ cells at different stages of disease in a patient with AML. A: Representative example of CD13/CD7 co-expression on normal CD34⁺ cells present in peripheral blood stem cells (leukapheresis material, LM) from a healthy donor.
B: Leukemia-associated phenotype (LAP, CD7⁺CD13⁺CD34⁺) of AML blasts at diagnosis from patient 35. Seventy-nine percent of the blasts were CD34⁺, 98% of these CD34⁺ blasts showed co-expression of CD13 and CD7. This LAP was used to detect residual leukemia cells at follow-up.
C: CD13/CD7 expression on CD34⁺ cells present in different follow-up samples. Only gated CD34⁺ cells are shown. For comparison, observe the low level of LAP expression on normal CD34⁺ cells present in control LM (A). The day of autologous peripheral blood stem cell transplantation (auto Tx) is designed as day 0.

tions is present at low frequencies. Retrospectively, this was possible in 17 cases for Pgp, 14 cases for MRP and 9 cases for BCRP. Activities of MRP and BCRP were not significantly different (p=0.552 and p=0.483) between paired CD34⁺ and CD34⁻ AML blasts. However, Pgp activity was significantly higher in 12/17 cases in the CD34⁺ fraction (p=0.002), irrespective of the AML CD34 percentage. A Pgp activity (mean±SD, n=17) of 2.3±1.8 was found in the CD34⁺ blast fractions, while the CD34⁻ blast fractions showed an activity of 1.7±1.6. The median factor difference in the 12 cases with higher Pgp activity in the CD34⁺ blast fraction was 1.3 fold. In the whole blast population, the CD34⁺ and CD34⁻ blast fractions were never seen as individual populations since pump activity differed by a maximum factor of 2.4 and we found that, in general, only populations whose fluorescence intensity differs by at least a factor of 5 can be identified reliably as separate populations (*not shown*).

iv) Since the leukemia stem cell in AML has been reported to have the immature phenotype CD34⁺ CD38⁻,[63] while the CD34⁺CD38⁺ and CD34⁺CD38⁺⁺ populations would contain more mature progenitors, a similar approach was applied comparing these subsets in five patients. The frequency of the CD34⁺CD38⁻ subset was low (median 0.3% of the CD34⁺ cells, range 0.1%-3.1%), and there was no indication that this subset had different Pgp, MRP or BCRP activity than other subsets (*not shown*). Overall, these findings indicate that except for small differences in Pgp activity between CD34⁺ and CD34⁻ blasts ABC transporter function of leukemia blasts at diagnosis is distributed quite homogeneously over all blasts within each patient. Very small sub-populations of blasts with considerably higher activities do not seem to be present.

Comparison of Pgp, MRP and BCRP function in leukemia cells present at follow-up and those at diagnosis

Samples from 45 AML patients were studied longitudinally for Pgp, MRP and BCRP function and in addition, for BCRP expression. Table 1 (last column) shows the type of follow-up sample studied for each patient. Figure 3 shows an example of the detection of malignant CD34⁺ cells at different stages of the disease. During the course of the disease an increasing percentage of CD34⁺ cells had the aberrant phenotype (CD7⁺CD13⁺CD34⁺). This example shows that, while the patient was still in complete remission as determined by morphology (day +152), LAP expression on the CD34⁺ cells (93% were CD7⁺ CD13⁺) present in the BM suggested a forthcoming relapse which did, indeed, occur at day 168. Pgp, MRP and BCRP function in the same patient during the course of disease is depicted in Figure 4.

Cytostatic drugs did not change pump function significantly in the majority of patients. Samples

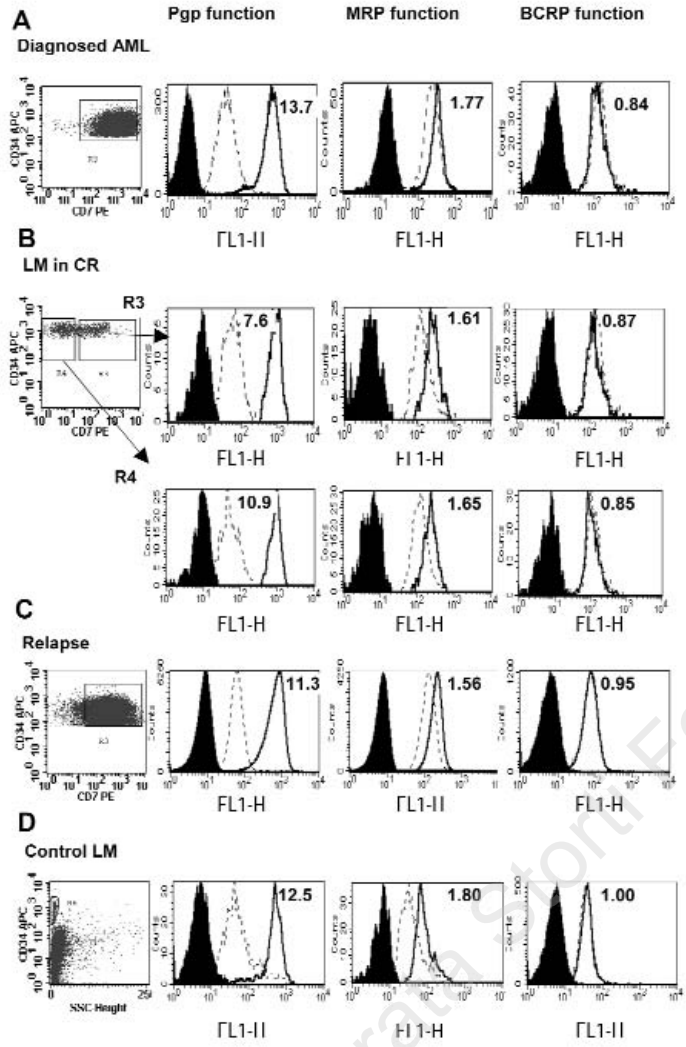

Figure 4. Pgp, MRP and BCRP function of leukemia cells at different stages of disease in an AML patient. Results from patient 35, the same as shown in figure 3 for MRD detection, are given as an example. The patient had high Pgp, intermediate MRP and no BCRP function in blasts at diagnosis and pump function in leukemia cells did not change significantly during follow-up. The left column of figures represents CD7 expression of gated CD34⁺ cells (A-C) and CD34 expression (D), in the next columns Pgp, MRP and BCRP function of gated cells, as shown in the first column. The filled curve represents the autofluorescence of the cells, the dotted line represents the fluorescence of cells incubated with Syto16 (for Pgp), calcein-AM (for MRP) and BODIPY-prazosin + PSC833 (for BCRP). The solid line represents the fluorescence of cells incubated with Syto16 + PSC833 (for Pgp), calcein-AM + probenecid (for MRP) and BODIPY-prazosin + PSC833 + Ko143 (for BCRP). A: AML at diagnosis showing CD7 expression on CD34⁺ cells, Pgp, MRP and BCRP function of these CD34⁺CD7⁺ cells was 13.7, 1.77 and 0.84, respectively. B: Autologous leukapheresis material with 0.51% CD34⁺ cells with CD7 expression present on 24% of the CD34⁺ cells. Regions were set around CD34⁺CD7⁺ (R3) and the CD34⁺CD7⁻ (R4) cell population. Pgp, MRP and BCRP function of these cell populations was 7.6, 1.61 and 0.87 (CD34⁺CD7⁺), and 10.9, 1.65 and 0.85 (CD34⁺CD7⁻), respectively C: Relapsed AML sample. Pgp, MRP and BCRP function of the CD34⁺CD7⁺ blasts was 11.3, 1.56 and 0.95, respectively. D: Representative example of Pgp, MRP and BCRP function of normal CD34⁺ cells present in leukapheresis material (LM) of a healthy donor. Note the similarity between Pgp, MRP and BCRP function in these cells and that in the presumed normal CD34⁺CD7⁻ cells shown in B. Pgp and MRP function was slightly, but not significantly, less (the difference was < 2×CV assays, see materials and methods), in LM in complete remission (B) than in AML samples taken at diagnosis (A) and at relapse (C).

taken during administration of such drugs showed no significant change of Pgp function in 10/11 cases, no change in MRP function in 10/11 cases and no change in BCRP function in 8/8 cases tested (Figure 5A). Pgp function increased significantly in one patient and MRP function in another but in no case was a change from no activity to detectable activity seen. Furthermore, BCRP expression did not change in the 7 studied patients (*data not shown*).

The samples obtained after chemotherapy course(s) revealed variable numbers of MRD cells, these accounting for a median of 0.24% (range 0.01%-4.05%) of total white blood cells. In those cases with more then one LAP present at diagnosis, all LAPs were used to detect MRD cells. No discrepancies were observed; the proportional frequencies of the different LAP-positive leukemia cells were similar after treatment and at diagnosis. Data on the level of MRD cells present in follow-up samples and correlations with clinical outcome are beyond the scope of this paper and will be presented elsewhere for a larger cohort of AML patients. After different courses of chemotherapy, in the majority of patients with MRD no significant changes were seen in Pgp (17/24), MRP (20/22) and BCRP (13/14) function when compared with drug efflux capacity at diagnosis (Figure 5B). The remaining patients did not show consistent changes; furthermore, none of the changes in Pgp and MRP function was observed from no activity to detectable activity in any of the samples. The patient with increased BCRP function (from 0.86 to 1.22) in BM obtained after the 1st course of chemotherapy also showed an increase in BXP-34 staining in this sample while BXP-21 staining remained unchanged. In the remaining 6 patients tested for BCRP expression, no consistent

changes were observed, the levels detected always being lower than those found in normal CD34+ cells and close to those found in BCRP-negative and low expressing cell lines. In addition, in six patients who expressed more then one LAP on blasts at diagnosis, we were able to study the drug efflux capacity of the different LAP-expressing MRD cells; in agreement with the findings of rather homogeneous drug efflux capacity of blasts at diagnosis, no significant differences (differences > 2 CV of assays) were detected in these different LAP-positive expressing MRD cells.

At relapse no changes were seen in 19/26 patients tested for Pgp function, 17/22 patients tested for MRP function and 10/10 patients tested for BCRP function (Figure 5C). In the remaining patients changes in Pgp, MRP and BCRP function and expression were not consistent and again in no case was a change from no activity to detectable activity seen in any of these samples. Furthermore, in agreement with findings in the samples taken at diagnosis, we did not detect subpopulations of blasts with considerably different ABC-transporter activity at relapse.

In a minority of patients we detected a significant change in pump function in leukemia cells present at follow-up (see thick lines, Figures 5A-C). In order to determine whether such changes were a transient or permanent phenomenon it was essential to study the patients longitudinally over time, including relapse samples. From 13 patients we were able to study leukemia cells both at diagnosis and in at least one follow-up sample obtained during chemotherapy and/or in minimal residual disease, and at relapse. Figure 6 shows the Pgp and MRP function of the leukemia cells studied serially in these individual patients. Importantly, in those cases with absence of pump activity at diagnosis no change towards activity was found. In a minority of cases increase or decrease of pump function was found, but overall no consistent and no permanent changes were observed at follow-up. Similar results were found for BCRP function and expression in longitudinally studied patients (*data not shown*). The results of all follow-up samples studied are summarized in Table 2, in which the mean activity or expression of ABC-transporters is given as a percentage of the corresponding value at diagnosis.

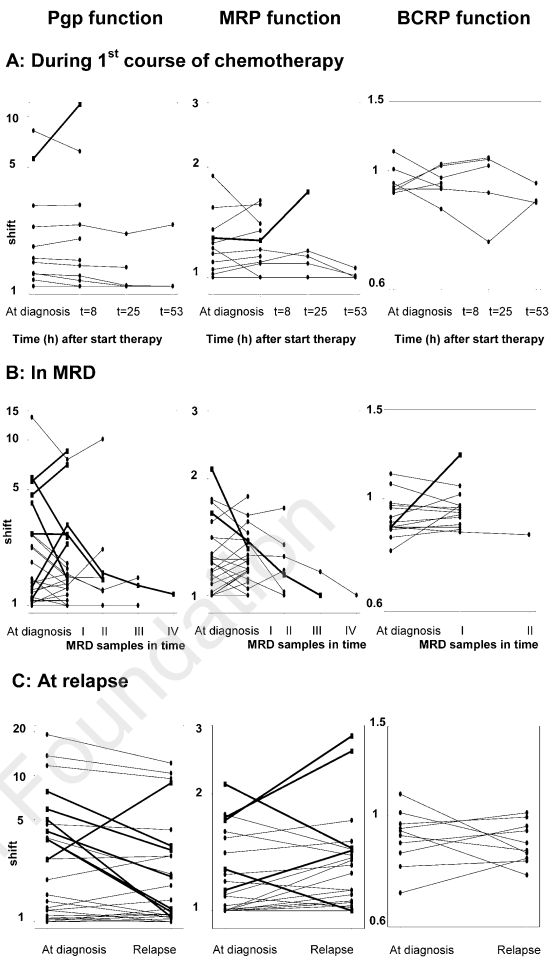

Figure 5. Comparison of Pgp, MRP and BCRP function of leukemia cells at follow-up and at diagnosis. The left column of figures represents Pgp function, the middle MRP function and the right BCRP function. A fluorescence shift > 1.0 indicates activity. The figures show paired samples of blasts at diagnosis and of blasts present at follow up. A significant change (>2 CV of assay, see materials and methods) in Pgp/MRP/BCRP function at follow-up compared with the corresponding function in the sample at diagnosis has been highlighted with thick solid lines. Thin lines represent follow-up samples without a significant change. A: During the 1st course of induction chemotherapy (8, 25 and 53 h after onset of therapy). Pgp, MRP and BCRP function was studied in 11, 11 and 8 patients, respectively, in a total of 20, 20 and 15 follow-up samples. B: In several MRD situations (after 1st, 2nd, and 3rd courses of chemotherapy, autologous peripheral blood stem cells, after autologous transplantation). Pgp, MRP and BCRP function was studied in 24, 22 and 14 patients, respectively, in a total of 37, 32 and 15 MRD samples. C: At relapse; Pgp, MRP and BCRP function was studied in 26, 22 and 10 patients, respectively.

Discussion

Relapse is a common occurrence in AML patients,[1] due to the persistence and outgrowth of MRD. The presence of the ABC-transporters Pgp and MRP, has been implicated in the observed poor treatment outcome in AML.[6-13] Whether the recently discovered new member of the ABC-transporters, BCRP,[16,18,64] which is present in many MDR cell lines,[65,66] contributes to this observed chemotherapy resistance in adult AML is still unclear. In addition, the exact mechanism(s) by which the above-mentioned ABC-transporters may mediate resistance in AML needs to be clarified. It may be that the level of drug efflux capacity at diagnosis determines treatment out-

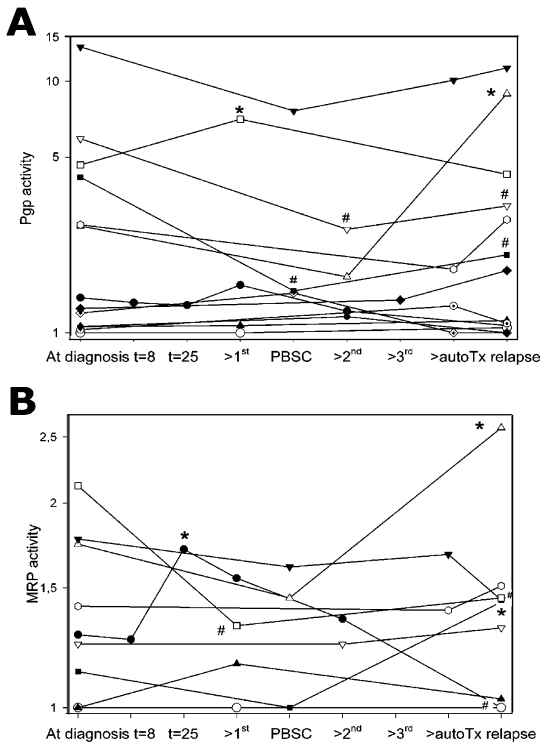

Figure 6. Pgp and MRP function of leukemia cells from longitudinally studied patients. Samples were tested at diagnosis, during the 1st course of induction chemotherapy (t=8 and 25 h after onset of therapy) and/or in minimal residual disease (after 1st, 2nd and 3rd courses of chemotherapy, autologous peripheral blood stem cell transplantation (PBSC), after autologous transplantation (auto Tx)) and at relapse. A fluorescent shift >1.0 indicates activity. The figures show paired samples, using different symbols for each patient, tested for Pgp function (A: 13 patients) and MRP function (B: 9 patients). A significant increase (*) or decrease (#) in pump activity at follow-up is depicted in the figures.

come but changes in the expression/function of transporters might occur during chemotherapy. The present study was aimed at elucidating whether the function of the ABC-transporters Pgp, MRP and BCRP, changes during the course of disease. We, therefore, studied leukemia cells from 45 AML patients, either during initial induction chemotherapy and/or in MRD situations and the MDR profile was compared with that found at diagnosis and relapse of the same patient. We investigated whether a transient or consistent upregulation of pump function occurs in AML, since a rapid upregulation of Pgp expression/function has been reported to occur after chemotherapeutic treatment in both *in vivo*[28] and *in vitro*[29,67] studies. In addition, we investigated whether there are subpopulations of blasts with different drug efflux capacities at diagnosis and, if so, whether the pre-existing highly resistant subpopulations might selectively survive chemotherapy.

Table 2. Activity and expression of ABC transporters at follow-up compared with their corresponding values at diagnosis of AML.

Time points studied[a]	*ABC transporter activity and expression*[b]				
	Pgp activity	*MRP activity*	*BCRP activity*	*BCRP expression*	
				BXP-34	*BXP-21*
During therapy[c]	100±8 (n=11)	102±4 (n=11)	102±5 (n=8)	97±6 (n=7)	105±5 (n=7)
MRD[c]	97±8 (n=24)	98±3 (n=22)	105±4 (n=14)	128±19 (n=7)	88±13 (n=7)
Relapse	93±12 (n=26)	108±5 (n=22)	98±4 (n=10)	112±17 (n=10)	80±10 (n=10)

[a]Follow-up samples were tested: during chemotherapy (8, 25 and 53 h after start 1st chemotherapy course), in minimal residual disease (after 1st, 2nd and 3rd course of chemotherapy, autologous peripheral blood stem cell transplant, BM after autologous transplantation) and at relapse. [b]Activity and expression depicted as mean ± SEM as a % of the corresponding values in the samples taken at diagnosis: the number of patients studied is given in parentheses. [c]Data from > 1 follow-up sample during the 1st course of chemotherapy and data from > 1 MRD sample per patient were pooled.

In the majority of patients we observed no significant change in Pgp, MRP and BCRP function at follow-up. Importantly, for Pgp and MRP function no changes were observed from *no activity* to *activity*, while for BCRP function only 1/40 follow-up samples tested showed a conversion from no activity to low activity. Patients whose Pgp and/or MRP or BCRP function changed at follow-up were not confined to a particular chemotherapeutic treatment protocol and in addition, did not have a particular immunophenotype. One remarkable observation was that two patients who, at diagnosis, had two populations of blasts with considerably different Pgp activity (one population without and one with Pgp activity) showed the same pattern of two populations with different Pgp activities in follow-up samples taken during the first course of chemotherapy and in MRD situations. This at least shows that there is no simple selective elimination of populations that can be predicted from observations on single resistance mechanisms.

The present results of the longitudinal MDR study in 45 AML patients show: (i) variable levels of Pgp and MRP function in blasts at diagnosis between patients; (ii) in the vast majority of patients, a rather homogeneous distribution of activity on all blasts within each individual patient; and (iii) no evidence for the presence of sub-populations of blasts with considerably higher pump activities. Our search for the presence of subpopulations of blasts in newly diagnosed AML, by examining whole blast populations, comparing $CD34^+$ versus $CD34^-$ and comparing $CD34^+CD38^-$ versus $CD34^+$ $CD38^+$ pairs in AML blasts, confirmed this view. The

levels of BCRP expression found in AML blasts at diagnosis were always lower than those found in normal CD34+ cells and close to levels found in BCRP-negative and low BCRP-expressing cell lines. Furthermore, no BCRP activity was detectable in the majority of samples; very low BCRP activity was found in only three samples. Similar levels of BCRP expression and function were found in the follow-up samples. The validation study in cell lines showed a nice correlation between BCRP expression and function; furthermore, BCRP function could be detected in cell lines that do exhibit low levels of BCRP expression (i.e. 8226/S, Figure 1). With the present techniques, it can be concluded that, despite low levels of BCRP protein, functional BCRP seems to be absent in blasts from most cases of newly diagnosed AML and follow-up samples. Our findings for BCRP are consistent with recent flow cytometry results from van der Kolk *et al.*,[22] who showed similar levels of BCRP expression and very low function in diagnosed AML as in the present study. Data on mRNA expression also showed low levels of BCRP expression in most AML patients, with only a few patients demonstrating high BCRP expression.[20,23]

Recently two other groups have reported data on paired diagnosis-relapse samples:[35-37] these data confirm the lack of differences in Pgp and MRP function or expression between diagnosis and relapse. Our data concerning Pgp and MRP function in paired diagnosis-relapse samples are in concordance with these reports. In contrast, results for BCRP are not that conclusive. Although we detected low levels of BCRP protein expression in blasts at diagnosis, detectable BCRP function was absent in the vast majority of samples. Like van der Kolk *et al.*,[22] in the present study we did not find higher BCRP function or expression in relapsed AML samples than in samples taken at diagnosis. Both these studies used a flow cytometric method. Two other studies which tested mRNA levels of BCRP found a significant increase of BCRP mRNA at relapse in adult AML[37] and in childhood AML.[19]

To our knowledge, this is the first study in which activity of ABC-transporters in leukemia cells has been studied longitudinally in AML patients on samples obtained during the first induction course of chemotherapy and samples obtained at consecutive time points in MRD. Together with our data in which we used paired diagnosis and relapse samples we can conclude that permanent increases of drug efflux pump activity are not of pivotal importance in AML. The fact that there is apparently neither chemotherapy-induced upregulation of Pgp/MRP function nor chemotherapy-induced selection of pre-existing highly resistant subpopulations indicates that the Pgp or MRP activity in a particular AML sample is likely an intrinsic property of the whole AML population. It is the differences in drug efflux in AML blasts at diagnosis between patients that cause differences in the proportion of cells that escape chemotherapy-induced cell killing. We thus hypothesize that if the emergence of MRD is causally related to Pgp and/or MRP function, then this is related to pump activity that is homogeneously distributed over all blasts at diagnosis of a patient, which in turn remains remarkably constant throughout disease. This hypothesis will be tested by correlating the number of MRD cells at follow-up with the levels of Pgp/MRP activity in the corresponding AML blasts at diagnosis. Preliminary evidence supporting this hypothesis was provided by two separate studies showing that Pgp positivity at diagnosis correlated with higher numbers of MRD cells after induction or consolidation treatment.[43,44] Findings of a direct correlation between Pgp/MRP activity at diagnosis and MRD frequency at follow-up might identify, already at diagnosis, those patients who will need additional treatment. Furthermore, based on our findings we propose that BCRP probably has a limited function as a drug transporter in adult AML but more extensive studies are needed to confirm this view.

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Pre-publication Report & Outcomes of Peer Review

Contributions

MAvdP was responsible for the data, analysis and wrote the manuscript. HJB, GJO and GJS designed and guided the study. JMP and MvdM carried out the efflux studies, NF and GWDW did the immunophenotyping; all contributed to analysis and interpretation. GLS and RJS helped in designing the BCRP expression assay and JDA and AvL in designing the BCRP activity assay. All the authors revised the paper. We thank G. Evertse and G. Westra for retrieving the patients' clinical characteristics and help with flowcytometry, respectively.

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In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

ATP-binding cassette (ABC) transporter superfamily members play crucial roles in normal and malignant hematopoiesis. The multidrug resistance-1 (MDR1) gene product, P-glycoprotein, and the breast cancer resistance protein (BCRP) are expressed differentially during hematopoiesis and may confer drug resistance.

What this study adds

This study shows that emergence of minimal residual disease is not accompanied by either up-regulation of ABC-transporter function under chemotherapy or by selection of pre-existing highly resistant subpopulations.