



P-glycoprotein in primary acute myeloid leukemia and treatment outcome of idarubicin/cytosine arabinoside-based induction therapy

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The expression of the drug transport protein, P-glycoprotein (Pgp/MDR1) has been found to be of prognostic significance for the achievement of complete remission (CR) or the duration of survival after daunorubicin (DNR)-containing induction therapy in acute myeloid leukemia (AML). This would suggest that the expression of Pgp in AML is high enough to have significant impact on intracellular DNR concentrations and on clinical therapy failure in AML. Recently, DNR has been replaced in many centers by idarubicin (IDA) as the first choice anthracycline in AML treatment. We have, therefore, performed a study in a group of 98 primary AML patients, who all received IDA, but not DNR during induction therapy in order to determine if the response to IDA-containing induction therapy might be related to the biologic characteristic of Pgp expression in AML. The AML samples were studied for Pgp expression by MRK16 antibody staining and for Pgp activity measured as the modulation of rhodamine 123 uptake by 2 μ M PSC 833. No correlation of Pgp with complete response rate, event-free survival or overall survival was found. In addition to Pgp, the expression of another protein that has been implicated by some studies in response failure to DNR-containing therapy, the major vault protein (Mvp/LRP), was studied. This marker did not correlate with CR or survival after IDA-containing therapy. The results of this patient study are consistent with model studies showing that the steady-state cellular accumulation of lipophilic anthracyclines such as IDA are little affected by Pgp. Therefore, putative beneficial effects of the inclusion of PSC 833 in IDA-containing therapy might rather be related to alternative mechanisms than to inhibition of Pgp-mediated IDA efflux. *Leukemia* (2000) 14, 1018–1024.

Keywords: acute myeloid leukemia; P-glycoprotein; major vault protein; LRP; idarubicin

Introduction

Resistance to chemotherapy is still a major problem for the successful treatment of acute myeloid leukemia (AML). The presence of resistant tumor cells in primary treatment refractory patients and their selection during induction therapy and subsequent regrowth are thought to contribute to the clinical failure of chemotherapy. The relative importance of intrinsic biological resistance of the leukemic cells compared to host factors or 'pharmacologic' resistance is not known. One way to explore the relative importance of various possible cellular resistance mechanisms in the outcome of therapy is to study the correlation between a particular resistance-related parameter and a treatment-related parameter (eg response). One of the most intensively studied resistance mechanisms in AML is the P-glycoprotein (Pgp) drug efflux pump, encoded by the *MDR1* gene.¹ It has been clearly established that a high Pgp expression in AML is associated with a decreased *in vitro* accumulation of the anthracycline daunorubicin in blast

cells.^{2–4} Also, a high *MDR1* or Pgp expression correlates with a poor prognosis.^{5–8} It is, however, still not proven that the mechanism of drug pumping by Pgp is responsible for this correlation.^{9–11} In phase I/II trials in AML patients Pgp inhibitors have been added to standard induction therapy containing at least one established Pgp substrate, mainly daunorubicin.¹² Phase III studies aimed at the reversal of clinical resistance by administration of a potent Pgp inhibitor, such as the cyclosporin analog PSC 833, will have to teach us whether ultimate therapeutic benefit of this approach can be obtained in AML.¹³

Another approach that might improve therapy is to use anthracyclines that are less susceptible to Pgp-mediated resistance than the classic drugs daunorubicin or doxorubicin. Such drugs are in general more lipophilic compounds such as aclacinomycin A,¹⁴ annamycin¹⁵ or idarubicin¹⁶ (IDA). The increased efficacy of these types of drugs are caused by their rapid passive transport over the cellular plasma membrane into the cytosol, whereas their Pgp-mediated efflux remains unaltered.^{17,18} Since IDA has in fact been shown to have clinically favorable properties compared to daunorubicin in induction regimens in AML,¹⁹ albeit the effect on long-term survival has not been established,²⁰ it is now replacing daunorubicin as standard therapy.^{19,20} Several *in vitro* studies suggest that Pgp inhibitors may have a small, but still significant effect on the net intracellular uptake of IDA and its cytotoxic metabolite idarubicinol,^{21–23} suggesting that clinical benefit might still be obtained by co-administration of idarubicin with a Pgp inhibitor, such as PSC 833.

In this study we have measured the Pgp activity in clinical specimens from patients with primary AML using a sensitive rhodamine assay in a population of patients receiving idarubicin-containing induction therapy. Another marker, that has been correlated to inferior response to daunorubicin-containing induction therapy, the major vault protein (Mvp/LRP), was also measured since no data on the correlation of this marker with response to IDA containing induction therapy are currently available.

Materials and methods

Patients

Peripheral blood or bone marrow from primary AML patients (mean age at registration 41.1 years; median and range are 43.5 and 15.6–63.1 years) was collected in heparinized glass tubes at initial diagnosis before therapy. Mononuclear cells were isolated by Ficoll–Paque density centrifugation. Patient characteristics are shown in Table 1. All patients were treated according to the HOVON-29 protocol of the Dutch–Belgian Hemato-Oncology Cooperative Group after having given written informed consent. Remission induction therapy consisted of two cycles of chemotherapy. The first cycle included idaru-

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Table 1 Patient characteristics

Total ^a	98
De novo AML	78
Secondary	8
Unknown	12
Males	42
Females	56
Age:	
<35 years	31
35–55 years	52
>55 years	15
WBC ($\times 10^9/l$) < 20	39
WBC 20–50	28
WBC >50	31
Risk class ^b	
good	8
–5/7 (q)	5
Rest	85
CR reached (ever)	
No	28
Yes	70
EFS:	
Continued CR	37
No CR	28
Relapse	25
Dead in CR	8
OS ^c	
Alive	43
Dead	55

^aFAB: M0 = 6, M1 = 13, M2 = 26, M4 = 23, M5 = 26, M6 = 4.^bGood risk is favorable cytogenetics with WBC <20^cMedian follow-up 17 months.

Cytogenetics

Cytogenetic analysis was performed on BM aspirates from patients by routine Giemsa-banding techniques.

Statistical analysis

Overall survival (OS) and event-free survival (EFS) are measured from the start of the first treatment cycle. All deaths were counted as failures in OS analysis. In EFS analysis patients were considered as failures in the case of no CR (with EFS duration of 1 day), and in the case of relapse or death in first CR. Actuarial probabilities were calculated by the method of Kaplan and Meier. Logistic regression analysis was used to test for an association of prognostic factors with the probability of reaching CR, while Cox regression analysis was used to test for associations with EFS and OS. All *P* values reported are two-sided. Prognostic factors considered in the analysis were risk classification, age, Pgp expression and activity and LRP expression. Patients were classified in risk classes based on cytogenetics and white blood cell count (WBC). Patients were considered good risk in the case of favorable cytogenetics, ie inv or del(16), t(8;21) or t(15;17) with WBC <20 $\times 10^9/l$. Patients with deletions of chromosome 5(q) or 7(q) were considered poor risk. All others, including those without known cytogenetics were considered at intermediate risk.

Flow cytometric analysis of P-glycoprotein expression and function and immunocytochemical analysis of Mvp/LRP expression

All patients treated according to the protocol from its activation on up to a beforehand set date of January 1998 were in principle also included in the present study. However, the number of tests done varies per assay because of factors which are related to availability of personnel to perform the various assays. Patients in whom at least one of the analyses below was done were included. No selection based on any patient characteristic other than inclusion in the protocol and with exclusion of M3 patients was done.

The rationale for the choice of the experimental methods used for the measurement of Pgp expression and function and Mvp/LRP expression have been extensively reported by us in three previous publications.^{9–11} Also, the experimental details are extensively described in these papers. In short, Pgp function is measured by flow cytometric determination of the increase in rhodamine 123 or DNR accumulation in AML samples by coinubation with 2 μM PSC 833. The results are expressed as the ratio of the mean fluorescence with/without PSC 833 (RHOratio and DNRratio, respectively). Pgp expression is flow cytometrically measured by staining viable cells with MRK-16 antibody and a FITC-labelled second antibody. The results are expressed as the ratio of the mean fluorescence of MRK16-labelled cells divided by isotype control-labelled cells (PgpMRK16). Mvp/LRP expression is measured by immunocytochemical staining of acetone-fixed cells with the antibody LRP56. The scoring is 0–4 compared to control cell lines as reported in detail before:¹⁰ 0 (all cells negative) and 1 (an occasional cell up to <10% positive cells) are taken as negative and 2, 3 and 4 (>10%, in almost all cases the great majority up to 100% positive cells in the order 2, 3 and 4 with increasingly intense staining) as positive.

bicin 12 mg/m² i.v. on days 1, 2 and 3 and cytarabine 200 mg/m² by continuous infusion on days 1–7. The second cycle included cytarabine 1000 mg/m² i.v. every 12 h on days 1–6 and amsacrine 120 mg/m² i.v. on days 4, 5 and 6. Concomitant administration of recombinant human granulocyte colony-stimulating factor (rhG-CSF) was determined by randomization. Patients with AML-M3 were excluded from this study, because they also received all-*trans* retinoic acid (ATRA). Following the induction therapy poor or intermediate risk patients in CR without an HLA-identical donor were randomized to receive either a third cycle of chemotherapy, consisting of mitoxantrone 10 mg/m² i.v. on days 1–5 and etoposide 100 mg/m² i.v. on days 1–5, or an autologous peripheral stem cell transplantation. Good risk patients in CR (t(15;17), t(8;21), inv(16) or del(16), with WBC <20 $\times 10^9/l$) received the third cycle of chemotherapy. The number of patients in this study with respect to treatment were as follows: 13 received course I; 30 received course I + II; 23 received course I + II + mitoxantrone/etoposide; 24 patients after induction received autologous stem cell transplantation and eight patients received allogeneic stem cell transplantation.

Response assessment

Response to treatment was assessed after each course of chemotherapy. A complete response (CR) required normal recovery of peripheral blood counts with a bone marrow aspirate containing less than 5% blasts.

Results and discussion

We have studied the expression and function of Pgp in 98 primary AML patients treated according to the HOVON 29 protocol, which consists of chemotherapy with or without rhG-CSF. All these patients received as a first course of remission induction therapy cytarabine together with IDA and as second course cytarabine plus amsacrine. The latter two drugs are not known to be substrates for Pgp, therefore IDA or its metabolite(s) is expected to be the only drug in the induction courses, of which the effect could be influenced to some extent by the drug transporter Pgp.

The number of patients with different outcomes subdivided by age, risk class and Pgp parameter classes are shown in Table 2. A complete response was reached in 71% of the patients. Risk class appeared to be the only predictive clinical parameter for CR ($P < 0.05$). None of the other factors (age, RHO ratio, DNR ratio, PgpMRK16 and LRPLRP56) adjusted for this risk factor showed a statistically significant association with the chance of reaching CR (logistic regression analysis, tests for trend). Event-free and overall survival curves are shown in Figures 1 and 2, respectively. The only significant association was seen between OS (median follow-up of 17 months) and risk class (logrank $P = 0.01$). After adjustment for this factor none of the other factors showed a statistically significant association with either OS or EFS (Cox regression analysis, tests for trend).

Since the type of consolidation treatment differed between patients we have done an additional analysis with endpoint DFS restricted to patients with CR. This analysis was adjusted for type of consolidation treatment (stem cell transplantation vs no stem cell transplantation). None of the MDR parameters showed a statistically significant association with DFS

adjusted for risk and type of consolidation treatment (not shown).

These results indicate that the outcome of induction therapy for primary AML, containing IDA instead of DNR as anthracycline is not likely to be influenced significantly by the expression of the resistance proteins Pgp and LRP. With regard to Pgp, the most extensively studied protein of both, this finding is in contrast to other studies of patient groups treated with the anthracycline DNR. Most of those studies indicated a prognostic difference between Pgp-positive and Pgp-negative AMLs, the former predicting poor outcome. This result was obtained despite large differences in methodology⁵⁻⁷ and despite the fact that the clinical outcome is determined not only by the anthracycline, but also by cytarabine, which is not a substrate for Pgp. Thus, there is a general notion that Pgp is an important biological factor in therapy for AML. This suggests that the Pgp activity in certain AMLs is high enough to pump out sufficient DNR to lower its intracellular concentration to subtoxic levels. Alternatively, Pgp may be a pseudo-marker for a generally more drug refractory cell population.²⁴ The effect of Pgp on DNR accumulation has been quantified by us before in AML samples. In untreated AML samples, blocking of the Pgp activity increased the intracellular DNR accumulation with a maximum of 90% in the highest Pgp expressing AML and with a mean increase of 8% and 11 cases higher than 25% increase (140 cases studied).¹⁰ Based on cell line data such increases of net DNR accumulation should indeed affect its anti-proliferative effect. Because the influence of Pgp expression on the cytostatic effect of DNR in the relevant malignant cell populations in AML has not been determined, it remains uncertain how to extrapolate these data to the *in vivo* antitumor effect.

The lack of correlation between Pgp function and response to IDA-containing therapy would be consistent with a number of cell line studies showing that the intracellular levels of the lipophilic IDA are much less affected by low expression levels of Pgp^{16,21-23} than are the intracellular DNR levels. This circumvention of the effect of Pgp pumping activity, which is caused by the rapid passive cellular influx of lipophilic drugs such as IDA¹⁴⁻¹⁸ may in fact contribute to the efficacy of such drugs in the clinic, although this remains to be proven.^{19,20} Also, our results do not seem to indicate that there is a detectable *in vivo* effect on the treatment outcome of the pumping by Pgp of the cytotoxic IDA metabolite idarubicinol, which is influenced by Pgp more like DNR.²³ If the idarubicinol contribution to the overall effect of the (IDA-containing) induction therapy was a major part of the effect, Pgp expression might have been expected to be more predictive for the outcome than when IDA itself is the main cytotoxic molecule. Of course, it remains possible that our patient group was too small to identify an overall small effect of Pgp on IDA or idarubicinol activity, which would most likely be smaller than that for DNR.

The expression of MRP1, which is another drug transporter implicated in tumor resistance to multiple agents of the same class as those subject to Pgp, including the anthracycline DNR, was measured only in a subgroup of patients in the present study and is therefore not reported here. Although no trend was seen for a prognostic value of MRP1, the number of patients was too small to draw definite conclusions. As shown by others, however, the variation in MRP1 expression in primary AML was small and varied little from the low expression levels present in normal hematopoietic cells.²⁵ Also, others have reported that MRP1 expression (by immunocytochemistry) had no impact on clinical outcome

Table 2 Results of tests and patient outcomes

	Total	Alive CCR	First event			All deaths
			No CR	Relapse	Dead in CR	
All patients	98	37	28	25	8	55
Age (years)						
<35	31	13	9	8	1	15
35-55	52	19	14	12	7	30
>55	15	5	5	5	0	10
Risk class						
Good	8	3	1	2	2	3
-5/7 q	5	1	4	0	0	4
rest	85	33	23	23	6	48
DNR ratio						
≤1.00	21	8	4	6	3	12
>1.00-1.04	22	9	6	6	1	12
1.05-1.09	18	9	4	4	1	8
>=1.10	18	4	7	5	2	12
RHO ratio						
≤1.1	46	18	15	11	2	26
>1.1-1.5	25	11	1	11	2	12
>1.5	19	5	9	3	2	12
PgpMRK16						
≤2.0	52	21	15	11	5	29
>2.0	38	12	12	12	2	22
LRPLRP56						
Neg 0-1	49	18	14	11	6	25
Pos 2-4	32	10	9	12	1	22

CCR, continued complete response.

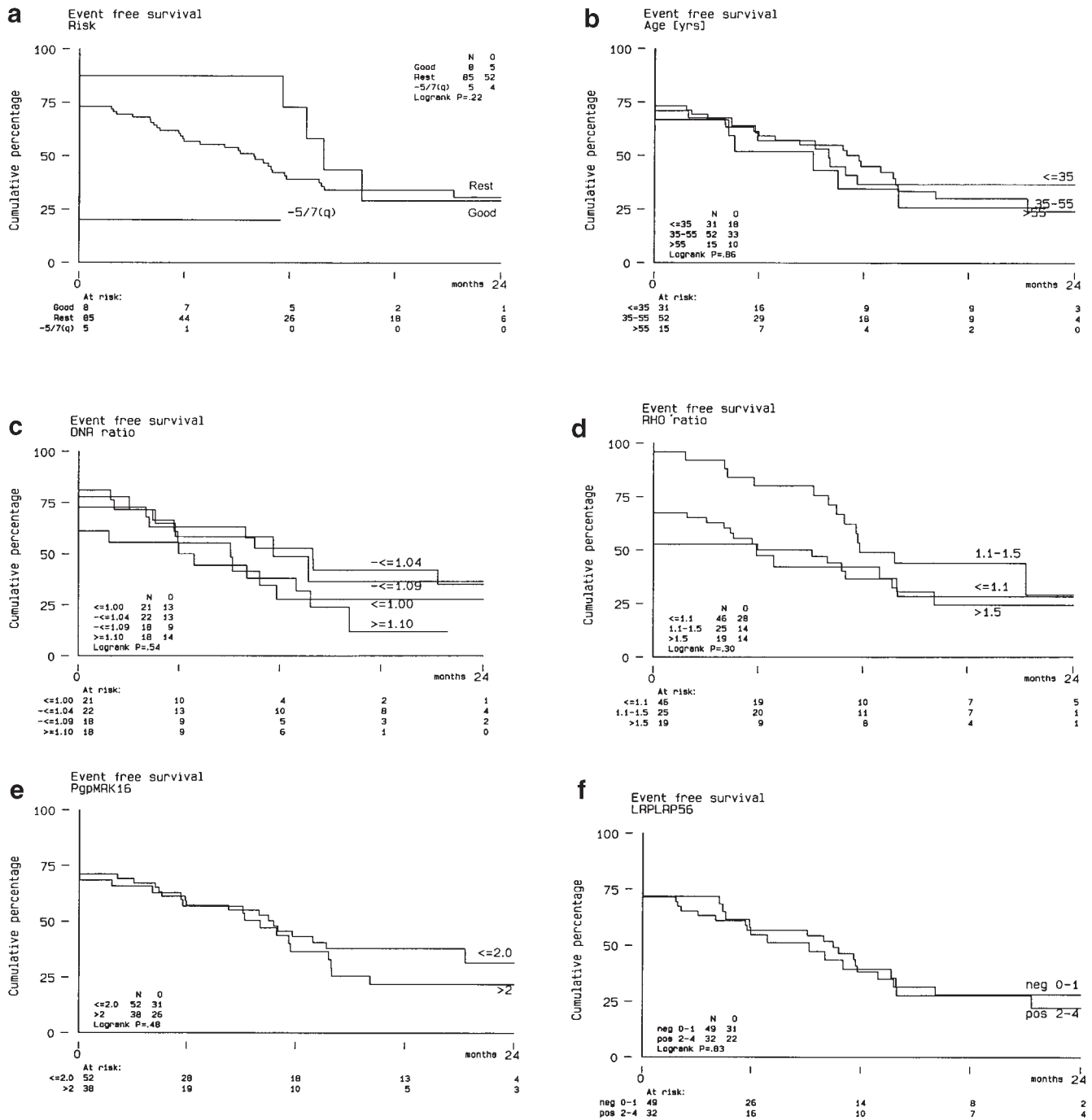


Figure 1 Survival curves for event-free survival according to risk (a), age (b), DNR ratio (c), RHO ratio (d), PgpMRK16 (e) and LRPLRP56 (f).

even in a DNR-based standard induction therapy of *de novo* AML in one study (80 samples), although in a recent update (127 patients) it was reported that intermediate or high MRP1 expression showed a trend ($P=0.09$) towards shorter OS.²⁶ In another study of 53 patients, with mixed treatment protocols, MRP1 function and MRP1 expression were determined by flow cytometry.²⁷ Despite rather similar results for both tests, only the MRP1 function test reached a significant difference for attaining CR, perhaps due to the relatively small patient group.²⁷ No results have yet been reported on the prognostic value of MRP1 and other MRP-family members in exclusively IDA (instead of DNR)-treated AML patients.

Another protein, the major vault protein Mvp/LRP, was

investigated in this study because its overexpression has been implicated as a strong prognostic factor in AML. This was initially reported by List *et al*²⁸ in a study of 87 AML specimens, including *de novo*, secondary and relapsed patients, receiving various chemotherapy regimens, including DNR, but also IDA. In this heterogeneous group, the LRP immunocytochemical staining (which was positive in 33% of *de novo* AML) was an independent adverse prognostic factor for response ($P = 0.0046$), whereas Pgp (measured by immunocytochemistry) was not. Differences in remission duration and OS approached significance only for LRP, but not for Pgp.²⁸ The LRP protein was investigated by List *et al* in relation to drug resistance because it was initially identified

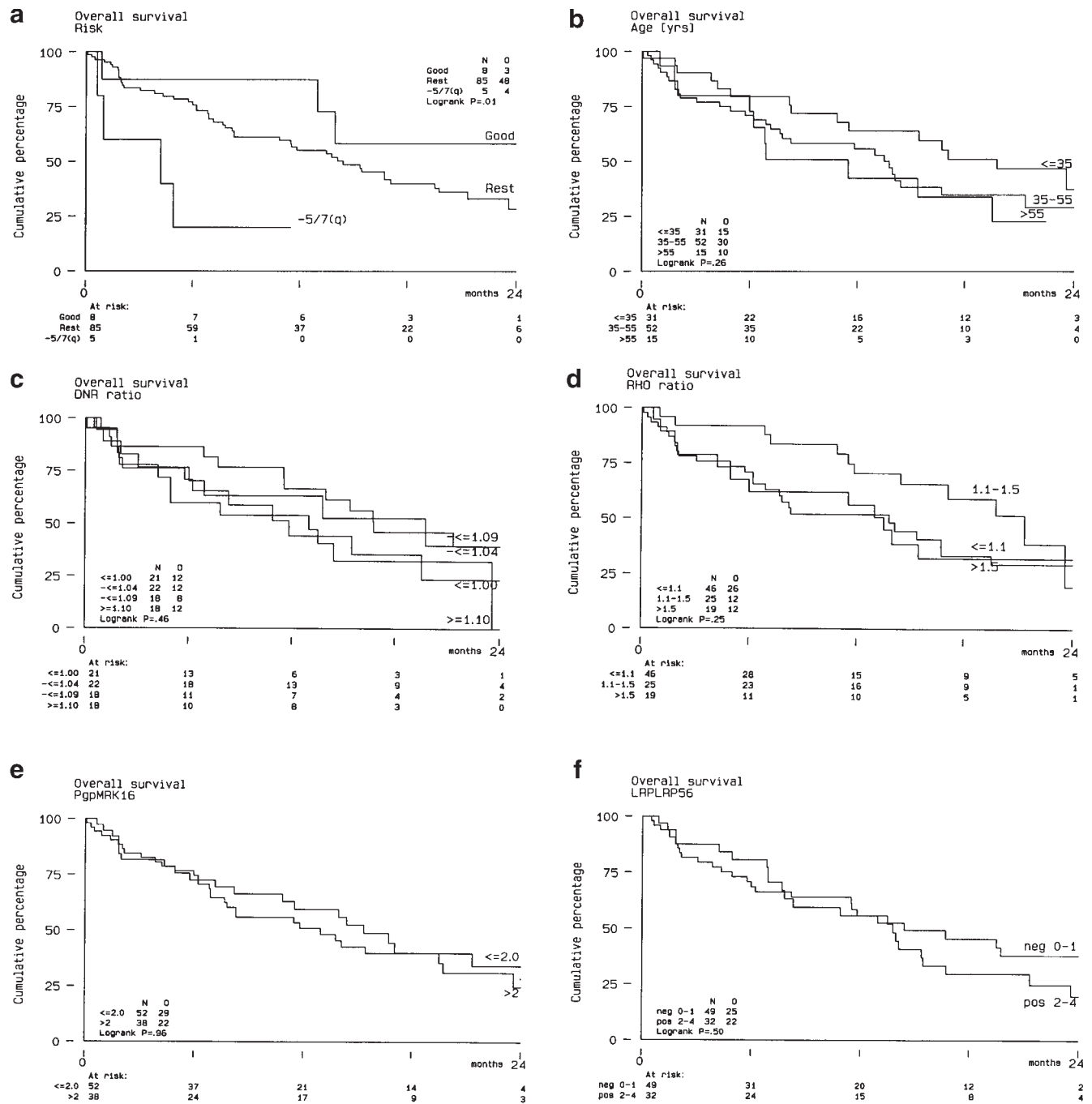


Figure 2 Survival curves for overall survival according to risk (a), age (b), DNR ratio (c), RHO ratio (d), PgpMRK16 (e) and LRPLRP56 (f).

as a protein, overexpressed in a non-Pgp overexpressing human lung cancer cell line, which accumulated a reduced amount of DNR.^{29,30} It has been determined recently, that in a number of multidrug-resistant tumor cell lines, indeed not only the major vault protein Mvp/LRP but also complete vault particles, which are protein and RNA containing structures, are upregulated.³¹ Surprisingly, the function of these structures, which are highly conserved among species, suggesting an essential function in cellular biology, is not known.³² We recently found that LRP expression in AML does not correlate with *in vitro* DNR accumulation in blast cells,¹⁰ leaving open the question of what vaults do in these cells.

In the present series of primary AML, treated uniformly with

an IDA-containing first course, we do not find a predictive value for LRP, determined by immunocytochemistry. Apart from the study of List *et al*, mentioned above, two other studies found that LRP predicted for poor outcome in AML in patients groups treated mainly, but not exclusively with a DNR-containing induction therapy.^{33,34} Both studies used an immunocytochemical method very similar to our method because of a better sensitivity than flow cytometry (our unpublished data and Ref. 34). Two other studies that used flow cytometry for LRP detection in AML found no predictive value for inferior induction rate^{27,35} or duration of remission.³⁵ One recent study found no predictive value for LRP expression (measured by flow cytometry) in 96 *de novo* AML patients,

treated with an IDA-containing regimen.³⁶ There is presently no reason to suspect that the different findings in the studies mentioned are primarily related to methodological differences in LRP detection, although this may remain a matter of controversy as long as no multilaboratory studies have been performed.³⁷ Rather, differences in patient groups and treatment protocols do not yet allow a definite conclusion with regard to the prognostic value of this marker.

In summary, we have studied two resistance markers in a group of primary AML patients, who were all treated with IDA-containing induction therapy. Although 23% of the patients had received mitoxantrone and etoposide as consolidation therapy, which are known Pgp substrates and therefore may theoretically contribute to any found correlation of Pgp expression with EFS or OS this apparently was not the case, since the markers Pgp and Mvp/LRP did not have predictive value for response rate, OS or EFS in the present study. This study complements a recent published study of a large group of AML patients treated with DNR in which P-glycoprotein expression and activity was studied with very similar methods with regard to sensitivity and specificity.³⁵ In that study Pgp was significantly correlated with CR. Our finding is of importance since IDA is replacing DNR in many treatment protocols for AML. Whereas there is some encouraging information for a beneficial effect of PSC 833 in refractory and relapsed AML,³⁸ the interpretation of clinical trials of PSC 833 given to patients treated with IDA-containing therapy such as planned by the Nordic Leukemia Group, should also take into account the present results that Pgp function does not seem to have an adverse effect on the outcome of IDA-based induction therapy. However, PSC 833 may have beneficial effects in combination with chemotherapy, independent of Pgp, as recently suggested.³⁹ This possibility is also suggested by our data that PSC 833 may largely increase the cytotoxicity of DNR in AML in an MTT-based viability assay, independent of Pgp function.¹⁰

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