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Prognosis for fludarabine therapy of chronic lymphocytic leukaemia based on *ex vivo* drug response by DiSC assay

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Summary. The cytotoxic antimetabolite fludarabine is a widely used active agent in chronic lymphocytic leukaemia (CLL). However, cost and occasional adverse side-effects necessitate careful use. Identifying before treatment patients not likely to benefit from fludarabine could advance disease management both clinically and financially.

We used the DiSC (differential staining cytotoxicity) assay, an *ex vivo* apoptotic drug response test, to identify the sensitivity or resistance to fludarabine of lymphocytes from B-cell CLL patients and compared the results with subsequent patient treatment, response and survival. Patients were grouped thus: those receiving fludarabine within 1 year of assay (± other cytotoxic drugs), and those receiving other chemotherapy (excluding fludarabine) within 1 year of assay.

Fludarabine-test-resistance was found in 12/100 (12%) of untreated patients and 45/143 (31%) of previously treated patients (17/32 (53%) of patients previously treated with fludarabine). Treating fludarabine-test-resistant patients with fludarabine resulted in poor response compared with fludarabine-test-sensitive patients (7% *v* 69%) and short survival (median 7·9 *v* 41·7 months; relative risk (RR) = 14·8; $P < 0·0001$). 81% of fludarabine-test-resistant patients were test sensitive to other regimens. If treated with chemotherapy other than fludarabine, test-resistant patients responded better and survived substantially longer than those treated with fludarabine (RR = 2·9; $P = 0·001$).

Not all CLL patients should receive fludarabine. Fludarabine-test-resistance by DiSC assay is a powerful independent prognostic factor. Pretreatment DiSC assay results could enable the toxic, clinical and financial costs of fludarabine treatment to be avoided in fludarabine-test-resistant patients. Disease management, response, survival and use of financial resources might be significantly improved if therapy choice in CLL patients was guided by DiSC assay.

Keywords: chronic lymphocytic leukaemia, fludarabine, prognosis, differential staining cytotoxicity assay, disease management.

The activity of fludarabine in previously treated and untreated patients with chronic lymphocytic leukaemia (CLL) has been established in open non-comparative studies (Grever *et al*, 1988), and most CLL patients will receive the drug sometime during the course of their disease (Sorensen *et al*, 1997; Keating *et al*, 1989). In prospective randomized trials it produced a higher response rate and longer duration of response than conventional alkylator or anthracycline based therapy (The French Cooperative Group on CLL, 1996; Rai *et al*, 1996). No significant increase in survival has been reported (The French Cooperative Group on CLL, 1996), although a small proportion of complete responses achieved with fludarabine are associated with elimination of residual clonal cells assessed by sensitive flow-cytometric or gene-rearrangement assays (Richardson *et al*, 1994).

The toxicity associated with fludarabine is usually modest, although neutropenia may occur at conventional doses (Sorensen *et al*, 1997). There have also been concerns about the prolonged suppression of helper T-cell numbers resulting in increased susceptibility to opportunistic infections (O'Brien *et al*, 1993). Occasional severe idiosyncratic toxicities have been noted, including acute tumour lysis syndrome (Frame *et al*, 1992), transfusion-associated graft-versus-host disease (Williamson *et al*, 1996), autoimmune haemolysis (Myint *et al*, 1995; The French Cooperative Group on CLL, 1996), Evans syndrome (Shvidel *et al*, 1997) and neurotoxicity (Spriano *et al*, 1994; Sorensen *et al*, 1997).

The clinical use of fludarabine in the treatment of CLL in the U.K. is partly limited by financial considerations. The cost of giving six cycles of fludarabine at the conventional dose

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schedule of 25 mg/m^2/d for 5 d is approximately 15 times higher than for chlorambucil (Best, 1995). Selection of patients to receive fludarabine is made by clinical criteria such as age and stage of disease but an additional process capable of identifying those patients who would not benefit from the drug would be valuable.

Many groups have investigated the newer short-term (4 d) *ex vivo* apoptotic drug response tests as methods of improving the disease management of individual patients (Bosanquet & Bell, 1996a, b; Bromidge *et al*, 1998; Morabito *et al*, 1998; Klumper *et al*, 1996; Bosanquet, 1991; Kaspers *et al*, 1995). Results correlate well with subsequent patient response and survival for a variety of diseases and drug regimens (Bosanquet, 1994; Fruehauf & Bosanquet, 1993; Mason *et al*, 1999). We therefore investigated the ability of the DiSC assay to prospectively identify subsets of CLL patients resistant or sensitive to fludarabine.

PATIENTS AND METHODS

Patients. Blood samples were from B-CLL patients: (a) entrants into the third U.K. Medical Research Council (MRC) CLL trial (most previously untreated), (b) entrants into phase II trials where parallel DiSC assays were performed (Bosanquet *et al*, 1999; Johnson *et al*, 1998; The French Cooperative Group on CLL, 1996), (c) those sent to Bath for *ex vivo* drug response testing (usually to aid disease management), or (d) accrued locally.

Treatment. In this study two groups of patients were delineated by treatment received: (a) patients starting fludarabine treatment within 1 year of test date (± other chemotherapy), referred to subsequently as 'patients receiving fludarabine', and (b) patients who began chemotherapy, excluding fludarabine, within 1 year of test, referred to subsequently as 'patients receiving other chemotherapy'. Patients who did not receive cytotoxic chemotherapy within 1 year of DiSC assay were not included in the analysis because drug response profiles may change over time (Bosanquet & Bell, 1996b; Bird *et al*, 1988). Fludarabine was administered at 25 mg/m^2/d on days 1–5 every 28 d for up to six courses. MRC criteria were used to assess clinical response to fludarabine. Partial response required all five of the following to be fulfilled: blood lymphocytes $<15\times10^9$/l; neutrophils $\geqslant2{\cdot}0\times10^9$/l; platelets $\geqslant100\times10^9$/l; Hb $\geqslant10$ g/dl (women), $\geqslant12$ g/dl (men) (neutrophils, platelets and Hb could also be 50% improvement over baseline); 50% reduction in size of lymphadenopathy, spleen or liver (Catovsky, 1990). For analysis, patients were divided into those who responded (complete or partial) to fludarabine and those who did not.

Ex vivo drug response. The methodology of the DiSC assay, whereby drug-induced apoptotic cell death is observed, has been described in detail (Bosanquet & Bell, 1996a). Briefly, mononuclear cells were isolated from blood over ficoll-hypaque, washed, counted, and incubated with selected drugs for 94 h. Cells from untreated patients were incubated with 7–10 standard CLL drugs: chlorambucil, cyclophosphamide, prednisolone, vincristine, doxorubicin, epirubicin, fludarabine (phosphate; Bosanquet, 1999), cladribine, pentostatin and methylprednisolone. Previously treated patients' cells were incubated with up to 25 other cytotoxics. At the end of incubation, fixed duck erythrocytes (as an internal standard) and fast green/nigrosin (to stain dead cells black) were added and the cells cytocentrifuged. Counterstaining with a Romanowsky stain enabled identification of remaining live cells which were evaluated morphologically to determine LC_{90}s, the lowest concentration of drug to produce a 90% reduction in tumour cell survival compared with control cells.

Data analysis. Fludarabine test resistance was defined as that giving a theoretical post-test response rate of <10% using the Bayes' Theorem curves of a test with 85% sensitivity and 80% specificity (Fruehauf & Bosanquet, 1993). Thus, treated patients (pre-test response rate $\leqslant$50%) were deemed test-resistant if their fludarabine LC_{90} was > mean LC_{90} of all treated patients, and untreated patients (pre-test response rate ~80%) were deemed *ex-vivo* resistant to fludarabine if their fludarabine LC_{90} was > mean LC_{90} + 1 SD of all untreated patients (Fruehauf & Bosanquet, 1993).

Statistical analysis. SPSS version 8.0.0 for Windows95 was used for statistical analysis. The association of fludarabine test result with patient response was analysed by univariate and multivariate logistic regression. Survival analysis was by Cox regression with backward elimination of non-significant covariates. All deaths were included in Kaplan-Meier curves; no qualitative differences were obtained when deaths due to causes other than CLL were classified as 'lost to follow-up'.

RESULTS

Patients

Between November 1989 and June 1995, specimens from 495 CLL patients were received from 122 physicians in 90 hospitals. Of the 385 (78%) patient specimens yielding LC_{90} results from fludarabine and six or more other CLL drugs (the remainder were tested with fewer than seven drugs or failed to survive the 4 d incubation), 131 (34%) did not receive chemotherapy within 1 year of DiSC assay (89 entered in the MRC CLL IIIA trial, i.e. registration only, no treatment until disease progression), 10 patients' notes could not be traced, and one patient was on treatment when assayed. Thus, this study comprises results from 243 CLL patients whose cells were tested for *ex vivo* response to fludarabine plus six or more other cytotoxic drugs and who subsequently received cytotoxic chemotherapy within 1 year of test. At analysis, patients had been followed-up for a median of 3·6 years (range 1·6–7·2) since DiSC assay and 148 (61%) had died.

Patient characteristics are presented in Table I. 66 patients received fludarabine and 177 received other chemotherapy. Parameters for these groups of patients were typical of CLL in general (Table I). 97 patients were part of the U.K. MRC CLL III trial, where non-responders to initial treatment received fludarabine; 66 patients were in other clinical trials. In 45 cases (18·5%) physicians received a DiSC assay report which may have influenced their choice of therapy (statistical

Table I. Patient characteristics.

Characteristic	Treatment		
	Fludarabine	Other chemotherapy	Total
Total	66	177	243
Age (mean ±SD)	62·3 ± 9·85	65·1 ± 10·2	64·4 ± 10·2
Stage A	15 (23%)	47 (27%)	62
B	20 (30%)	41 (23%)	61
C	31 (47%)	89 (50%)	120
Sex			
M	47 (71%)	129 (73%)	176
F	19 (29%)	48 (27%)	67
Previous chemotherapy			
Yes	46 (70%)	97 (55%)	143
No	20 (30%)	80 (45%)	100
Prior fludarabine	4 (6%)	28 (16%)	32
Prior regimens (mean number)	0·76	0·71	0·72

analyses performed excluding these cases gave results very similar to those presented below). At assay set-up, median lymphocyte count was 95×10^9/l and median cell viability was 96%.

DiSC assay results

Fludarabine LC_{90} values ranged from <0·25 μg/ml to 64·0 μg/ml with an overall median of 0·791 μg/ml. There was no significant difference in mean LC_{90} value between the fludarabine and other chemotherapy groups. Results identified 57 patients (23%) whose cells were test-resistant to fludarabine: 12/100 (12%) untreated patients and 45/143 (31%) previously treated patients (17/32 patients (53%) who had previously been treated with fludarabine were test-resistant).

Response of patients treated with fludarabine analysed by DiSC assay result

Of 66 patients who received fludarabine within a year of DiSC assay, 36 responded: a response rate of 55% (Table II). A very much lower response rate was observed for fludarabine-test-resistant patients compared with fludarabine-test-sensitive patients (7% (1/15) *v* 69% (35/51)). Although the 95% confidence intervals were large where numbers are low, there was still a highly significant difference between the response in the two groups having allowed for the effect of previous chemotherapy ($P<0·0001$; Table II).

Table II. Patients responding to fludarabine (% response rate, 95% confidence intervals (CI)) by test result and previous chemotherapy.

Previous chemotherapy	Fludarabine-test-sensitive	Fludarabine-test-resistant
No	12/15 (80%, CI 52–96%)	1/5 (20%, CI 0–72%)
Yes	23/36 (64%, CI 48–80%)	0/10 (0%, CI 0–31%)
Total	35/51 (69%, CI 56–81%)	1/15 (7%, CI 0–32%)

Response rate was lower among test-resistant patients, after stratification by previous chemotherapy ($P<0·0001$).

Analysis by univariate logistic regression identified a significant association between patient characteristics and response for sex ($P=0·02$), Binet stage ($P=0·02$), treatment with knowledge of test result ($P=0·01$), but not for previous chemotherapy or age. However, the greatest association was between fludarabine test resistance and response ($P=0·0015$). By multivariate logistic regression, stage and sex remained statistically significant predictors of patient response to fludarabine, but the most significant parameter continued to be fludarabine test resistance ($P=0·002$).

Survival of patients treated with fludarabine analysed by DiSC assay result

Fifteen of the 66 patients who received fludarabine within a year of test were fludarabine test resistant. None survived to 17 months (Fig 1). Median survival was 7·9 months (95% confidence interval (CI) 3·2–12·6 months). For fludarabine-test-sensitive patients, median survival was 41·7 months (CI 26·0–57·3 months).

Cox regression analysis with backward elimination of non-significant covariates was performed for patients treated with fludarabine (Fig 1). The covariates included were sex, age, stage, previous therapy, treated with knowledge of test result, and fludarabine test sensitivity. Two covariates remained in the final equation: previous therapy (relative risk 1·9 (CI 0·98–3·7); $P=0·06$) and fludarabine test resistance (relative risk 14·8 (CI 6·2–35·6); $P<0·0001$). The relative risk for fludarabine test resistance was not substantially altered if all the factors were kept in the model (relative risk 12·4; CI 5·0–30·6).

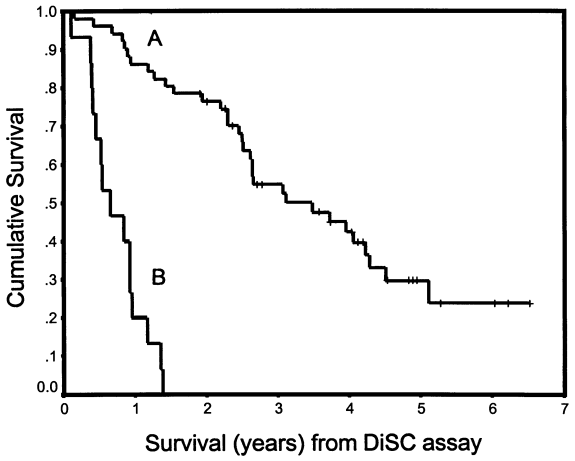

Fig 1. Kaplan-Meier survival curves of patients who received fludarabine: (A) 51 fludarabine-test-sensitive patients; (B) 15 fludarabine-test-resistant patients.

Survival of patients treated without fludarabine analysed by DiSC assay result

The relative risk in fludarabine-test-resistant versus test-sensitive patients treated with other chemotherapy was much less: 2·65 (CI 1·64–4·26). In this group, age, sex and treatment with knowledge of test result were also significant covariates.

Cause of death in fludarabine-test-resistant fludarabine-treated patients

Of the 15 fludarabine-test-resistant patients treated with fludarabine, the main cause of death could be identified in 14. The majority (10) died of resistant disease, one died after transformation to Richter's syndrome, and three due to possible complications of fludarabine treatment.

Survival of fludarabine-test-resistant patients analysed by treatment group

Fifty-seven of the 243 patients (23·5%) were fludarabine test resistant. This comprised 31·5% (45/143) of the previously treated patients, but also 12% (12/100) of patients who had received no prior therapy. Within this group of 57 fludarabine-test-resistant patients there was a significant difference in survival between the group receiving fludarabine and the group not receiving fludarabine, despite there being no statistical difference in age, stage, sex and previous chemotherapy between these two groups (Table III). As noted above, fludarabine-treated patients had a short life expectancy (median survival 7·9 months; CI 3·2–12·6), and few had the opportunity to be treated with other chemotherapy due to the morbidity and mortality caused by ineffective therapy. Patients not treated with fludarabine, however, had a significantly longer survival: median 16·3 months (CI 9·4–23·1 months; $P = 0{\cdot}001$; Fig 2). Relative risk for death of patients treated with fludarabine compared with those treated with other chemotherapy was 2·9 (CI 1·5–5·8).

Cox regression analysis of fludarabine-test-resistant patient survival allowing for age, sex, stage and previous chemotherapy still left treatment as the most significant factor ($P = 0{\cdot}002$). The survival curves were very similar when split into previously treated and previously untreated patients. Thus, the median survival of the five previously untreated fludarabine-test-resistant patients given first-line fludarabine was less than 12 months.

Table III. Characteristics of fludarabine-test-resistant patients by treatment group.

	Treatment	
Characteristic	Fludarabine	Other chemotherapy
Total	15	42
Age (mean ±SD)	63·7 ± 12·3	63·7 ± 11·0
Stage, %A/%B/%C	20%/13%/67%	19%/17%/64%
Sex, M/F (%F)	10/5 (33%)	30/12 (29%)
Previous chemotherapy		
Yes/no (%yes)	10/5 (67%)	35/7 (83%)
Fludarabine	1 (7%)	16 (38%)
Prior regimens (mean number)	0·73	1·21
Ex vivo sensitivity to:		
Fludarabine	0%	0%
Other CLL drugs (including those detailed below)	80%	81%
Methylprednisolone	53%	37%
Doxorubicin	15%	49%
Pentostatin	17%	47%
Vincristine	25%	33%
Cladribine	29%	29%

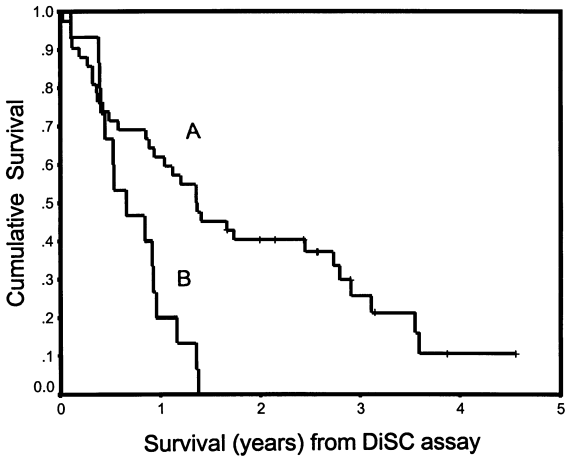

Fig 2. Kaplan-Meier survival curves of fludarabine-test-resistant patients: (A) 42 patients who received other chemotherapy; (B) 15 patients who received fludarabine (from Fig 1).

Survival of fludarabine-test-sensitive patients analysed by treatment group

During the first year post-assay, survival of fludarabine-test-sensitive patients was better in those treated with fludarabine. However, overall survival was similar for the group of patients treated with fludarabine within a year of test when compared with the group treated with other chemotherapy (2-year survival 76% in both cases, $P = 0{\cdot}4$). Cox regression analysis showed survival in this group to be dependent on the usual factors of age, stage, sex and previous therapy.

Ex vivo sensitivity of fludarabine-test-resistant patients to other cytotoxic therapy

Cells from the 57 fludarabine-test-resistant patients were tested against a median of 16 cytotoxic drugs. 46 patients (81%) were test-sensitive to other CLL drugs (Table III), the most active being methylprednisolone, doxorubicin, pentostatin, vincristine and cladribine. Only 11 patients (19%) were resistant to all standard CLL drugs tested; but in more than half of these, non-CLL drugs showed *ex vivo* activity including ifosfamide, thioguanine, mercaptopurine, 8-chloro-cAMP (Bosanquet *et al*, 1997) and asparaginase, providing a basis for trying a novel therapy where indicated.

DISCUSSION

For decades chlorambucil has been the mainstay for treating CLL (Catovsky *et al*, 1991; Dighiero *et al*, 1998). Various alternative treatments have been investigated, mostly based on cyclophosphamide plus an anthracycline, but none has proved superior in response or survival (Catovsky *et al*, 1991). The introduction of fludarabine (and possibly the other new antimetabolites cladribine and pentostatin) has improved this situation. Fludarabine induces more responses and a longer disease-free survival (Sorensen *et al*, 1997; Keating *et al*, 1991; The French Cooperative Group on CLL, 1996) with the possibility of increased quality of life, but its effect on overall survival is still not clear (The French Cooperative Group on CLL, 1996). However, it is expensive (U.K. treatment cost per course £6810 (approx. $11000)) (Best, 1995) when compared with the traditional first-line therapy, chlorambucil (£410 ($700)). Although its side-effects are usually moderate, occasional 'idiosyncratic' side-effects can be life-threatening and there is prolonged immune suppression in some patients.

This study demonstrated that the fludarabine DiSC assay result was a prognostic factor independent of stage, age and sex. It has identified patients who will die prematurely when given fludarabine, and it may be that the expected, but as yet unidentified, increase in survival from fludarabine treatment has not been observed because of these patients. Although it does not prove that the use of the assay to choose treatment would improve survival, there are several factors which suggest this may be true, notably the survival difference between test-resistant and test-sensitive patients was greater among the group of patients treated with fludarabine than it was in those treated with other drugs.

For patients on either fludarabine or other chemotherapy, fludarabine DiSC assay results were a prognostic indicator. This may be in some small part due to the use of fludarabine in other chemotherapy later on and in part due to drug cross-resistance attributable to pleiotropic drug sensitivity in some untreated patients and pleiotropic drug resistance (but not multidrug resistance, MDR (Bosanquet *et al*, 1996)) in patients with advanced CLL. (Thus, patients test-resistant to fludarabine are more likely to be test-resistant to other CLL therapies.) However, it may be that fludarabine DiSC assay results detect a more fundamental underlying characteristic of the malignant cells. This could be, for instance, the ability of chemotherapy to induce fas (CD95, APO1) or fas ligand on the surface of sensitive cells (Krammer, 1997; Debatin, 1997) and so be killed by cytotoxic T-lymphocytes. Alternatively, sensitive cells might express wild-type p53 whilst in resistant cells p53 is mutated or deleted (Dohner *et al*, 1995; Bowen *et al*, 1999; unpublished observations). If the mode of action of fludarabine was p53-dependent whilst the action of, say, methylprednisolone (a drug which is more active in advanced than in untreated CLL (Bosanquet & Bell, 1996b) and not cross-resistant with fludarabine) was via a different pathway, this could help to explain the molecular events underlying the results illustrated in Figs 1 and 2. In cells where p53 was deleted or mutated (i.e. in fludarabine-test-resistant patients), a non-p53-mediated pathway to apoptosis would need to be activated to achieve clinical response. We have observed good responses to methylprednisolone in fludarabine-test-resistant patients with advanced CLL (Bosanquet *et al*, 1995).

In this study the DiSC assay identified a group of patients who had a low response rate (7%) to fludarabine and poor survival (<18 months; Fig 1). 31% of previously treated and 12% of untreated patients were in this fludarabine-test-resistant category. The median survival of recently diagnosed patients who were fludarabine-test-resistant and given fludarabine was less than 12 months, comparable to the poor survival of heavily pretreated patients.

The survival of fludarabine-test-resistant patients who

received fludarabine was much worse when compared with those who received other chemotherapy (Fig 2). Most of these patients died of progressive disease. Giving fludarabine to these patients, of whom 46/57 (81%) showed test-sensitivity to other CLL therapy, increased morbidity and often precluded subsequent treatment with other chemotherapy. Although the choice of treatment was not randomized, so that comparisons must be treated with caution, it would seem preferable to avoid treatment with fludarabine for these patients.

Possible alternative strategies for treating fludarabine-test-resistant patients include the use of high-dose methylprednisolone (Bosanquet *et al*, 1995). The benefits of steroids in end-stage disease have long been known, and recent DiSC assay results have provided some rationale for this in that treatment with chlorambucil induces a sensitivity to steroids in CLL lymphocytes which can persist to the end stages of the disease (Bosanquet & Bell, 1996b; Bosanquet *et al*, 1995).

Traditionally, treatment in the clinic has been based on clinical trial results and targeted at cohorts of patients with individual variation being catered for empirically. Designs for future disease management involve increased individualization of therapy, for instance including pharmacogenetics (Boddy & Ratain, 1997) and need to embrace *ex vivo* drug resistance to optimize patient response and survival. This would target expensive treatments at patients most likely to benefit.

An economic assessment of the DiSC assay in CLL, using results from a group of patients similar to those whose results are presented here, has recently been published (Mason *et al*, 1999). It concluded that the DiSC assay may be a cost-effective technology for improving patient outcome: the estimated incremental cost effectiveness was £1470 ($2400) per life year gained (Mason *et al*, 1999). Since potential savings of drugs costs were not included, the study could not assess any direct financial benefit arising from the use of the DiSC assay in clinical management to rationalize the use of expensive drugs. Nevertheless it is reassuring that the cost per life-year gained with the DiSC assay compares favourably to many currently funded health-service activities.

The work presented here is one of the largest series of *ex vivo* drug response assays investigating a single agent in one malignancy. It parallels many similar experiments in other cancers and leukaemias using short-term (2–4 d) apoptotic drug response tests that show similarly reduced survival in test-resistant patients (Fruehauf & Bosanquet, 1993; Klumper *et al*, 1996). It provides a compelling rationale for a randomized clinical trial in previously treated patients (where in this series 32% were fludarabine-test-resistant) comparing disease management strategies – physician choice versus DiSC assay-guided physician choice – to establish whether the use of the DiSC assay could really provide the major benefit for a subset of patients suggested by this study. The new MRC CLL IV trial will include a randomization to DiSC assay guided physician choice versus physician choice of therapy for patients who relapse off chlorambucil or fludarabine ± cyclophosphamide.

This study suggests that not all CLL patients should be treated with fludarabine. DiSC assay guided therapy could enhance disease management and treatment options, and possibly improve survival rates, for this intractable leukaemia.

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