Quantitative analysis of minimal residual disease by PCR in childhood acute lymphoid leukemia

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Chapter 6

NO CLEAR RELATION BETWEEN IN VITRO DRUG RESISTANCE AND LEVEL OF MINIMAL RESIDUAL DISEASE (MRD) AS DETECTED BY PCR AT THE END OF INDUCTION THERAPY IN CHILDHOOD ALL

Submitted for publication
No clear relation between in vitro drug resistance and level of Minimal Residual Disease (MRD) as detected by PCR at the end of induction therapy in childhood ALL

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ABSTRACT

Still 20-30\% of children with acute lymphoblastic leukemia (ALL) suffer from a relapse. Measurement of drug resistance by the colorimetric Methyl Thiazol Tetrazolium (MTT) assay determines the cellular response to chemotherapy in vitro. The level of minimal residual disease (MRD) at the end of induction therapy as determined by quantitative PCR measures drug response in vivo. Both techniques have shown their significant value to predict relapse and can thus be used to modify treatment. However, the correlation between the data obtained by these different techniques is unknown. We retrospectively studied the correlation between these two predictors of outcome in 20 patients, diagnosed with B-precursor- (n=17) or T-ALL (n=3).

MRD levels ranged between less then $1.0 \times 10^{-5}$ and $1.0 \times 10^{-1}$ (median $2.6 \times 10^{-4}$). LC50 values (drug concentration lethal to 50\% of the cells) differed more than 1000-fold for prednisolone and L-asparaginase, and more than 100-fold for vincristine between individual patients. We were not able to show a correlation between the combined or single drug sensitivity and the level of MRD at the end of induction. For prednisolone sensitivity, a trend was seen for a correlation between the MTT assay and MRD (rho 0.45, p=0.08). We hypothesize that this correlation reflects the strong influence of prednisolone on the initial treatment response, which can be used to determine the risk for relapse. This lack of a correlation between the results of both assays warrants a prospective clinical study to investigate whether a combination of both approaches will increase the predictive value of the individual assay.
INTRODUCTION

Currently therapy in children with ALL has resulted in a survival rate of over 80%, however still 20-30% of children ultimately suffer from a relapse. In order to reduce long-term effects, using less intense therapy in low-risk patients, individualization of treatment is needed. Also identification of resistant leukemia cases is needed to increase survival rates. Therefore more sensitive methods to provide information on the efficacy of cytostatic treatment have been developed. The clinical response of leukemia cells to chemotherapy can be predicted by in vitro drug resistance assays and can be measured in vivo by the kinetics of MRD at the end of induction therapy and during early follow-up.

Accurate measurement of cellular drug resistance by in vitro drug resistance assays, e.g. the colorimetric MTT assay, provides prognostically significant information. It has also been shown that relapsed leukemia samples are more drug resistant in vitro, in agreement with clinical experience.

It has been demonstrated that the level of MRD measured by PCR, using antigen rearrangements as a target, at the end of induction therapy predicts long term outcome in ALL. Precise quantification of cell reduction after initiation of therapy to document the speed in reduction of leukemia burden, may give even more specific information about the effectiveness of therapy. Both tests might give the same estimation on relative therapy resistance. The MTT assay is non-laborious and results are available within one week after obtaining the leukemic cells, which allows an early change of therapy. Quantitative detection of MRD using antigen rearrangements as targets is quite laborious, although real-time quantitative RQ-PCR techniques have decreased the time expenditure considerably. However, it is not known yet how the results of the two tests correlate and whether both tests recognize the same patients.

Therefore, bone marrow samples of in total 20 ALL patients were analyzed in this retrospective study, using the MTT-assay as well as the PCR-based MRD detection to determine the correlation between their results.

MATERIALS AND METHODS

Patients and Materials

Initially, bone marrow samples from 31 children were obtained at diagnosis and at the end of induction therapy (42 days of treatment). In eight patients, information on in vitro drug resistance was not obtained because of a too low OD signal in drug-free control cultures or too low (<70%) percentage of ALL cells in drug-free control cultures after 4 days. In three patients, it was not possible to develop an allele-specific primer that reached a minimal
sensitivity of $10^4$. The remaining 20 patients were successfully analysed in both assays. Seventeen patients were diagnosed with B-precursor ALL and three patients had T-ALL. Median follow-up time was 75 months (ranging from 24 to 186 months).

The patients were selected on the basis of presence of sufficient bone marrow samples at diagnosis and at the end of induction. The mononuclear cell fractions were isolated by Ficoll-Hypaque (1.077 g/cm3; Pharmacia, Uppsala, Sweden) density gradient centrifugation prior to cryopreservation. After thawing, one part of the leukemic cells (minimal $10^6$) was used in the MTT-assay, whereas from the other part DNA-isolation was performed on $5.10^6$ cells in order to develop allelic specific (ASO)-primers. Genomic DNA was extracted from mononuclear cell fraction from normal lymphocytes (MNC) with the QIAamp Blood kit (Qiagen, Chatsworth, CA, USA), as described previously. Immunophenotyping was routinely performed at diagnosis using standard techniques. Southern Blot analysis was performed on samples of each patient to detect IGH or TCR-gene rearrangements.

Sixteen patients were treated according to the BFM strategy-based protocol ALL-8 of the Dutch Childhood Leukemia Study Group (DCLSG), three patients according to protocol ALL-9 and one patient according to protocol ALL-6 (Table 1). The risk-group classification slightly differed between the treatment protocols. Induction therapy included prednisolone, vincristine, and L-asparaginase for protocol ALL-8, whereas in protocol ALL-6 and ALL-9 prednisolone was replaced by dexamethasone. The DCLSG provided us with ten bone marrow samples from ALL patients; the other ten patients were all treated in the Emma Kinderziekenhuis AMC.

**In vitro drug resistance**

In vitro drug resistance was measured using the cell culture MTT-assay. Thawed cryopreserved leukemic cells from 20 patients and fresh leukemic cells from 11 patients, taken at diagnosis, were cultured in RPMI 1640 (GIBCO, Uxbridge, UK), supplemented with fetal calf serum, penicillin, streptomycin, fungizone, gentamycin, glutamine, insulin, transferrin and sodium selenite. Three drugs (vincristine, prednisolone and L-asparaginase), each at six concentrations, were tested as previously described. Leukemic cells were incubated with each drug at each concentration in duplicate in wells of microculture plates at 37°C in humidified air with 5% CO₂, at a final concentration of $1.6 \times 10^6$ cells per ml. Six wells contained leukemic cells in drug-free medium to determine the survival of the control cells to compare with survival of leukemic cells in presence of various drugs. Six wells containing medium only were used as blank specimens for spectrophotometric analysis. After 4 days, $10^{-1}$ (5mg/ml) MTTsalt (Sigma Chemical Corp, St Louis, MO) was added for 6 hours. MTT is reduced by living cells only into colored formazan crystals. The MTT
crystals were dissolved with 100 μl of acidified isopropanol, and formazan production was quantified using a spectrophotometer at 562 nm. Since the optical density (OD) is linearly related to the cell number, the leukemic cell survival (LCS) was calculated at each drug concentration using the equation LCS = (OD treated well/mean OD control wells) x 100%, after correction for the blank. The drug concentration lethal to 50% of the ALL cells (LC50) was used to express the level of drug resistance. Samples were considered evaluable if the drug-free control wells contained more than 70% leukemic cells after 4 days of culture and if the control optical density at day 4 exceeded 0.050. The MTT assay gives reliable results under these conditions. The percentage of leukemic cells was determined by May-Grunwald-Giemsa staining and light microscopy. Previous studies have shown that the use of cryopreserved versus fresh samples in the MTT assay, did not alter the drug sensitivity results.

Identification of antigen receptor PCR targets at diagnosis

Complete V_H-D-J_H rearrangements of the IGH gene were amplified with family specific Framework Region 1 primers (V_H1/7, V_H2, V_H3, V_H4/6, V_H5) or consensus Framework Region 3 (FR3), in combination with the consensus Jh21 reverse primer. Immunoglobulin Kappa Deleting rearrangements (IGK) were amplified using either VK family primers or the intron RSS primer in combination with a Kde primer. For the rearrangements of the TCR-delta (TCRD), respectively TCR-gamma (TCRG) receptor gene, the consensus forward V82-3, respectively V_y family primers, were used in combination with the D83-3, respectively J_y reverse primer. Generally, major PCR bands on ethidium bromide stained polyacrylamide gels were excised and eluted in 10mM Tris pH8.0. After a second round PCR with the same primer combination, the PCR product was directly used for the sequence analysis using the Big Dye terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 377 Automated Sequencer (PE Biosystems, Foster City, CT, USA). Ig/TCR gene rearrangements were identified using DNAPLOT software (W. Muller, H-H. Althaus, University of Cologne, Germany)(http://www/mrc-cpe.cam.ac.uk/imt-doc/).

Quantification of MRD by Real-time Quantitative PCR (RQ-PCR)

On basis of the sequence data of the Ig and TCR gene rearrangements, primers and probes were designed using Primer Express (PE Biosystems) and Oligo 6 software (W. Rychlik, National Biosciences, Plymouth MN USA), according to the manufacturer's guidelines. Allele-specific oligonucleotides (ASO's) were developed complementary to the V_H-D_H, V_k-Kde, V82-D83 or V_y-J_y junctional regions. The forward primer always contained the
N-region of the junctional region. The reverse primer was designed complementary to the intron downstream of each J_\text{H} \text{ segment respectively} K\text{de}, D83, J_\gamma.\text{22,23} For RQ-PCR analysis the TaqMan 1000 reactions Gold with Buffer A kit (PE Biosystems) was used. Reaction mixtures and conditions were as described previously.\text{40} Real-time information was obtained using ABI-PRISM 7700 Sequence Detection System containing a 96-well thermal cycler (PE Biosystems). A standard curve for quantifying the leukemia-specific-quantities in follow-up samples, is established using a serial dilution of diagnosis DNA. To correct for the quantity and amplifiability of DNA in the follow-up samples, the albumin gene was used as a control.\text{40} An albumin standard curve was established using MNC DNA diluted in water in 10-fold dilutions. Corrections on MRD quantities in follow-up samples were performed according to the manufacturer’s guidelines (PE biosystems). Briefly, the quantities established by the leukemia-specific target were divided by that of albumin. Subsequently, the quantity at diagnosis was set at 1.0 and leukemia-specific quantities in follow-up samples were related to the value at diagnosis, in order to obtain an accurate determination of the tumor load. Standard deviations were corrected accordingly. For the standard curve, all RQ-PCR experiments were carried out in duplo and the follow-up samples were analysed in triplicate. Several negative controls were included.

**Quantification of MRD in a Limiting Dilution Assay (LDA)**

To quantify residual leukemia cells, limiting dilution assays were performed with a seminested patient-specific PCR using a consensus primer set FR3/Jh21 or a consensus primerset V\delta2-3 or D\delta2-3/D\delta3-3 in the first reaction, and a clone specific sense primer, designed complementary to each J_\text{H} segment, in the second round on two-fold serial diluted DNA samples as described previously.\text{76} Reactions were performed in mixtures (total volume 50\text{$\mu$L}) as previously described.\text{76} The PCR protocol took place in a thermal cycler (Perkin Elmer Cetus Model 9600; Norwalk, CT). The second-round of the heminested PCR had an input of 2\text{$\mu$L of PCR product from the first PCR reaction. After testing the ASO-primers at various temperatures, it turned out that 60°C was the ideal annealing/extension temperature for most of these primers. In preliminary experiments, five-fold serial dilutions of DNA samples in water were tested in five-fold replicates to investigate at which dilution the PCRs became negative. Subsequently, two-fold serial dilutions in 20-fold replicates were tested ranging from the highest preliminary dilution showing amplification to the lowest preliminary dilution not showing amplification in the five-fold diluted samples. After size separation each PCR product was visualized by ethidium bromide staining. Specificity was tested by amplification of 1\text{$\mu$g of normal DNA from peripheral blood mononuclear cells. Sensitivity was tested by performing PCR on serial dilutions of
leukemia DNA mixed with DNA isolated from normal mononuclear cells. The number of
cells at each dilution was verified by amplification of the FcγReceptorIIIb gene, present in
each cell. The ratio of leukemia cells to total bone marrow cells was calculated from the
ratio of leukemia to Fc-receptor (FCR) targets, considering the presence of one rearranged
Ig allele and two FCR genes per cell. Because both nested PCRs are able to detect a single
cell, the number of positive PCR reactions at a certain dilution is distributed according to a
Poisson distribution, and as such quantification of malignant cells is possible. The
mean number of targets required to give a positive reaction was determined by the Taswell
method. A computer program, developed at the University of Tilburg and Maastricht by
Leo Strijbosch was used to perform the necessary calculations. We have previously
compared this limiting dilution analysis with the RQ-PCR using germline Ig TaqMan
probes, both approaches gave comparable results.

Statistical analysis

Correlation for the continuous variables was studied using non-parametric tests, i.e.
Spearman test. A drug sensitivity profile was made for each patient by combining the
results of in vitro prednisolone (PRD), vincristine (VCR) and L-asparaginase (ASP)
cytotoxicity and resulted in an individual “PVA-score” that varied between 3 (sensitive to
all three drugs) and 9 (resistant to all three drugs). The MRD level \( \leq 1.0 \times 10^{-4} \) was defined
as low MRD, MRD level between \( 1.0 \times 10^{-4} \) and \( 1.0 \times 10^{-2} \) as intermediate MRD and finally
an MRD level \( \geq 1.0 \times 10^{-2} \) was described as high MRD, based on results of previous
(semi)quantitative MRD studies. Statistical analysis for the different groups was
performed by the Wilcoxon matched pairs signed-ranks and Mann-WhitneyU tests. Both
tests were used two-tailed at the level of significance 0.05.

RESULTS

20 Patients were successfully analyzed in both MRD detection and MTT assay.
Quantification of MRD by PCR was done for 10 patients using a limiting dilution assay and
for 10 patients by real-time quantitative RQ-PCR. In nine patients an \( IGH^- \), in four patients
an \( IGK^- \), in five cases a \( TCRD^- \) and in two cases a \( TCRG^- \)-rearrangement was used as a
target in the PCR reaction.

Results of drug resistance testing

LC50 values for all drugs varied markedly between the patient samples as shown in table1.
The LC50 for VCR ranged from less than 0.049 to 28.182 \( \mu \text{g/ml} \) (median 0.65 \( \mu \text{g/ml} \)). The
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LC50 for L-ASPA ranged from less than 0.002 to >10.0 IE/ml (median 0.10 IE/ml). PRD LC50 values ranged from 0.029 to more than 250.0 g/ml (median 0.925 g/ml).

Table 1: Results of MTT assay for PRD, VCR and ASPA and MRD detection by LDA/RQ-PCR

<table>
<thead>
<tr>
<th>Pt</th>
<th>Immuno-phenotype</th>
<th>Treatment</th>
<th>LC50 PRD (µg/ml)</th>
<th>LC50 VCR (µg/ml)</th>
<th>LC50 ASPA (iu/ml)</th>
<th>PVA score*</th>
<th>MRD Level</th>
<th>MRD #</th>
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<tbody>
<tr>
<td>1</td>
<td>Pre BALL</td>
<td>8</td>
<td>2.880</td>
<td>2.273</td>
<td>0.033</td>
<td>6</td>
<td>5.0 x 10^-4</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Pre BALL</td>
<td>8</td>
<td>13.020</td>
<td>28.182</td>
<td>0.748</td>
<td>7</td>
<td>1.0 x 10^-3</td>
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</tr>
<tr>
<td>3</td>
<td>Pre BALL</td>
<td>8</td>
<td>0.363</td>
<td>10.795</td>
<td>1.179</td>
<td>8</td>
<td>1.0 x 10^-4</td>
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</tr>
<tr>
<td>4</td>
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<td>0.480</td>
<td>0.679</td>
<td>1.314</td>
<td>7</td>
<td>6.0 x 10^-5</td>
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<tr>
<td>5</td>
<td>T-ALL</td>
<td>8</td>
<td>1.370</td>
<td>0.195</td>
<td>0.015</td>
<td>4</td>
<td>9.0 x 10^-5</td>
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</tr>
<tr>
<td>6</td>
<td>Pre BALL</td>
<td>8</td>
<td>24.740</td>
<td>1.333</td>
<td>1.644</td>
<td>7</td>
<td>3.0 x 10^-4</td>
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</tr>
<tr>
<td>7</td>
<td>T-ALL</td>
<td>8</td>
<td>251.0</td>
<td>0.629</td>
<td>0.054</td>
<td>6</td>
<td>1.0 x 10^-1</td>
<td>3</td>
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<tr>
<td>8</td>
<td>Pre BALL</td>
<td>8</td>
<td>251.0</td>
<td>0.900</td>
<td>0.054</td>
<td>7</td>
<td>4.0 x 10^-2</td>
<td>3</td>
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<tr>
<td>9</td>
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<td>251.0</td>
<td>2.188</td>
<td>11.000</td>
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<td>0.029</td>
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<td>1.685</td>
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<td>0.550</td>
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<td>0.365</td>
<td>0.012</td>
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<td>&lt;1.0 x 10^-5</td>
<td>1</td>
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<td>251.0</td>
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<td>0.050</td>
<td>4.625</td>
<td>1.265</td>
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</tr>
<tr>
<td>20</td>
<td>Pre BALL</td>
<td>6</td>
<td>251.0</td>
<td>0.040</td>
<td>0.002</td>
<td>5</td>
<td>8.4 x 10^-3</td>
<td>3</td>
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</table>

* PVA-score represents the combined score of in vitro prednisolone, vincristine and L-asparaginase cytotoxicity: varying from 3 (sensitive to all agents) to 9 (resistant to all three drugs).

** MRD profile: #1=low level<10^-4, #2 intermediate level 10^-2 – 10^-4 and #3=high level >10^-2

Results of PCR-based MRD detection

In all PCR reactions a minimal sensitivity of 10^-4 was reached. Levels of MRD, as determined at the end of induction therapy, ranged from < 1.0 x 10^-5 to 1.0 x 10^-1. The mean MRD at this time-point was 9.0 x 10^-3 (median 2.6 x 10^-4). Distribution was not according to a Gaussian pattern. In five out of 20 cases, the MRD level was higher than 8.4 x 10^-3. The other patients had low MRD levels below 1.5 x 10^-3. Considering the three groups with different levels of MRD, eight patients had low MRD, eight patients had intermediate levels of MRD and four patients were having high MRD.

Comparison of MTT and PCR results

The MRD level at the end of induction therapy was not significantly related to the combined PVA-score (p=0.48). Analysis of the individual drug sensitivity of VCR and L-
In vitro drug resistance and MRD

ASPA did not correlate at all with the MRD level at the end of induction therapy (p=0.8; p=0.5 respectively). For prednisolone sensitivity, a trend was seen for correlation between the MTT-assay and MRD assays, but the correlation was too weak to become significant in this small group (rho 0.45, p=0.08). Analysis of all 20 patients (although differently treated) showed a significant correlation between the LC50 for PRD and the MRD level (rho 0.49, p=0.015)(figure). The rho-values for the other drugs were not influenced.

Also looking at the drug sensitivity for PRD, VCR and ASPA versus the patients with low or high MRD level, a correlation between both assays could not be demonstrated (p=0.77, p=0.62; p=0.72 respectively).

![Figure](image.png)

**Figure.** Correlation between LC50 prednisolone and MRD level, as determined by RQ-PCR and LDA in the total group of patients (p=0.015). The four differently treated patients are indicated by (*).

**DISCUSSION**

A considerable number of ALL patients relapses and these relapses can only partially be predicted with the current risk factors such as age, white blood cell count, immunophenotype and genotype. An additional prognostic factor to determine the risk for relapse is the response to chemotherapy, which can be measured both in vivo and in vitro. The two used techniques in this study, i.e. the determination of in vitro drug resistance by the MTT-assay at diagnosis and in vivo detection of MRD by PCR using gene rearrangements as a target during follow-up, have individually shown strong prognostic significance.\(^3\)\(^4\)\(^7\)\(^9\)\(^21\)\(^87\)\(^96\) It seems likely, considering the high prognostic significance of both factors, that the same patients are identified as low or high risk. We compared both
techniques to examine whether they measure the same relative therapy resistance or whether they may complement each other.

This study with both tests successfully performed in 20 children with ALL, showed no significant correlation between in vitro drug resistance and the level of MRD as detected by PCR at the end of induction therapy for the group of 16 equally treated patients. Both the combined drug resistance, as defined in the PVA-score, and the drug sensitivity to prednisolone, vincristine and L-asparaginase as single drugs, were not correlated to the MRD kinetics. Also comparison of low/high MRD levels in PRD, VCR and ASP sensitive versus resistant cases showed no significant differences.

A previous study in a small number of children with ALL (n=29) as well, has shown a significant correlation between the LC50 for PRD with the MRD level. In our study with 16 patients, we also see a trend for correlation between the LC50 for PRD as a single agent and the MRD level at the end of induction (rho 0.45, p=0.08). Moreover, if we included four extra patients in our analysis, we were able to show a significant correlation (rho 0.49, p=0.015). Nevertheless, we have to remark that these four patients were differently treated from the other 16 patients, with dexamethasone instead of prednisolone during induction therapy. Glucocorticoids are highly cytotoxic to lymphoblasts and are thus important in the treatment of childhood ALL. A poor in vivo clinical response to initial PRD monotherapy was shown to be associated with poor prognosis. Acute Lymphoblastic Leukemia protocols of the BFM Study Group, some of which were also used by the Dutch Childhood Leukemia Study Group (ALL-7 & 8), used the clinical response to one week PRD monotherapy as one of the determinants of the risk-group stratification. Previously, a significant correlation was reported between the anti-leukemic activity of PRD in vitro and the in vivo response to PRD monotherapy. Similarly, study of MRD during induction therapy has shown that the rapid decrease of MRD in the first month of treatment, as determined by quantitative RQ-PCR at two and four weeks, has important consequences for prognosis and may be highly informative. This may be reflected in the PRD resistance data. We hypothesize that in early treatment a patient group can be defined, characterized by PRD resistance and high MRD levels, which have a high relapse risk. The lack of correlation in our equally treated patient group of patients may be due to the low number of patients. The correlation seen in our total patient group of 20 patients, indicates that at least a larger group of patients has to be studied to see if a real correlation between LC50 for PRD and MRD level exists.

In our study no correlation was found between the in vitro resistance to VCR and ASP as single agents versus MRD level at the end of induction therapy, while both drugs play a major role in induction treatment for ALL. In vitro resistance to the single agents PRD and ASP, but also to VCR, of ALL cells obtained at initial diagnosis, predicted the
occurrence of relapse.\textsuperscript{21,87} We have no explanation for the lack of a significant correlation between in vitro resistance to VCR and ASP with MRD levels, although patient numbers were small in this retrospective study.

It is important to stress that although both techniques are influenced by drug sensitivity, they are fundamentally different, as they take hold of two different mechanisms. Whereas the MTT assay reflects the in vitro drug resistance to a standard set of cytostatic drugs of a subset of leukemic cells that survive during a four-day culture period, the number of leukemic cells as detected by PCR is an in vivo measurement of kinetics of all leukemic cells and is dependent upon multiple factors like pharmacokinetics, dosage and administration of drugs, and proliferation of vital leukemic cells. These differences between the two techniques might explain the lack of a strong correlation between the two tests. Because both tests have been shown to predict relapse-risk in patients treated with this induction regimen, our results might implicate that a combination of both tests will give an even better correlation with disease outcome. However, it should be stressed that a relatively large group of patients had to be excluded from our initial patient group, because of a too low survival in control cultures.

In conclusion, we have not shown a correlation between in vitro drug resistance and the post-induction MRD level as detected by PCR. Both techniques, using tests that each have proven independent prognostic significance, may identify at least in part different patients at lower or higher risk of relapse, and combining the information obtained by both techniques may have additional prognostic significance. As these techniques may be implemented in routine clinical diagnostics and used for individualization of treatment, studies that show which of both techniques is the most informative, or whether both techniques, if used in tandem, will give the most relevant information, are important. A prospective study on a large number of equally treated patients, in which both techniques are performed and evaluated with regard to clinical outcome is warranted. Such a study is currently being performed in the Netherlands by the DCLSG (ALL-9) and in Germany by the COALL group (ALL-97). Within a few years, combined clinical and laboratory data will become available from these treatment protocols.

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