

## P-gp Expression and Rh 123 Efflux Assay Have no Impact on Survival in Egyptian Pediatric Acute Lymphoblastic Leukemia Patients

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### ABSTRACT

**Purpose:** In a previous work we have studied MDR status in terms of P-glycoprotein (P-gp) expression and Rhodamine 123 efflux assay in Egyptian acute leukemia patients. We have reported results comparable to the literature as regards ANLL both in pediatric and adult cases. However, higher figures were encountered for the functional assay in ALL. As our ALL cases especially in pediatric age group show worse prognosis compared to literature, we hypothesized that the higher percentage of cases with positive Rh123 efflux assay might be a contributing factor.

**Material and Methods:** A total of 108 cases were studied including 80 ALL and 28 ANLL. ALL cases included 48 male and 32 female with an age range of 6m to 18 yrs and a median of 7 yrs. ANLL cases included 18 male and 10 female with an age range of 6m to 18 yrs and a median of 8 yrs. P-gp expression was evaluated using 4E3 and UIC2 mAb, analyzed by Coulter XL flow cytometer and expressed as a ratio at a cut off of  $\geq 1.1$  and/or  $\geq 5\%$  positive cells. For the evaluation of MDR function Rh123 efflux assay using cyclosporine as a blocker and expressed as a ratio at a cutoff of  $\geq 1.1$  and/or  $\geq 10\%$  positive cells was performed. MDR expression and function were correlated to age, Hb, TLC, CD34 expression, immunophenotype and DNA index in ALL, FAB subtypes in ANLL as well as to CR, DFS and EFS in ALL.

**Results:** In ALL, P-gp expression was encountered in 26.4% of cases. Positive Rh efflux was reported in 61.5%. No correlation was encountered between neither expression nor functional assay with age, Hb, TLC, CD34 expression or immunophenotype. CR was achieved in 89.8%; neither P-gp expression nor Rh123 efflux had an impact on CR except for Rh123 efflux in T-ALL where a cutoff of 1.25 could predict CR at a total accuracy of 70.6%. DFS was 92.3% while EFS was 72.2% for the whole group. No significant difference was encountered

neither between cases expressing or lacking P-gp nor between cases with negative or positive Rh123 efflux assay. In ANLL P-gp expression was encountered in 47.6% of cases, while positive Rh123 efflux assay was encountered in 75% of cases. No correlation as encountered between neither expression nor Rh123 efflux assay and neither age, Hb, TLC, CD34 expression nor FAB subtypes.

**Conclusion:** Neither P-gp expression nor Rh123 efflux assay has any impact on survival in pediatric ALL. Rh123 ratio of 1.25 is predictive of CR in TALL.

**Key Words:** MDR1 - Rh 123 efflux - ALL - ANLL.

### INTRODUCTION

Failure of chemotherapy due to the presence at diagnosis or the emergence after therapy of drug resistant tumor cells is a major obstacle in cancer therapy [1].

Multiple drug resistance (MDR) is a well known phenomenon of cross resistance involving resistance to a broad spectrum of natural products used in chemotherapy including alkaloid compounds and bacterial and fungal antibiotics such as anthracyclins and etoposide [2]. An accepted mechanism of MDR is a reduced cellular accumulation and an altered subcellular distribution of cytotoxic drugs. In many instances this is mediated by increased expression at the cell surface of MDR1 gene product, P-glycoprotein (P-gp), a 170-KD energy - dependent efflux pump [3,4]. The traditional model for P-gp function is one where P-gp acts as a "drug pump" to export drugs out of a cell against a concentration gradient; P-gp can remove a

range of structurally diverse drugs without an apparent substrate specificity [5]. P-gp is not the only MDR-related protein; several proteins of the ATP binding cassette family are involved in the intracellular transport of a variety of molecules. Besides P-gp, the other two major members are multidrug associated protein (MRP) and the lung resistance protein (LRP) [1].

As all these and possibly other MDR related proteins, may be involved in drug efflux, MDR functional assay has proved superior to P-gp expression in evaluating the MDR status of leukemic cells [2,6]. It reflects the degree of drug retention by the cells regardless the underlying mechanism. P-gp, however, was claimed to have functions other than drug efflux, which might have an impact on leukemic cells, mainly in protecting them against caspase dependent apoptosis [2,7] through modulation of a sphingomyelin-ceramide apoptotic pathway [8].

Evaluation of MDR functional assay by flow cytometry is the standard method. There are 2 ways to express the MDR status, either as the percentage cells showing Rh123 efflux or as the Rh123 efflux ratio [9].

P-gp is tested by the use of different mAbs including 4E3, UIC2 and MRK16. Percentage of expression can be tested by either flow cytometry or immunohistochemistry. However, flow cytometric evaluation of expression by calculating the relative fluorescent intensity in relation to isotypic control is claimed to be superior [10].

MDR +ve functional assay is reported in about 50-67% of ANLL cases being associated with CD34 expression [11]. ALL, on the other hand, is reported to show a low incidence of the MDR phenotype (8-20%), though recently it was proved to be an independent bad prognostic factor in adult ALL [12,13]. P-gp expression was reported in 20 to 40% of ANLL and in 20 to 30% of ALL [14]. A lot of data are available on the impact of either on the clinical outcome with marked controversy with one or the other claimed to have bad prognostic implication on achieving CR and/or on survival.

In a previous work we reported an incidence of +ve Rh123 efflux in 59% of ANLL and 56% of ALL [15]. The latter is considered a relatively

high incidence especially for pediatric ALL. Previous reports from the NCI, Cairo University have documented survival rates that are still lower than that internationally reported for pediatric ALL [16]. Accordingly we hypothesized that, among other causes, the relatively high incidence of MDR might be a contributing factor.

In this work we prospectively analyzed 28 ANLL and 80 ALL pediatric cases for Rh efflux MDR functional assay and P-gp expression. We correlated Rh123 efflux and P-gp expression to CD34 expression in all, to FAB subgroups in ANLL and to immunophenotype and DNA index as well as to CR, DFS and EFS in ALL.

## PATIENTS AND METHODS

This study was performed on 108 acute leukemia (AL) cases including 28 ANLL and 80 ALL. The 28 ANLL included 18 male and 10 female with an age range of 6 months to 18 years, a mean of  $8.1 \pm 4.7$  and a median of 8 years. As regards FAB subtypes, the cases included 20 M1/M2, 4 M3 and 4 M4/M5. The 80 ALL cases included 48 male and 32 female with an age range of 6 months to 18 years, a mean of  $7.61 \pm 4.65$  and a median of 7 years. As regards immunophenotype, the cases included 25 T (12 comp. I, 4 comp. II, 9 comp. III), 7 Pro-B (CD19+, Cyt CD22+, CD10 -ve), 22 CALL (CD10 +ve, cyt  $\mu$ -ve) and 26 Pre-B (cyt  $\mu$ +ve, sIg, -ve).

All cases were tested for CD34 expression and DNA ploidy was determined in ALL cases.

### *P-glycoprotein (MDR) functional assay:*

Cyclosporin modulation assay for rhodamine -123 uptake [9]:

Leukemic blast cells were resuspended at  $10^6$ /ml and incubated with 200 ng/ml Rh123 (Sigma) with and without 5  $\mu$ g/ml cyclosporin A at 37°C for 75 min and then pelleted at 4°C. Cells were then rinsed in cold PBS and resuspended in propidium iodide 10  $\mu$ g/ml to be able to exclude dead cells.

*Flow cytometric analysis for Rh123 efflux assay (Fig. 1):*

- Percentage cells with +ve efflux: A sample is considered +ve if  $\geq 10\%$  of the cells stained with Rh123 alone revealed a lower Rh123

fluorescence intensity as compared with cyclosporin + Rh123.

- Rh123 efflux ratio:

$$\frac{\text{Mean channel number of cyclosporin + Rh123}}{\text{Mean channel number of Rh123 alone}}$$

A case is considered positive at a ratio of  $\geq 1.1$ .

*P-glycoprotein (MDR) expression [10]:*

Indirect fluorescent staining was performed using 4E3 or UIC2 mAb. One ml hemolyzing solution was added to 100ul heparinized blood, shaken, centrifuged, the cell pellet resuspended in PBS and incubated with 10ul primary Ab for 1hr. Secondary Ab (goat antimouse IgG FITC) was added, incubated for 30 minutes in the dark at 4°C; cells were washed and resuspended in PBS. An isotype control was run in parallel using mouse IgG2A as the primary Ab. Test and control were analyzed using Coulter XL flow cytometry.

*Percentage cells with P-gp expression:* A sample is considered +ve if  $\geq 5\%$  of the cells were stained with the mAb.

*Relative fluorescent intensity (RFI) or ratio:*

$$\frac{\text{Mean channel number of specific Ab}}{\text{Mean channel number of isotypic control}}$$

A case is considered positive at a ratio of  $\geq 1.1$ .

*Statistical analysis [17]:*

SPSS statistical package (version 10.0) was used for data analysis. Mean and standard deviation were estimates of quantitative data and median (range) of non-normally distributed data. Two-Sample Kolmogorov-Smirnov test compared 2 independent groups and Kruskal Wallis ANOVA compared more than 2 independent groups. Chi-square/Fischer exact were tests of proportion independence. Spearman rank test (non-parametric correlation analysis) was used to measure association of quantitative variables. ROC (receiver operating characteristic curve) was used to show the relationship between sensitivity and specificity for MDR expression with different cut-off points. Kaplan-Meier method was used for estimating survival and Breslow test to compare curves. *p* value is significant at 0.05 level.

## RESULTS

P-gp expression was encountered in 19/72 (26.4%) ALL cases and in 10/21 (47%) ANLL cases. Positive Rh123 efflux was encountered in 48/78 (61.5%) ALL and 21/28 (75%) ANLL cases.

In ALL cases, there was no relation between neither P-gp expression nor Rh123 efflux on one side and immunophenotype on the other (Table 1). DNA ploidy studies showed that 28 cases were diploid (DI 0.99-1.01), 14 near diploid (DI > 0.95-0.98 or 1.02-1.04), 5 hyperdiploid with DI  $\geq 1.16$ , 10 hyperdiploid with DI < 1.16 and 5 hypodiploid (DI < 0.95). No association was encountered between DNA ploidy and neither P-gp expression nor Rh123 efflux.

In ANLL, positive Rh123 efflux was detected in 15/20 (75%) M1/M2 cases, 4/4 (100%) M3 and 2/4 M4 and M5 cases. The corresponding figures for P-gp expression were 12/19 (63.2%), 2/4 (50%) and 1/3 (33%) respectively. The differences were statistically insignificant.

The association of CD34 expression with P-gp expression and Rh123 efflux is shown in Table (2). The only association was a higher incidence of P-gp expression in CD34 +ve compared to CD34 -ve cases (64.3 vs 33.3%) but statistical significance was not attained.

Strong positive correlation was obtained between Rh123 efflux ratio and % cells with positive efflux ( $r$  0.827,  $p$  < 0.0001, Fig. 2). Strong positive correlation was, as well, obtained between P-gp expression expressed as % positive cells and as relative fluorescent intensity ( $r$  0.641,  $p$  < 0.002).

Disconcordance in Rh123 efflux assay between efflux ratio and % cells with positive efflux was encountered in 11 cases while disconcordance in P-gp expressed as % positive cells and as ratio was encountered in 8; disconcordance in both was encountered in one case (Tables 3,4).

No correlation was encountered between P-gp expression and Rh123 efflux with a disconcordance of 43.5% in ANLL and 64.7% in ALL; the majority showed +ve function and -ve expression (Table 5).

CR was achieved in 89.8% of ALL patients. No difference was encountered in CR rate neither between patients with -ve or +ve P-gp expression nor between patients with -ve or +ve Rh123 efflux. Comparing patients who achieved to those who did not achieve CR, statistically significant difference was not obtained neither for P-gp expression nor for Rh123 efflux. TALL patients who achieved CR (10 cases) had a lower Rh123 ratio with a mean of  $1.23 \pm 0.46$ , median of 1.14 and range of 0.64-23 compared

to a mean of  $1.64 \pm 0.48$ , median of 1.6 and range of 1.03-2.5 in those who did not achieve CR (7 cases), but the difference was found to be statistically insignificant. However, using continuous variable analysis, CR could be predicted in TALL at a Rh123 efflux cutoff ratio of 1.25 with a sensitivity of 80% and specificity of 62.5% with a total accuracy of 70.6%. DFS at 2 years was obtained in 92.3% and EFS in 72.2% in ALL cases. There was no impact of P-gp expression or Rh123 efflux on neither.

Fig. (1): Eh123 efflux assay.

- A: Gating on the living cells.
- B: Rh123 efflux ratio (curves superimposed), log changed into linear by software Coulter software used (XL versionII) and WinMidi software.
- C: Gating on Rh123 and cyclosporin A.
- D: Previous gate is posed on Rh123 alone showing efflux +ve assay.

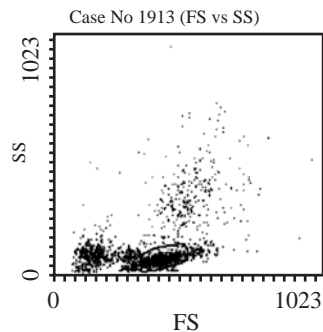


Fig. (1-A)

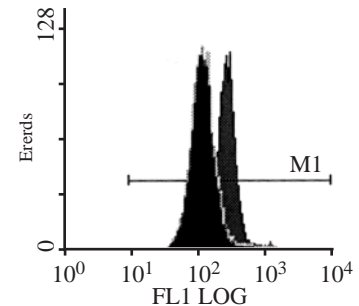


Fig. (1-B)

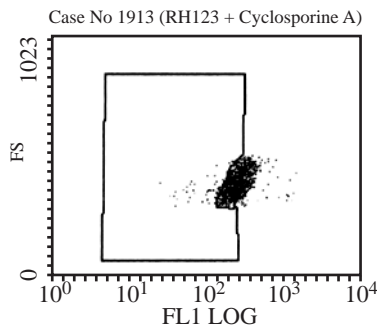


Fig. (1-C)

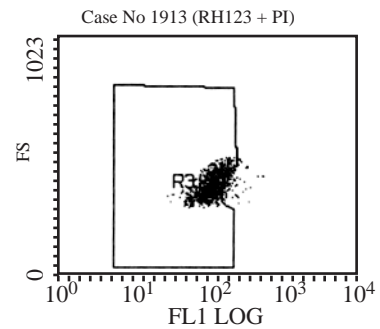


Fig. (1-D)

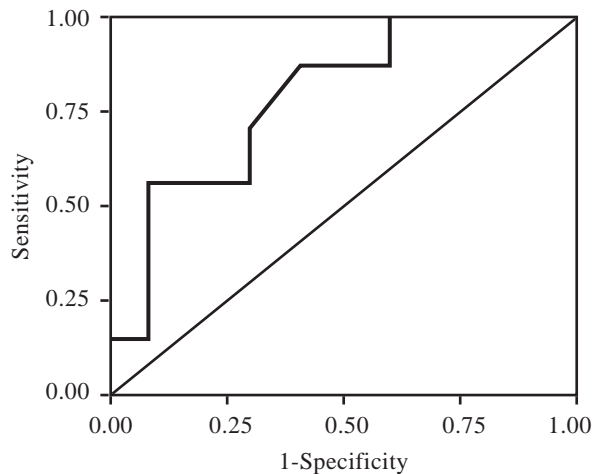


Fig. (2): Roc curve for predicting CR in T ALL through Rh123 efflux ratio.

Table (1): Rh123 efflux assay and P-gp expression in pediatric ALL cases in relation to immunophenotype.

Phenotype	Rh efflux		GP 170 expression	
	No.	%	No.	%
Pro B	4/7	57.1	3/7	42.9
CALL	15/21	71.4	6/20	30
Pre B	14/26	53.8	6/26	23.1
T ALL	15/24	62.5	4/19	21.1
Total	48/78	61.5	19/72	26.4

Table (2): Rh123 efflux assay and P-gp expression in relation to CD34 expression.

	CD34 +ve		CD34 -ve	
	No.	%	No.	%
<i>Rh efflux:</i>				
ANLL	13/17	76.5	8/11	72.7
ALL	22/29	75.8	28/48	58.3
<i>P-gp expression:</i>				
ANLL	9/14	64.3	3/9	33.3
ALL	7/27	25.9	10/34	29.4

Table (3): Disconcordance in Rh efflux assay between efflux ratio and % cells with +ve efflux in various ALL and ANLL cases.

Case	Cells %	Efflux ratio	CD 34%	Case	Cells %	Efflux ratio	CD 34%
M1	3	1.14	77	Pre B	21	1.08	8
Pro B	6.5	1.37	62	Pre B	9	1.1	1
CALL	3	1.17	70.5	T Early	20	1.08	25
CALL	21	1.09	0.3	T CD8+	8	2.5	1
CALL*	7	1.28	64.7	T CD8+	6	1.2	0.4
Pre B	16	1.08	23.5				

\* Showed disconcordance in P-gp expression.  
 N.B.: +ve efflux ≥ 10% +ve cells and/or ratio ≥ 1.1.

Table (4): Disconcordance between % +ve cells and florescent ratio of P-gp expression in various ALL and ANLL cases.

Case	Cells %	Ratio	CD 34%	Case	Cells %	Ratio	CD 34%
AML:M1	6.8	0.6	23	CALL*	1.9	1.39	64.7
AML**	2.5	1.13	0.4	Pre B	5	0.6	0.1
AML**	10	1	6	Pre B	0.2	1.1	24
AML:M4	0.26	1.75	21	B/M	5	0.9	59

\* Showed disconcordance in Rh123 efflux assay.  
 \*\* FAB subtype was not available.  
 N.B.: +ve P-gp ≥ 5% +ve cells and/or ratio ≥ 1.1.

Table (5): Disconcordance between MDR function (Rh123) and expression (P-gp).

	No.	R+/E+	R+/E-	R-/E+	R-/E-
ANLL	23	13 (56%)	8 (34.8%)	2 (8.7%)	0 (0.00%)
ALL	68	7 (10.3%)	37 (54.4%)	7 (10.3%)	17 (25%)

R: Rh123 efflux. E: P-gp expression.

### DISCUSSION

In this work we performed MDR functional assay and P-gp expression in 108 pediatric AL cases including 28 ANLL and 80 ALL. We used both % cells with +ve Rh123 efflux and Rh123

efflux ratio to evaluate MDR function and both % +ve cells and relative florescent intensity to evaluate P-gp expression.

As expected, there was positive correlation between the 2 methods for each in different cases. The correlation, however, was not absolute and a certain degree of disconcordance was encountered. As regards the functional assay it was encountered in 11 cases of which 5 were positive by percentage and negative by ratio, the other 6 showed the reverse. A similar situation was encountered with P-gp expression where disconcordance was encountered in 8 cases, 4 of them were +ve by percentage and negative by ratio and 4 showed the reverse. This finding raises a question about which would be more relevant. A large fraction of cells with a relatively low efflux power might be overcome by a high therapeutic dose. A small fraction of cells with high efflux power, on the other hand, may not pose an initial problem in response to therapy but might ultimately expand as a resistant clone with growth advantage and causes relapse. In this work no correlation was encountered between Rh123 efflux and P-gp expression with disconcordance of 43.5% and 64.7% in ANLL and ALL cases respectively. Though many similar reports are encountered in the literature [18,19], our figures are higher. A disconcordance rate of 27-37% was previously reported [18-21]. Studying a large number of 352 AML cases [21], Rh123 +ve/P-gp -ve phenotype was encountered in 15% of the cases, Rh123 +ve/P-gp -ve/MRP -ve/LRP -ve in another 18% while P-gp +ve/Rh123 -ve was encountered in 4% with an overall disconcordance rate of 37%. The disconcordance was reported to be independent of leukemia type and to be comparable in children and adults [18].

On the other hand, correlation between MDR1 expression and Rh123 efflux was reported by other authors [20,22,23] but even within these reports concordance was not absolute. In our series, the disconcordance was mainly in the form of Rh123 +ve/P-gp -ve phenotype (49.5% vs 9.9%). Comparable results were obtained by Moeloose et al. [18] (25% vs 5%) but not by Poulain et al. [24] (9% vs 18%); but these latter authors reported on 34 cases only. Working on 60 cases, Leith et al. [20] reported relative balance between the two phenotypes (25% vs 30%) but working on a larger series of 352 AML cases, the same authors [21] reported

Rh123 +ve/P-gp -ve and Rh123 -ve/P-gp +ve in 33% and 4% respectively which is comparable to our results.

In general the discordance may be explained by the presence of a nonfunctioning P-gp. The other way round, proteins other than P-gp are involved in drug efflux. The relatively high incidence of +ve Rh123 efflux and lack of P-gp expression suggests that the latter is not the main contributor to +ve efflux assay in our cases especially ALL. This suggestion is supported by the findings of Den Boer et al. [25] who reported that accumulated Daunorubicin concentration was inversely correlated with LRP but not with P-gp or MRP. It is also supported by the presence of 18% among a large number of 352 AML cases with Rh123 +ve/P-gp -ve/LRP -ve/MRP -ve phenotype [21] as previously mentioned. This necessitates studying the 2 other candidate proteins namely LRP and MRP as well as the recently described breast cancer resistance protein (BCRP) claimed to play a major role in drug resistance [26].

In this work the incidence of MDR +ve functional assay in ANLL is in concordance with the literature, though we failed to detect correlation neither to CD34 expression nor to FAB subtypes. Data in the literature about P-gp and Rh123 efflux association with FAB subtypes are controversial. Similar to our results, lack of correlation was previously reported [27]. On the other hand higher expression of P-gp was reported in M1/M2 compared to M3, M4 and M5 (37% Vs 10%) [21]. Lower expression of P-gp in M3 was documented in another series [28]. In harmony with these findings Rh123 efflux was reported to be higher in M1 and M2 [7] and to be absent in M5 [29]. In contrast, lower +ve Rh123 efflux was reported in M1 and M2 compared to M4 and M5 [12,26]. The marked controversy may be mainly attributed to the small numbers studied in most series including ours. Still an appreciable difference is the Rh123 efflux encountered in the four M3 cases tested in our study; a larger number of cases is needed to verify.

Unlike the FAB subtypes, association of P-gp expression and Rh123 efflux with CD34 expression is documented in most reports [12,20,23,29]. The only association with CD34 in our series was encountered with P-gp expression in ANLL. A similar, also insignificant, associa-

tion was encountered by Suarez et al. [30], while Thorova et al. [27] reported CD34 association only in cases with > 50% P-gp expression. The lack of association of Rh123 efflux with CD34 expression was also detected in our previous series [16] which makes it a consistent finding in our patients. However, double labeling for CD34 and Rh123 was not performed and hence direct correlation at the individual cell level was not investigated.

The high incidence of MDR +ve functional assay in our pediatric ALL cases was not previously reported. We assumed that this finding might explain, at least partly, the low survival rates obtained in our patients compared to those in the literature. However, the results obtained in this work showed no impact on CR or survival in ALL of neither MDR function nor P-gp expression except for an impact of Rh123 efflux on CR in TALL. Lot of controversy is reported in the literature about the prognostic value of MDR function and/or P-gp expression. P-gp expression was claimed to be associated with lower CR rate in both ANLL and ALL [31]. In adult ANLL P-gp expression was reported to be associated with lower CR rate [10,24,27,31,32] and shorter CR duration [27]. It was claimed to be a better predictor for CR than the functional assay [10,24] suggesting an additional role of P-gp in mediating drug resistance besides that of drug efflux pump [10]. Such an impact was denied by some authors [7] and was attributed to another MDR associated protein namely LRP by others [23]. Rh123 efflux was also claimed to have impact on CR in adult ANLL patients [7,26,34] as well as on survival and relapse rate [7]. Impact on survival, however, was denied by other authors [13,26, 34,35,36].

Studies of P-gp and Rh123 efflux in ALL are more limited. P-gp expression was claimed to have an impact on CR in adult ALL [31], to be significantly associated with lower CR, lower OS and EVS and higher relapse independent of age, TLC, phenotype and karyotype [13,37]. Such an impact has been denied by other authors [7]. In this study an impact of Rh123 efflux, but not of P-gp expression, on CR in TALL was encountered. One recent study has reported an impact of P-gp expression on OS and EFS in TALL, mostly in adults; using multivariate Cox analysis, it was claimed to be the only significant prognostic factor [35].

In Conclusion, Rh123 efflux assay is the standard method to evaluate MDR status; the relative importance of the % Rh123 efflux +ve cells and efflux ratio needs to be evaluated. P-gp expression is not correlated to Rh123 efflux, other relevant proteins involved in MDR need to be studied. Rh123 efflux might be a predictor of CR only in TALL. The high incidence of MDR +ve functional assay in our pediatric ALI cases is not among the factors contributing to bad treatment outcome.

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