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The use of individualized tumor response testing in treatment selection: second randomization results from the LRF CLL4 trial and the predictive value of the test at trial entry

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The choice of treatment according to *in vitro* drug response testing was developed for chronic lymphocytic leukemia (CLL) as the Differential Staining Cytotoxicity assay.¹ This demonstrated that fludarabine-test-resistant patients treated with fludarabine had a poorer response rate and worse median survival than fludarabine-test-sensitive patients.² A simplified method, the tumor response to anti-neoplastic compounds (TRAC) assay, was developed and its value documented in CLL patients.³

We report the largest study in patients entered into a randomized trial, in three contexts:

- (1) As a randomized variable in nonresponders and relapsed patients to assess its value in the choice of second-line therapy.
- (2) Assessing *in vitro* sensitivity to therapy received, as a prognostic variable after second-line treatment.

- (3) At study entry, to ascertain whether the *in vitro* sensitivity to treatment received could predict outcome, and to assess correlation with other prognostic factors.

The LRF CLL4 trial was a multicenter study including 777 CLL patients requiring treatment, randomized from 1999–2004 to receive chlorambucil, or fludarabine alone or with cyclophosphamide. Response rates, progression-free survival (PFS), overall survival (OS), and the prognostic impact of clinical and biological variables, have been published.^{4,5} A second randomization (2000–2008) was incorporated for patients who were nonresponders, progressed while on first-line treatment, or relapsed within a year of remission. The trial was approved by a UK multicenter research ethics committee.

The TRAC assay was performed on blood samples. At trial entry, the result was not available to patients or physicians. The second randomization, using a new sample, was between 'TRAC-assay-guided' and 'protocol-guided' therapy.

Randomization was balanced with respect to age, gender, disease status and planned treatment. Before second randomization, physicians specified what treatment they planned to give. TRAC assay reports were only sent to the physician for patients allocated to assay-guided therapy. TRAC methodology was previously described.³ Drugs tested were chlorambucil, cyclophosphamide (mafosfamide *in vitro*), methyl-prednisolone, prednisolone, vincristine, doxorubicin, mitoxantrone, cladribine, fludarabine and pentostatin; and the drug concentrations lethal for 90% of cells (LC₉₀s) were determined. The report sent to doctors showed drug sensitivities as a drug sensitivity index—a percentage rank of LC₉₀s whereby 0% indicated the most resistant patient and 90% indicated very sensitive.³ For analysis, trial entry LC₉₀ results were compared with response. Cutoffs were determined for sensitive, intermediate and resistant. For drug combinations, all drugs needed to show resistance *in vitro* for the combination to be considered TRAC-resistant. For TRAC-sensitivity, at least one drug needed to show *in vitro* sensitivity. Patient response was assessed using the National Cancer Institute criteria. PFS was defined as the time from first randomization to relapse, progression or death. OS from randomization was the primary outcome. χ^2 was used for testing associations between categorical variables. Analyses of outcome used Kaplan–Meier curves and log-rank comparisons between groups. Cox regression was used for multivariate analysis. The follow-up was up to 31st October 2010 (median follow-up from trial entry was 7 years 6 months; from second randomization 5 years 9 months). *P*-values were two-sided.

(1) At second randomization, 84 patients were allocated to protocol-guided and 84 patients to TRAC-assay-guided treatment. Most (73%) had initially received chlorambucil; 40% were nonresponders and 60% had relapsed. There were no significant differences between the two groups regarding patient/disease characteristics (Supplementary Table 1). Fifty-two percent of physicians planned to give fludarabine as second-line treatment. The proportion of patients who did not receive the planned treatment did not differ significantly between the planned treatments (fludarabine 25%, cyclophosphamide, doxorubicin, vincristine and prednisolone 47%, fludarabine with cyclophosphamide \pm / – rituximab or mitoxantrone 30%). As expected, fewer TRAC-assay-guided patients received their planned treatment compared with protocol-guided (51 vs 83%, *P* < 0.0001). In the protocol-guided arm there was no difference in the proportion receiving their planned treatment by TRAC assay result (10/13 (77%) TRAC-resistant vs 51/60 (85%) TRAC nonresistant, *P* = 0.5). In the TRAC-assay-guided arm 0/10 (0%) of the TRAC-resistant patients received their planned treatment compared with 41/66 (62%) TRAC nonresistant (*P* = 0.0002) (Supplementary Table 2). This suggests that the report, when available, was used by the physicians to alter the planned treatment when drug resistance was indicated.

There were no significant differences between assay-guided vs protocol-guided in response rate (Supplementary Table 3) or OS (Figure 1a). However, survival at 1 year was better (*P* = 0.04). Results were not altered if stratified by age, stage, gender or risk group.⁵ Within the TRAC-resistant subgroup there was no significant difference in OS between the randomized arms (odds ratio = 1.04; 95% confidence interval: 0.72–1.49; *P* = 0.8).

(2) No patient in the TRAC-assay-guided arm received a treatment to which they were resistant, compared with 17% in the protocol-guided arm (*P* = 0.0002). Patients with greater *in vitro* sensitivity to the given treatment were more likely to respond (sensitive 57/68 (84%), intermediate 39/55 (71%), resistant 5/10 (50%)); (*P*(trend) = 0.01), independently of the randomized arm. There was no significant difference in survival from second randomization by TRAC assay (sensitive/intermediate/resistant) to the given treatment, *P*(trend) = 0.4 (Figure 1b); however, the 13 resistant patients appeared to fare worse than the others, odds ratio = 1.75 (95% confidence interval: 0.82–3.72), *P* = 0.2.

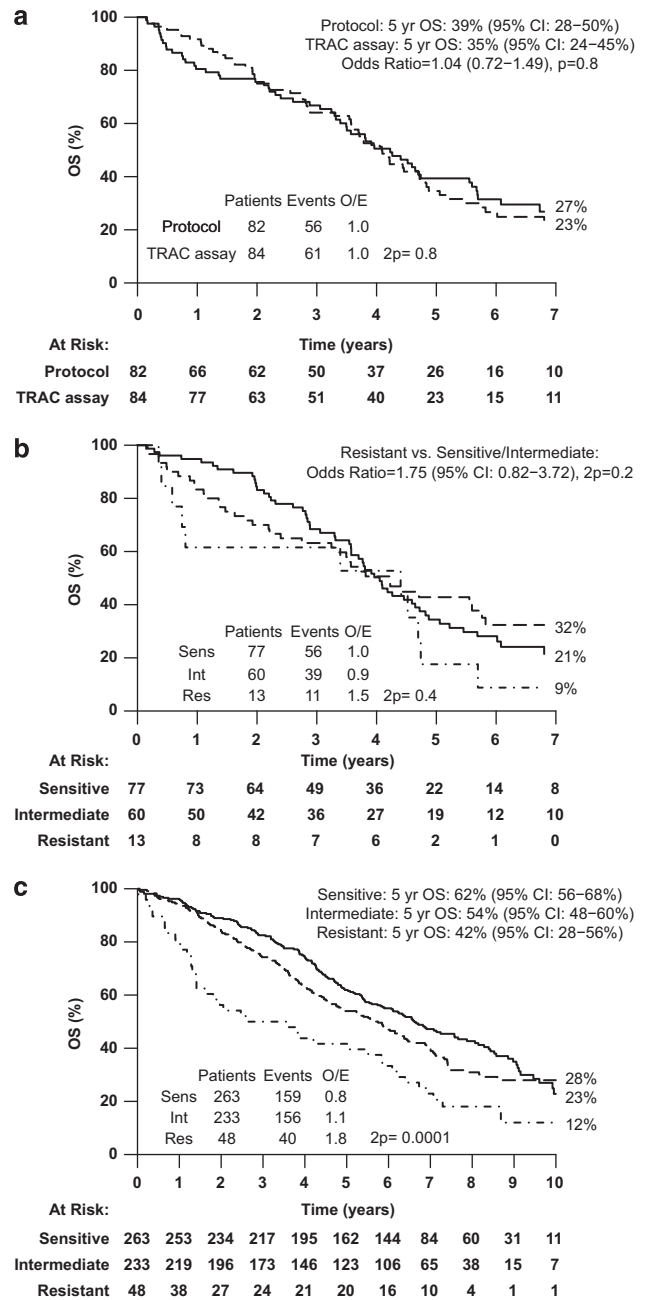


Figure 1. (a) OS from second randomization by TRAC-assay-guided (hashed line) vs protocol-guided arm (solid line). (b) OS by *in vitro* sensitivity to the given second-line treatment (sensitive = solid line, intermediate = hashed line, resistant = hashed/dotted line). (c) OS from first randomization by *in vitro* sensitivity to the randomized treatment (sensitive = solid line, intermediate = hashed line, resistant = hashed/dotted line).

(3) At trial entry, the TRAC assay was performed in 544/777 randomized patients, and this group was broadly representative. Higher LC₉₀s for fludarabine, chlorambucil and mafosfamide were associated with 17p deletion and 11q deletion and for chlorambucil and mafosfamide, but not fludarabine, with unmutated *IGHV* mutational status (Supplementary Table 4).

TRAC-assay-sensitivity was predictive of improved OS (Figure 1c). In univariate analyses, LC₉₀s for fludarabine, chlorambucil and

mafosfamide were statistically significant for both PFS and OS (Table 1). When the TRAC assay result was added to a multivariate OS model it was not significant ($P=0.8$). For PFS, the LC_{90} for fludarabine added independent prognostic value ($P=0.002$), though the LC_{90} for chlorambucil was of borderline significance ($P=0.05$) and for mafosfamide was nonsignificant. When including LC_{90} results for both fludarabine and chlorambucil, only the fludarabine result was significant (Table 1).

Our results suggest that *in vitro* drug sensitivity correlates with patients' outcome. When considering all relapsed/refractory patients, there was an association between *in vivo* response and greater sensitivity by TRAC assay. PFS from second randomization was not measured, but survival at 1 year was significantly better in the TRAC-assay-guided arm—a result which is of particular interest given that no chemotherapy-only protocol has been shown to increase OS.

Several studies have investigated the *in vitro* drug sensitivity of CLL cells^{2,6–10} and its relationship with clinical and biological parameters.^{11–14} These results suggested that *in vitro* drug sensitivity of CLL cells is independent of *IGHV* gene mutation status.^{11,14} However, as expected from clinical experience, *in vitro* sensitivity to fludarabine and chlorambucil was lower in patients with *ATM/TP53* deletions^{13,14} and higher in patients with low-beta-2 microglobulin, longer lymphocyte doubling-time and lack of *TP53* deletion.¹² The *in vitro* prednisolone sensitivity

of *TP53*-deleted cases correlated with the reported *in vivo* sensitivity.¹⁵ These studies reported only a small number of cases, with little information concerning the relationship between the *in vitro* and *in vivo* findings. Morabito *et al.*,⁹ using the MTT assay, concluded that *in vitro* testing for fludarabine predicted response. Castejon's study,¹² using an *in vitro* apoptotic test in 50 patients, showed that sensitivity to fludarabine correlated with a clinical response but, unlike in our study, there was no correlation between *in vitro* sensitivity and *in vivo* response to chlorambucil.

Information on the significance of *in vitro* drug sensitivity assays in CLL is limited to a few nonrandomized studies.^{2,3} The main findings were a correlation between *in vitro* and *in vivo* sensitivity to fludarabine, which translated into longer survival for the fludarabine-sensitive group,² and a lack of *in vitro* cross-resistance between fludarabine and pentostatin, suggesting that pentostatin could be a salvage drug in fludarabine-resistant patients.³

The present study is the only one that analyzes the value of the *in vitro* drug assay in untreated CLL patients entered into a randomized clinical trial, and in whom clinical and biological prognostic factors were considered. *In vitro* drug resistance correlated with the presence of 17p and 11q deletion, as documented in other small studies,^{13,14} but also with unmutated *IGHV* genes. The TRAC assay LC_{90} (fludarabine), together with 17p and 11q deletion and *IGHV* gene mutation status was an independent variable predicting PFS. Although the TRAC assay result was associated with OS in univariate analysis, it was not significant in multivariate analysis. This might be because most relapsed and refractory patients had been treated with chlorambucil and were rescued with alternative therapies.

In summary, the TRAC assay may be of value in practice, particularly in untreated patients who are unfit for intensive regimens and for whom treatment with chlorambucil or fludarabine is planned. The use of these agents in patients with *in vitro* drug resistance predicts nonresponse. In the relapse setting, short-term survival may be improved, while further studies including new agents (for example, bendamustine, lenalidomide) in the panel of drugs tested may give additional information on the clinical value of testing. At diagnosis, the TRAC assay provided prognostic information additional to other known factors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. Cox regression analyses for LC_{90} TRAC assay results^a at trial entry

Variable	Events/ Patients	HR and 95% confidence interval	P-value
<i>OS—univariate analysis^{b,c}</i>			
Log ₁₀ LC_{90} -flu	356/544	1.35 (1.15 – 1.58)	0.0003
Log ₁₀ LC_{90} -chl	355/543	3.16 (2.25 – 4.44)	<0.0001
Log ₁₀ LC_{90} -maf	354/536	1.82 (1.25 – 2.65)	0.002
<i>PFS—univariate analysis^b</i>			
Log ₁₀ LC_{90} -flu	500/ 544	1.65 (1.42 – 1.91)	<0.0001
Log ₁₀ LC_{90} -chl	499/ 543	2.48 (1.86 – 3.32)	<0.0001
Log ₁₀ LC_{90} -maf	493/ 536	1.65 (1.19 – 2.48)	0.003
<i>PFS—multivariate analysis^e</i>			
Addition of LC_{90} -flu to model with other known prognostic factors (excluding <i>del17p</i>) ^d			
<i>Treatment</i>			
Flu	261/ 284	1.00	<0.0001
Chl		1.30 (0.97 – 1.74)	
Flu with cyclophosphamide		0.53 (0.38 – 0.75)	
11q deletion		1.53 (1.14 – 2.05)	0.005
β2 microglobulin ≥4 mg/l		1.35 (1.06 – 1.74)	0.02
<i>IGHV</i> unmutated and/or V3-21 usage		2.03 (1.54 – 2.68)	<0.0001
Log ₁₀ LC_{90} -flu		1.50 (1.17 – 1.93)	0.002

Abbreviations: chl, chlorambucil; flu, fludarabine; HR, hazard ratio; maf, mafosfamide; OS, overall survival; PFS, progression-free survival; TRAC, tumor response to anti-neoplastic compounds. ^a LC_{90} values modeled instead of TRAC categories as definitions of both TRAC resistance and PFS use response. ^bHR for LC_{90} results are for a unit increase. ^cNone of the LC_{90} values retained significance in multivariate analysis for OS. ^d LC_{90} -chl and LC_{90} -maf were not significant after the inclusion of these factors and so have been removed from the model. ^eMultivariate analyses tested whether the TRAC assay results added value to the previously identified prognostic factors,⁵ with models fitted both with and without the inclusion of the TRAC assay to the baseline models, and the change in deviance examined between the two models. Final model presented in table.

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Whole-genome-amplified DNA as a source for mutational analysis underestimates the frequency of mutations in pediatric acute myeloid leukemia

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Acute myeloid leukemia (AML) is the most common acute leukemia in adults, whereas it constitutes only ~15–20% of childhood leukemias. AML is a biologically and clinically heterogeneous disease, for which cytogenetic findings have proved essential for stratifying patients into prognostic subgroups. Although a large proportion of AML display normal karyotype (adults 35–50%, children 25%),¹ recent developments have revealed molecular markers, such as the mutational status of the FMS-related tyrosine kinase 3 (*FLT3*), Nucleophosmin (*NPM1*) and *CEBPA* genes, to aid further subdivision of AML patients with normal karyotype. To date, these molecular prognosticators are well established in adult AML,² whereas they have been less studied in pediatric AML. In a recent study, we performed an extensive analysis of DNA-based markers (for example, *FLT3*, *NPM1*, *CEBPA* and *WT1* genes) and RNA-based markers in a large cohort of uniformly treated patients from the Nordic countries, further supporting *FLT3* and *NPM1* as key prognostic markers also in pediatric AML.³

One of the major obstacles when working with DNA samples with great clinical/scientific value, such as tumor samples from leukemia patients, is the limited amounts of DNA for genetic analysis. Whole-genome amplification (WGA) has therefore emerged as an important method for reproducing abundant quantities of DNA as an alternative source of DNA for genetic analysis. In recent years, implementation of WGA DNA has increased drastically for different

type of applications, such as genome-wide analysis of single-nucleotide polymorphisms (SNP) and copy number variations (CNV).^{4,5} However, the usefulness of WGA method is mainly based on the presumption that the entire genome is amplified with minimal amplification bias. One of the most commonly used WGA methods is the multiple displacement amplification (MDA), which replicates the genome isothermally using random hexamer primers and DNA polymerase, such as Phi29, followed by strand displacement. Even though several studies on Phi29 have demonstrated that it has high genome coverage, little amplification bias and high accuracy compared with genomic DNA controls,^{6,7} controversial results have also been reported.^{8,9}

In our aforementioned study on pediatric AML, we initially planned only to perform the DNA mutational analysis using WGA DNA, and later confirm the identified mutations using original DNA samples in order to save original DNA, as this sample cohort is one of the rare and larger population-based collections in the world. However, to study the impact of WGA DNA as a source of DNA, we instead decided to perform WGA for all 216 AML samples using the GenomiPhi V2 DNA Amplification Kit (Amersham Biosciences, Uppsala, Sweden), and in parallel carried out DNA mutational analysis for the *FLT3* and *NPM1* genes using original and WGA DNA.³ To rule out the possibility of any sample misplacement or the WGA process itself, all samples underwent WGA two times (set I and set II) using the same kit. *FLT3*-ITD and *NPM1* mutations were analyzed by PCR amplification, followed by subsequent fragment analysis as described previously.^{3,10} To