bjh research paper

Improving the prediction of acute myeloid leukaemia outcomes by complementing mutational profiling with *ex vivo* chemosensitivity

Esther Onecha,[1,2] Yanira Ruiz-Heredia,[2,3] David Martínez-Cuadrón,[4] Eva Barragán,[4] Pilar Martinez-Sanchez,[1,2,5] María Linares,[1,2,5] Inmaculada Rapado,[1,2,6] Jaime Perez-Oteyza,[7] Elena Magro,[8] Pilar Herrera,[9] José Luis Rojas,[3] Julián Gorrochategui,[3] Jesús Villoria,[10] Blanca Boluda,[4] Claudia Sargas,[4] Joan Ballesteros,[3] Pau Montesinos,[4] Joaquín Martínez-López[1,2,5,6] and Rosa Ayala[1,2,5,6]

[1]Hematology Department, Hospital Universitario 12 de Octubre, Madrid, Spain, [2]Hematological Malignancies Clinical Research Unit, CNIO, [3]Vivia Biotech, Tres Cantos, [4]Department of Hematology, Hospital Universitari i Politècnic La Fe, Valencia, [5]Complutense University, [6]CIBERONC, Instituto Carlos III, [7]Hematology Department, Hospital Universitario Sanchinarro, [8]Hematology Department, Hospital Universitario Principe de Asturias, [9]Hematology Department, Hospital Universitario Ramon y Cajal and [10]Medicxact, Madrid, Spain

Received 2 September 2019; revised 31 October 2019; accepted for publication 1 November 2019

Correspondence: Rosa Ayala, Hematology Department, Hospital Universitario 12 de Octubre, Edificio de Atención Ambulatoria, Planta 6 Bloque D, Avenida de Córdoba s/n, 28041, Madrid, Spain.
E-mail: rosam.ayala@salud.madrid.org

Summary

Refractoriness to induction therapy and relapse after complete remission are the leading causes of death in patients with acute myeloid leukaemia (AML). This study focussed on the prediction of response to standard induction therapy and outcome of patients with AML using a combined strategy of mutational profiling by next-generation sequencing (NGS, $n = 190$) and *ex vivo* PharmaFlow testing ($n = 74$) for the 10 most widely used drugs for AML induction therapy, in a cohort of adult patients uniformly treated according to Spanish PETHEMA guidelines. We identified an adverse mutational profile (*EZH2, KMT2A, U2AF1* and/or *TP53* mutations) that carries a greater risk of death [hazard ratio (HR): 3·29, $P < 0·0001$]. A high correlation was found between the *ex vivo* PharmaFlow results and clinical induction response (69%). Clinical correlation analysis showed that the pattern of multiresistance revealed by *ex vivo* PharmaFlow identified patients with a high risk of death (HR: 2·58). Patients with mutation status also ran a high risk (HR 4·19), and the risk was increased further in patients with both adverse profiles (HR 4·82). We have developed a new score based on NGS and *ex vivo* drug testing for AML patients that improves upon current prognostic risk stratification and allows clinicians to tailor treatments to minimise drug resistance.

Keywords: acute myeloid leukaemia, sequencing, *ex vivo* sensitivity test.

Refractoriness to induction therapy and relapse after achieving complete remission (CR) is a common cause of death among patients with acute myeloid leukaemia (AML). Cytogenetic and molecular alterations at diagnosis and response to therapy are the best predictors of the relative risk of AML relapse and are helpful in deciding between chemotherapy and haematopoietic stem cell transplantation (HSCT) at first CR (Onecha *et al.*, 2018). In this respect, next-generation sequencing (NGS)-based studies have yielded important insights into the molecular pathogenesis of AML, and the mutational profile of AML is now better defined. For example, a single patient can carry up to 400 genomic variants, of

First published online 18 February 2020
doi: 10.1111/bjh.16432

which 13 (on average) have been found to reside in the coding region of genes (Pemovska *et al.*, 2013b). Some of these genes have been identified as potential drivers with prognostic implications, such as *NPM1, CEBPA, DNMT3A, TET2, RUNX1, ASXL1, IDH1* and *KMT2A*, whereas others, including *FLT3*, *IDH1*, *KIT* and *RAS*, are considered important therapeutic targets (Pemovska *et al.*, 2013b). The clinical prognostic relevance of many recurrent somatic mutations has been demonstrated and, accordingly, mutational profiling was recently incorporated as a prognostic factor in AML European LeukaemiaNet (ELN) guidelines (Patel *et al.*, 2012; Papaemmanuil *et al.*, 2016).

Most clinical protocols include a nucleoside analogue (cytarabine) in combination with a topoisomerase II inhibitor (daunorubicin or idarubicin) as standard induction therapy. This scheme has remained largely unchanged over the last four decades despite the five-year overall survival (OS) of AML patients remaining low at ~ 30% (Rowe & Tallman, 2010). A small number of new drugs targetted to mutational events of AML have been developed, such as the fms-related tyrosine kinase 3 (*FLT3*) inhibitors for *FLT3* mutational events, which occur in 20–30% of patients (Gale *et al.*, 2008). Unfortunately, responses of only 2–6 months were reported when these drugs were used as single agents (Tyner *et al.*, 2018). Clinical benefits have also been shown with drugs targeting the *IDH1* and *IDH2* genes, namely ivosidenib and enasidenib, respectively (Tyner *et al.*, 2018), the hypomethylating agent 5-azacytidine in *TET2*-mutated patients (Itzykson *et al.*, 2011), or the nucleoside analogue decitabine in *TP53*-mutated patients (Welch, Petti & Ley, 2017).

Because resistance to clinical treatment can underlie therapeutic failure, *in vitro* sensitivity studies have been used for preclinical screening prior to drug planning in clinical trials (Mollgard *et al.*, 2008; Sato *et al.*, 2011). Similarly, *ex vivo* drug sensitivity testing such as PharmaFlow [Pharmacology by flow cytometry, a cell-based flow cytometry pharmacology testing platform (Vivia Biotech S.L., Tres Cantos, Spain)]of clinically available FDA-approved drugs has been used to identify the most suitable combination therapy for precision medicine strategies in patients with AML (Bennett *et al.*, 2014; Swords *et al.*, 2018; Martinez-Cuadron *et al.*, 2019). Indeed, individual *ex vivo* drug sensitivity testing has been used to assign personalised antibiotic and antifungal treatments for decades, and this tailored approach could also be applicable to anticancer drugs (Bennett *et al.*, 2014; Swords *et al.*, 2018; Martinez-Cuadron *et al.*, 2019) However, the prediction of individual patient response before treatment delivery remains challenging, and the number of biochemical and genetic biomarkers associated with drug sensitivity is very limited (Gale *et al.*, 2008; Tyner *et al.*, 2018).

In a descriptive study involving AML patients, large sequencing assays were used to identify mutations in *FLT3*, *IDH1*, *KIT* and *RAS* that result in significant changes in drug sensitivity (Swords *et al.*, 2018). This study, nevertheless, did not evaluate the clinical significance of combining sequencing assays with *ex vivo* chemosensitivity profiling for the detection of resistant subjects and the survival of patients with AML.

In the present study, we assessed the clinical relevance of a combined strategy based on both mutational profiling by NGS in 190 patients and *ex vivo* PharmaFlow in 74 patients using the 10 most widely used drugs for AML induction therapy. We focussed on the prediction of responses to standard induction treatment and outcome of patients with AML who were uniformly treated according to Spanish PETHEMA (Programa español de tratamientos hematológicos) group guidelines.

Patients and methods

Patients

A multicentre, non-interventional cohort study was carried out in five Spanish institutions of the PETHEMA group. A total of 190 patients with AML (non-M3 type) diagnosed between 2005 and 2015 were included in the NGS study, of whom 74 were also available for the *ex vivo* PharmaFlow study. In total, 57 (77%) patients were treated with a 3 + 7 schedule whereas 17 (23%) were treated with the FLUGA scheme (fludarabine plus low-dose cytarabine); see details in supplementary Data S1, *Induction treatments*.

Prominent clinical parameters [white blood and blast cell counts, age, gender, cytogenetics risk group (by ELN-2010 criteria), AML type, haematopoietic stem and progenitor cell type, induction treatment, and survival data] were incorporated into the clinical database, which is summarised in Table I.

The study was conducted according to Spanish law 14/2007 on biomedical research and was approved by the Research Ethics Board of each participating institution. All patients provided informed consent. Data for this study were provided by PETHEMA trials registered at as NCT01296178, NCT00952588 and NCT02607059.

Mutational profile workflow

For the sequencing study, DNA from total bone marrow or peripheral blood samples was evaluated. The samples were stored in the biobanks of the Hospital 12 de Octubre, Madrid (n = 132) and the Hospital Politécnico La Fe, Valencia (n = 58). All patient samples were genetically characterised at the time of diagnosis by conventional G-band karyotyping and fluorescence *in situ* hybridisation.

The mutational screening was performed using a custom NGS panel (Table SI) consisting of 32 genes recurrently mutated in myeloid diseases (Cedena *et al.*, 2017). In addition, the detection and quantification of mutated *NPM1* sequences was performed by an allele-specific quantitative polymerase chain reaction (q-PCR) according to the method of Gorello and colleagues (Cedena *et al.*, 2017), using RNA as the biological sample and *ABL1* or *β-GUS* as the

Table I. Patient characteristics. Table presents the clinical data of patients included in the NGS and *ex vivo* studies

		NGS Assay n = 190	*Ex vivo* Assay n = 74
Sample	BM	173 (91%)	74 (100%)
	PB	17 (9%)	–
Gender	Male	100 (53%)	44 (60%)
	Female	90 (47%)	30 (40%)
Age at diagnosis	Years, median (range)	57 (18-91)	58 (19-91)
Blasts at diagnosis	%, median (range)	63 (4-99)	67 (20-99)
WBC at diagnosis	10^9/l, median (range)	17 (1–300)	20,2 (1–242)
AML origin	*de novo*	152 (80%)	62 (84 %)
	AML-MDS	20 (11%)	8 (11 %)
	tAML	18 (9%)	4 (5 %)
Cytogenetics	Normal	101 (53%)	35 (47 %)
	Altered	89 (47%)	39 (53 %)
Cytogenetics Risk Group ELN 2010	Low	14 (7%)	11 (15%)
	Intermediate	131 (69%)	47 (63%)
	High	45 (24%)	16 (22%)
HSCT	Autologous	45 (24%)	15 (20%)
	Allogenic	32 (17%)	15 (20%)
	No done	113 (59%)	44 (60%)
Induction treatment*	(3 + 7) scheme	154 (81%)	57 (77%)
	Azacitidine	2 (1%)	–
	Decitabine	1 (0,5%)	–
	FLUGA scheme	26 (14%)	17 (23%)
	Support	7 (3,5%)	–
Response to induction	CR	110 (58%)	42 (57%)
	PR	30 (16%)	19 (26%)
	Resistence	19 (10%)	13 (17%)
	Death	31 (16%)	—
Time to 1st CR	Days, median (range)	39 (13-130)	40 (19–87)
Cases of relapse		60 (32%)	24 (32%)
Time to first relapse	Months, median (range)	14 (1-96)	20 (1–45)
Cases of death		117 (62%)	46 (62%)
Follow-up time	Months, median (range)	26 (1–150)	20 (0,5–70)

AML, acute myeloid leukaemia; BM, bone marrow; CR, complete remission; ELN, European leukaemiaNet (2010); FLUGA, fludarabine plus cytarabine; HSCT, haematopoietic stem cell transplantation; MDS, myelodysplastic syndromes; NGS, next generation sequencing; PB, peripheral blood; PR, partial remission; WBC, white blood cells.

*3 + 7 regimen of chemotherapy: one or two induction cycles of cytarabine and idarubicin during seven and three days, respectively; and two or three consolidation cycles at high doses of cytarabine, twice daily for three alternate days followed by allo- or auto-HSCT. The remainder of patients were included in other clinical trials (Mylotarg, NTC0104104; Flugaza (NCT02319135); Panobidara, NCT00840346). Clinical data were collected in the following Spanish AML epidemiological registries: NCT01700413, NCT02006004, NCT00464217, NCT02607059, NCT01041040 and NCT01296178.

expression reference genes for normalisation (Gorello *et al.*, 2006). Internal tandem duplications were detected with PCR and GENSCAN, as previously described (Burge & Karlin, 1997).

PharmaFlow chemosensitivity profiling

For *ex vivo* drug sensitivity assays, fresh bone marrow samples from 74 patients were collected in heparinised tubes and were received by the processing laboratory within 24 hours from extraction. Sample incubation with the corresponding drugs was performed maintaining the native microenvironment, as previously described in detail (Bennett *et al.*, 2014; Hernandez *et al.*, 2017; Martinez-Cuadron *et al.*, 2018). After incubation for 48 hours, red blood cells were lysed and remaining cells were washed, labelled with fluorescent conjugated antibodies, and injected into the flow cytometer. Briefly, after drug incubation, cells were labelled with 20 µl of 1 × binding buffer plus Annexin-V fluorescein isothiocyanate and at least two of the best monoclonal antibodies that unequivocally identified the pathological cells, referred to as backbone reagents, according to EuroFlow panels (van Dongen *et al.*, 2012; Kalina *et al.*, 2012). A series of 10 drugs were selected for screening: cytarabine (Cyt, n = 74), idarubicin (Ida, n = 74), fludarabine (Flu, n = 73), clofarabine (Clo, n = 54), daunorubicin (Dau, n = 48), mitoxantrone (Mit, n = 49), etoposide (Eto, n = 42), amsacrine (Ams, n = 29), 6-thioguanine (Thi, n = 24) and decitabine (Dec,

n = 23). The response effect was measured as the number of live pathological cells (LPCs) remaining in the presence of varied drug concentrations after incubation for a given time period, which was used to calculate dose–response curves.

Statistical analysis

The pharmacodynamics of each drug was evaluated *via* non-linear models for repeated measurement data, based on Hill's equation using an individualised fitting approach. A four-parameter version of Hill's equation was used for model fitting: the number of LPCs after incubation in the absence of drug (E_0), the maximum fractional decrease in LPC number that the drug can elicit (E_{max},), the drug concentration exerting half of the E_{max} (EC_{50},), and a slope parameter (γ) of the drug concentration curve. The E_{max} values were constrained within the range 0–1, and this was the range of individual cell responses. After fitting, the data were normalised with respect to the individually fitted E_0 values. This normalisation allowed for the calculation of the normalised area under the curve (AUC_{norm}) of individual response profiles (the fitted Hill curves) by numerical integration between the concentration points representing the 20th and 80th percentiles of the distribution of the estimated individual EC_{50} values. These AUC values were used to describe the *ex vivo* drug effects. Larger AUC_{norm} values are indicative of resistance (as they derive from higher EC_{50} values or lower E_{max} values). In addition, patients and tested drugs were clustered through a bidimensional unsupervised procedure as per Ward's method using half-squared Euclidean distances as measures of dissimilarity. The patients were grouped into three (multisensitive, neutral and multiresistant) groups that were further aggregated into two [sensitive (multisensitive and neutral), multiresistant] categories after the visual inspection of the dendrograms and scree plots.

More details are given in supplementary Data S1, *Statistical analysis.*

Results

Mutational screening by NGS prediction of outcomes

A total of 264 non-recurrent somatic variants were detected in 164/190 patients (86%) included in the sequencing study with the NGS custom panel. A total of 217 of these variants (82·2%) were single nucleotide variants (SNV), and the remaining 47 (17·8%) were insertion/deletion variants. The most frequently mutated genes in this series were *NPM1* (28·6% of patients), *DNMT3A* (26·5%), *TET2* (21·1%), *NRAS* (19·5%) and *FLT3* (25%) (Figure S1). Fifty-two of the 264 variants (19·7%) were predicted to be pathogenic as per the Sorting Intolerant From Tolerant, Polymorphism Phenotyping v2 or CONDEL algorithms (Ng & Henikoff, 2003; Adzhubei, Jordan & Sunyaev, 2013), and 151 out of 264 (57·2%) are described in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Tate *et al.*, 2019).

We found significant associations between the presence of specific mutations and OS (data available for n = 185). The risk of death was significantly greater in patients with mutations in *EZH2* [hazard ratio (HR): 2·44, 95% confidence interval (CI): 1·27–4·86, P = 0·011], *KMT2A* (HR: 2·21, 95% CI: 1·20–4·05, P = 0·011), *U2AF1* (HR: 3·19, 95% CI: 1·47–6·88, P = 0·003) and *TP53* (HR: 2·92, 95% CI: 1·78–4·79, P < 0·001) (Fig 1A–D) than in patients with non-mutated

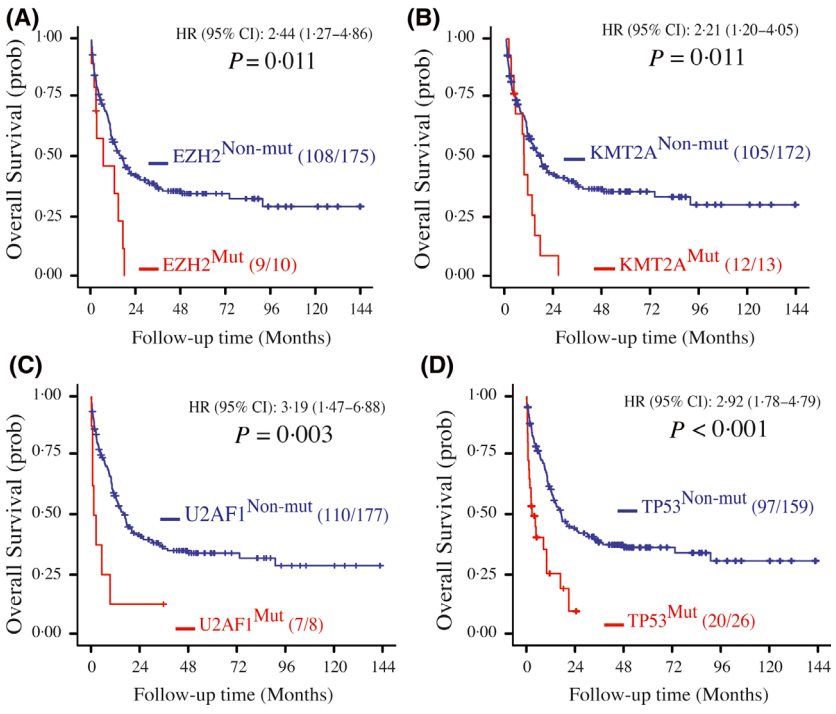

Fig 1. Kaplan–Meier overall survival curves in acute myeloid leukaemia (AML) patients classified by mutational profile. Kaplan–Meier curves of patients with individual adverse mutational profile [mutation in the *EZH2* (A), *KMT2A* (B), *U2AF1* (C) or *TP53* (D) genes] *versus* non-mutated patients. Number of censored patients with respect to the stratified groups and the number at risk is indicated. *P* values are considered significant (<0·05). [Colour figure can be viewed at wileyonlinelibrary.com]

genes. Patients with at least one mutation in any of these genes were considered to have an adverse mutational profile. We evaluated the association of having an adverse mutational profile (at least one mutation in *EZH2*, *KMT2A*, *U2AF1* and/or *TP53* genes) in the subgroup of patients who were included in the chemosensitivity study with OS and we consistently found a significantly greater risk of death (HR: 3·29, 95% CI: 1·78–6·08, $P < 0·0001$) (Figure S2).

Cytogenetic classification (ELN 2010 criteria) was also evaluated, and no significant differences were found in OS for the complete cohort ($n = 190$, $P = 0·08$) or for the *ex vivo* study ($n = 74$, $P = 0·88$).

Cytarabine ex vivo assay, clinical response and OS

Since all patients included in the *ex vivo* PharmaFlow study had received cytarabine, albeit with a second drug, the correlation between the activity of cytarabine in these patient samples, patients' clinical response and OS was studied. We found a significant correlation between the *ex vivo* classification and clinical response to induction therapy: 22/35 (63%) patients classified as resistant *ex vivo* did not respond clinically to induction therapy, and 29/39 (74%) patients classified as sensitive *ex vivo* showed a clinical response. The overall correlation was correct for 51 out of 74 patients (69%) and the association was significant ($P = 0·001$, Table II).

In total, 35 patients were classified as resistant to cytarabine in the PharmaFlow study. The risk of death was significantly higher among these patients than in those classified as sensitive to cytarabine (HR: 1·91; 95% CI: 1·06–3·43; $P = 0·030$; supplementary Figure S3).

Interrelationships between mutational and ex vivo sensitivity profiles

The presence of specific gene mutations was compared between groups of patients classified as resistant or sensitive to particular drug assays, to search for relationships between these two experimental readouts. As expected, there were large differences in AUC_{norm} values between sensitive and resistant patients in selected drug–gene pairs. For example, patient samples containing *KMT2A* mutations were more sensitive to idarubicin (median AUC_{norm} values: 7·78 vs. 40·12, $P = 0·001$, Fig 2A) and fludarabine (12·16 vs. 38·45, $P = 0·044$, Fig 2B) than non-mutated samples. Similarly, patient samples with the *FLT3*-ITD mutation were more sensitive to daunorubicin than non-mutated *FLT3*-ITD samples, with significant differences in AUC_{norm} between the groups (9·85 vs. 35·88, $P = 0·007$, Fig 2C). Also, patient samples with the *FLT3*-SNV mutation were more sensitive to 6-thioguanine than non-mutated patient samples (9·69 vs. 39·6, $P = 0·044$, Fig 2D). In addition, patient samples with mutated *NPM1* had a greater sensitivity to mitoxantrone than non-mutated *NPM1* samples (14·29 vs. 42·58, $P = 0·029$, Fig 2E) and a similar finding was observed for amsacrine (26·22 vs. 57·20, $P = 0·031$, Fig 2F).

Conversely, the presence of some mutations was associated with *ex vivo* drug resistance. For example, patients with *TP53* mutations had significantly higher AUC_{norm} values than non-mutated patients for fludarabine (52·36 vs. 26·97, $P = 0·044$, Fig 2G) and mitoxantrone (90·83 vs. 37·43, $P = 0·045$, Fig 2H). Also, *U2AF1* mutations were associated with significantly higher AUC_{norm} values for amsacrine (70·33 vs. 33·77, $P = 0·032$, Fig 2I) and 6-thioguanine (89·22 vs. 22·43, $P = 0·047$, Fig 2J). Likewise, patients with mutations in *IDH2* or *EPOR* had significantly higher AUC_{norm} values for cytarabine than patients with non-mutated genes (*IDH2*: 56·80 vs. 19·19, $P = 0·049$, Fig 2K; *EPOR:* 57·20 vs. 19·48, $P = 0·043$, Fig 2L).

Overall, these results suggest that the differences of *ex vivo* chemosensitivity can be partially explained by the presence of certain somatic mutations in malignant cells.

Multiresistance in the chemosensitivity ex vivo assay confers an adverse prognosis

The inter-individual variation of the pharmacodynamic parameters derived from the standard Hill dose–response curves was large. For example, the $\log EC_{50}$ values for cytarabine ranged from −1·89 to 4·07 (median: 0·70). A complete description of the estimated pharmacologic parameters is shown in supplementary Table SII. This large variability

Table II. Cytarabine *ex vivo* assay correlation.

		Clinical response			
		RESISTANT	SENSITIVE		
Ex vivo response	RESISTANT	22 (30 %)	13 (17·5%)	PPV 63 %	$n = 35$ (47 %)
	SENSITIVE	10 (13·5 %)	29 (39 %)	NPV 74·4 %	$n = 39$ (53 %)
		$n = 32$ (43 %)	$n = 42$ (57 %)	69 %	
		SENSITIVITY	SPECIFICITY		
		68·8 %	81·5 %		

Correlation of *ex vivo* response with clinical outcome. For clinical response, complete remission was considered as sensitive response; and partial remission, refractoriness or death by toxicity was considered as resistant response. For *ex vivo* evaluation, an AUC value <21·4 (cut-off obtained from ROC curve) is considered as a sensitive response and an AUC value >21·4 as a resistant response. PPV, positive predictive value; NPV, negative predictive value.

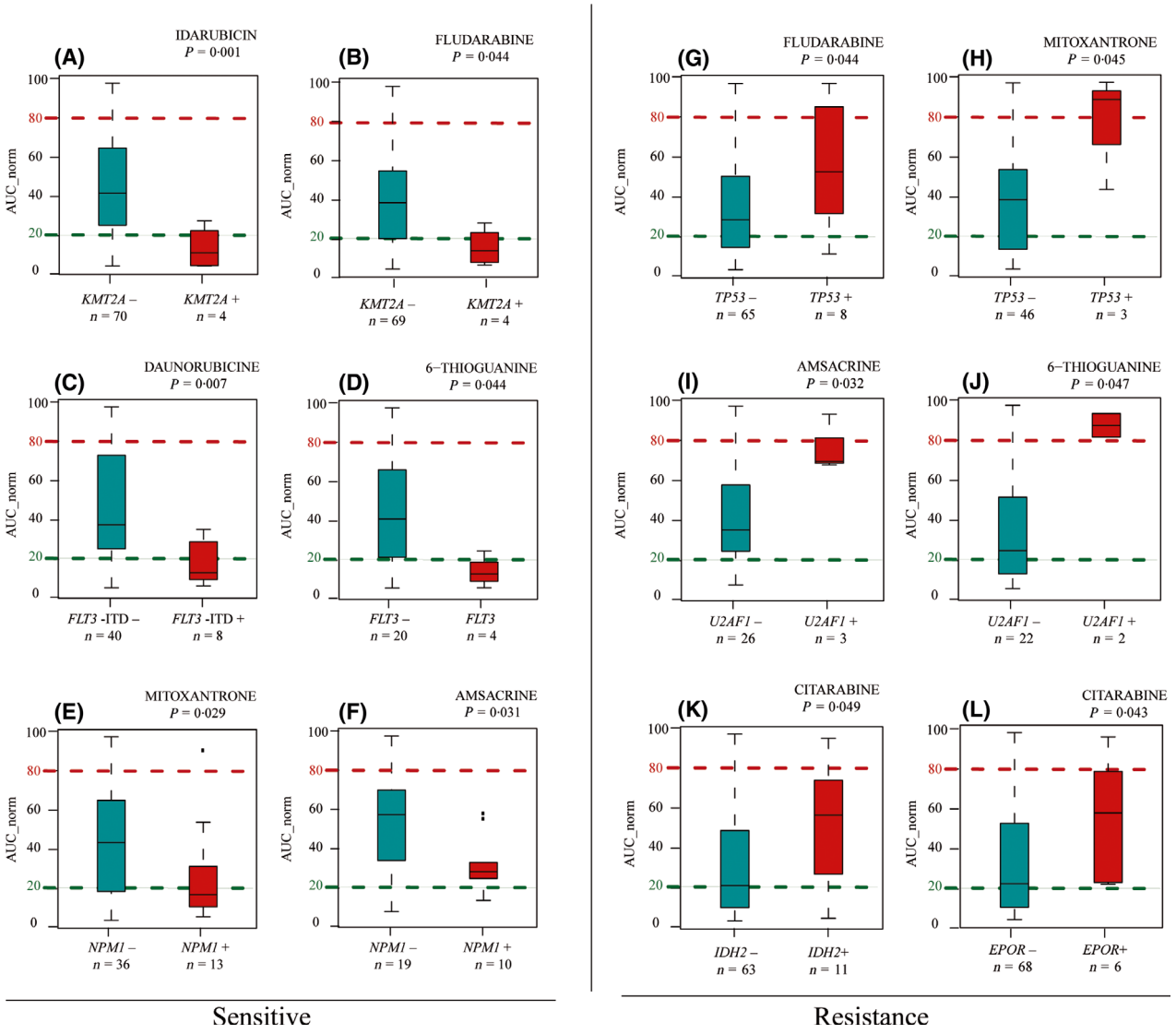

Fig 2. Mutational status-modified pharmacological response. On theleft, box plots of AUC_{norm} values focus on sensitive patients in selected drug–gene pairs. Patients with a mutation in *KMT2A* (red) have greater sensitivity to idarubicin (A) with a median AUC_{norm} of 40·12 vs. 7·78 (P = 0·001, n = 74) and fludarabine (B) with a median AUC_{norm} of 38·45 vs. 12·16 (P = 0·044, n = 73) with respect to non-mutated patients for *KMT2A* (green). Patients with a mutation in *FLT3*-ITD (red) are more sensitive to daunorubicin (C) with an AUC_{norm} median of 35·88 vs. 9·85 (P = 0·007, n = 48) with respect to non-mutated patients for *FLT3*-ITD (green). Patients mutated in *FLT3*-SNV (red) have a higher sensitivity to 6-thioguanine (D) with a mean AUC_{norm} of 39·6 vs. 9·69 (P = 0·044, n = 24) with respect to non-mutated patients for *FLT3*-SNV (green). Patients mutated in *NPM1* (red) have a greater sensitivity to mitoxantrone (E) with an AUC_{norm} median of 42·58 vs. 14·29 (P = 0·029, n = 49) and amsacrine (F) with a mean AUC_{norm} of 57·20 vs. 26·22 (P = 0·031; n = 29) compared with non-mutated patients for *NPM1* (green). On the right, box plots of AUC_{norm} values focus on resistant patients in selected drug–gene pairs. Patients mutated in *TP53* (red) have lower sensitivity to fludarabine (G) with an AUC_{norm} median of 26·97 vs. 52·36; (P = 0·044, n = 73) and mitoxantrone (H) with a mean AUC_{norm} of 37·43 vs. 90·83 (P = 0·045; n = 49) with respect to non-mutated patients for *TP53* (green). Patients mutated in *U2AF1* (red) have lower sensitivity to amsacrine (I) with an AUC_{norm} median of 33·77 *vs.* 70·33 (P = 0·032, n = 29) and 6-thioguanine (J) with a median AUC_{norm} of 22·43 vs. 89·22 (P = 0·047; n = 27) with respect to non-mutated patients for *U2AF1* (green). Patients mutated in *IDH2* have lower sensitivity to cytarabine (K) with AUC_{norm} median of 19·19 vs. 56·80 (P = 0·049; n = 74) compared with non-mutated patients for *IDH2*. Patients mutated in *EPOR* have lower sensitivity to cytarabine (L) with a median AUC_{norm} of 19·48 vs. 57·20 (P = 0·043; n = 74) with respect to non-mutated patients for *EPOR*. [Colour figure can be viewed at wileyonlinelibrary.com]

facilitated the classification of sensitive and resistant samples for each drug, as samples showed an extreme sensitive or extreme resistant profile if the individual AUC_{norm} value was above the 80th AUC_{norm} population percentile for this group or below the 20th percentile, respectively. Because the 10 drugs studied were not evaluated in all patient samples systematically, we used clustering analysis to study the activities of several drugs evaluated in different samples.

The clustering analysis identified 23 patients with a multiresistant (MR) profile, labelled with an adverse

chemosensitivity profile. These patients had a significantly greater risk of death as compared with the remaining 51 patients who had a favourable chemosensitivity profile (multisensitive or neutral) (HR: 2·09, 95% CI: 1·14–3·82; $P = 0·017$; Fig 3).

This cluster analysis also identified three homogeneous groups of drugs that were generally consistent with their mechanisms of action (Fig 4, left). Accordingly, the topoisomerase inhibitors daunorubicin, mitoxantrone, etoposide, amsacrine and idarubicin grouped together in the same branch, whereas the nucleic acid inhibitors cytarabine, fludarabine and clofarabine grouped into another branch. By contrast, the topoisomerase inhibitor decitabine and the nucleic acid inhibitor 6-thioguanine were located together in a separate branch.

To assess whether mutational status and clinical data correlated with clustered drug response groups, the most relevant mutated genes in AML were examined (Fig 4, right). Mutations in *FLT3* (*ITD* and/or *SNV*) *NPM1*, *DNMT3A*, *IDH1/2*, *KIT*, *NRAS*, *TET2* and *TP53* were found equally in all three groups. However, the multisensitive group was the only group that presented mutated *KMT2A* and the only group without mutations in *EPOR*, *EZH2*, *RUNX1*, or *U2AF1*. Analysis of clinical characteristics showed there were no differences between *ex vivo* groups with regards to AML type, prognosis or cytogenetic risk. However, we observed that the multiresistance *ex vivo* group included patients who showed partial remission or resistance to induction therapy.

Mutational and pharmacological profiles in combination improve clinical outcome prediction

As described previously, adverse mutational or adverse chemosensitivity profiles were associated with adverse outcomes (shorter OS and drug resistance). The relative contributions of mutational and pharmacological data to predict OS were explored in a multivariate Cox model. Having an adverse chemosensitivity profile (multiresistance group) was independently associated with mortality risk (HR: 2·09, 95% CI: 1·14–3·82, $P = 0·017$), as was the presence of mutations in *KMT2A* (HR: 3·49, 95% CI: 1·16–10·45, $P = 0·026$) or *TP53* (HR: 3·23, 95% CI: 1·36–7·70, $P = 0·008$) genes. The *U2AF1* and *EZH2* genes were not significantly associated with OS and were not included in the final model.

The prognostic index derived from the Cox model was regarded as an overall risk custom score that integrated the information from both chemosensitivity and mutational profiles. This overall risk score was dichotomised after constructing the ROC curve against the observed clinical response, and the optimal cut-off point for such dichotomisation was established at 2·02. Patients with risk scores above and below this threshold were considered to have an overall adverse and favourable profile, respectively. The risk of death was greater among patients with an overall adverse profile than in patients with an overall favourable profile (HR: 3·40, 95% CI: 1·69–6·84, $P < 0·001$; Fig 5A).

Finally, Fig 5B shows the OS and risk of death classification combining both mutational profile (MUT^+/MUT^-) and PharmaFlow multiresistant profiles (MR^+/MR^-). The risk of death was greater in patients who had adverse mutational and multiresistant profiles (MUT^+/MR^+; HR: 4·82, 95% CI: 1·76–13·25, $P = 0·002$) than in the group of patients without mutational or multiresistant features, separately or together. Also, patients showing only an adverse mutational profile had a greater risk of death than patients without an adverse mutational profile (MUT^+/MR^-; HR: 4·19, 95% CI: 1·89–9·27, $P < 0·001$), and patients with the presence only of an MR profile (MUT^-/MR^+) showed a greater risk of death than patients without an MR profile (HR: 2·58; 95% CI: 1·24–5·34, $P = 0·011$).

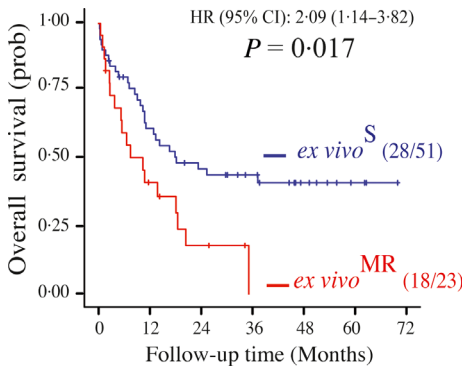

Fig 3. Kaplan–Meier overall survival curves in acute myeloid leukaemia (AML) patients classified by *ex vivo* profile. AML patients were classified as sensitive (*ex vivo*^{S}, $n = 51$) or multiresistant (*ex vivo*^{MR}, $n = 23$) depending on the response of a series of 10 anti-leukaemia drugs. The number of censored patients with respect to the stratified groups and the number at risk and hazard ratio (HR) of risk with a 95% confidence interval is indicated. *P* values are considered significant (<0·05). [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

Precise methods to predict resistance to standard therapy are needed to properly evaluate alternative treatments for AML. (Walter *et al.*, 2015) The present study has established that the identification of somatic mutations by NGS in patients with AML can be combined with the information from *ex vivo* chemosensitivity assays to help predict clinical response in AML before starting induction therapy, to identify patients resistant to induction treatment as well as those with a worse outcome. In addition, the parallel evaluation of *ex vivo* drug-resistant patterns and genetic variations might reveal new insights into the mechanisms of drug resistance.

The use of allelic variants as molecular markers with predictive value is increasing as a criterion to monitor the progression of tumours and the emergence of resistance, both at the beginning and during the treatment. Indeed, the

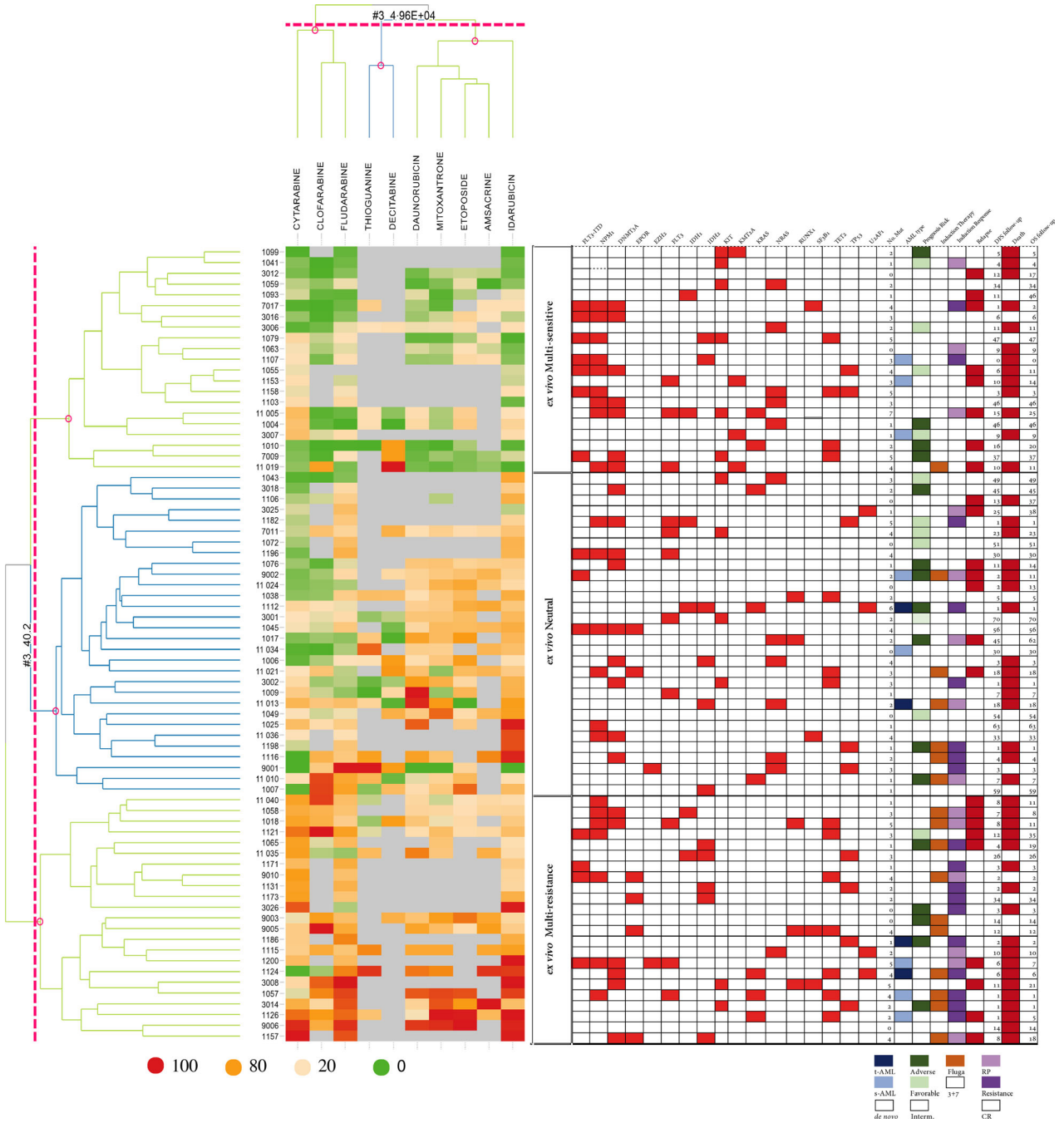

Fig 4. Drug response profile, mutational profile and clinical features connection. On the left, the drug response profile is represented by a heat-map showing the level of response to 10 drugs through AUC_{norm} values. *Ex vivo* samples from acute myeloid leukaemia (AML) patients at diagnosis (rows) and drugs (columns) were ordered according to level of response. At the top, clustering drugs are shown grouped by mechanism of action. Patients were clustered by drug response in three groups: multisensitive, neutral and multiresistant. The level of response was graduated from 0 to 100, as indicated in the legend. On the right, mutational and clinical features of AML patients at diagnosis (rows) are shown by integrated table data. The 17 most recurrent genes are shown, as well as the number of mutations (No.Mut), AML type (dark blue is secondary AML from therapy: t-AML; light blue secondary AML from myelodysplastic syndrome: s-AML; blank *de novo*), prognosis group by ELN-2010 criteria (dark green is the adverse group, light green the favourable group and blank the intermediate group), induction therapy (orange is the FLUGA scheme and blank the 3 + 7 scheme), induction clinical response [dark purple stands for resistance and light purple for partial remission (PR)], relapse, follow-up of disease-free survival (DFS) in months, death and follow-up of overall survival (OS). [Colour figure can be viewed at wileyonlinelibrary.com]

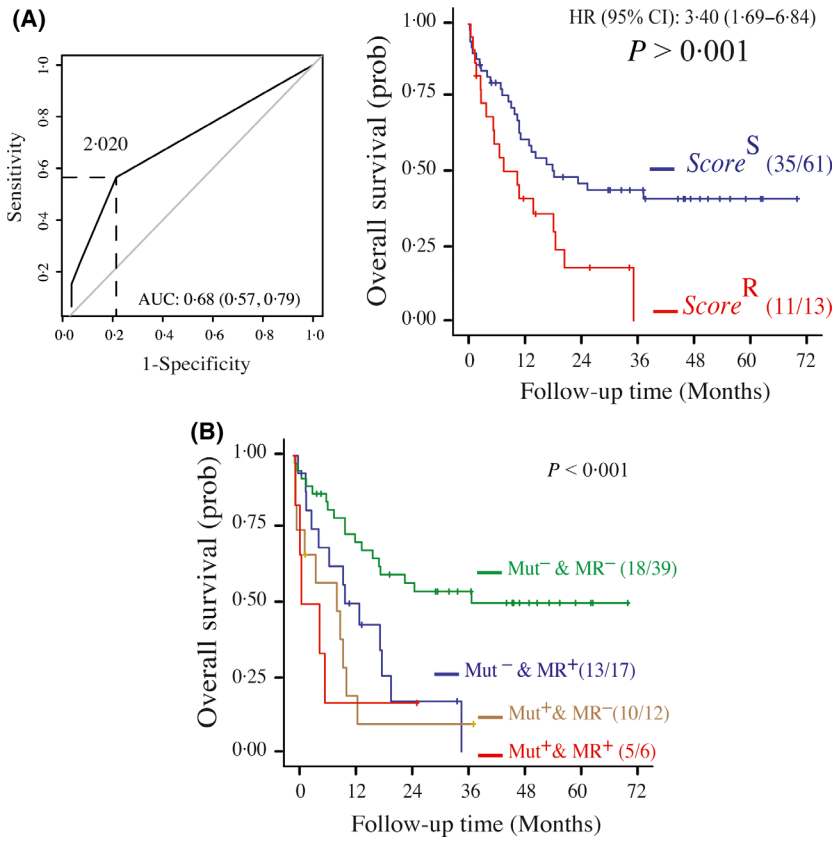

Fig 5. Overall survival according to custom score. (A) AML patients were classified based on a score including mutations in *TP53* or *KMT2A* detected by next-generation sequencing, and a multiresistant profile detected by drug assay. The score was built using the hazard ratio value for each parameter and the cut-off was established using ROC curve analysis. Sensitive patients ($Score^S$, n = 61) showed higher overall survival (OS) than resistant patients ($Score^R$, n = 13). The number of censored patients with respect to the stratified groups and the number at risk and hazard ratio (HR) of risk with a 95% confidence interval is indicated. P values are considered significant (<0·05). (B) The graph displays overall survival according to the presence of mutations in the *EZH2*, *KMT2A*, *U2AF1* and/or *TP53* genes (Mut^+) or not (Mut^-) in combination with the *ex vivo* multiresistant profile status (MR^+ or MR^-) determined by 10-set assay, at diagnosis. The number of censored patients with respect to the stratified groups and the number at risk is indicated. P values are considered significant (<0·05). [Colour figure can be viewed at wileyonlinelibrary.com]

advances in genomics focussed on cancer allow the identification of mutations even when they are present at very low allelic frequency, both at the beginning of the disease or acquired later, which confer resistance to various drugs. Molecular markers along with the patients' age, secondary AML, high white blood cell count and cytogenetic alterations are all associated with resistance to treatments (Walter *et al.*, 2015). In this regard, the latest ELN recommendations incorporate the mutations in six genes (*FLT3, NPM1, CEBPA, RUNX1, TP53* and *ASXL1*) among the genetic prognostic factors of AML (Dohner *et al.*, 2017). Nevertheless, prognostic assessment of AML remains challenging given the high degree of molecular complexity and heterogeneity, and the identification of patients with chemo-refractory disease is difficult (Walter *et al.*, 2015). On the other hand, the inter-individual variability of *ex vivo* chemosensitivity profiling has previously been related to genomic changes in AML, suggesting a complex interdependence between the genomic makeup and drug response related to variations of clonal composition of the tumoural load following drug exposure, although functional studies are warranted. (Pemovska *et al.*, 2013a).

We observed interrelationships between mutational and *ex vivo* sensitivity profiles. In particular, higher *ex vivo* sensitivity was observed in patients mutated in *KMT2A* in idarubicin and fludarabine assays, in patients mutated in *FLT3* in daunorubicin and 6-thioguanine assays, and in patients mutated in *NPM1* in mitoxantrone and amsacrine assays. Conversely, lower sensitivity *ex vivo* was observed in patients mutated in *TP53* in fludarabine and mitoxantrone assays, in patients mutated in *U2AF1* in amsacrine and 6-thioguanine assays, in patients mutated in *IDH2* in cytarabine assay, and in patients mutated in *EPOR* in cytarabine assays. These results are novel as previous studies have only found relationships with targetted treatments (not approved in monotherapy) as opposed to our study using standard treatments approved in clinical protocols (Pemovska *et al.*, 2013b). This leads us to postulate that functional changes identified by known biomarkers might play a key role in the mechanisms underlying drug resistance. However, one limitation in the present study is that the mechanisms of action of the evaluated drugs are not exclusive to a specific molecular target and functional studies become complex, similar to the reported functional studies where interrelated new clinical drugs with the respective target landscape are needed (Klaeger *et al.*, 2017).

Remarkably, we were able to develop a custom integrated risk score based on both pharmacological and mutational information that could identify a subgroup of patients with an adverse prognosis. To our knowledge, this is the first study where a combined prognostic score was derived in a series of AML patients treated homogeneously, obtaining

both correlations with clinical and outcome results (unlike previously published works) (Pemovska *et al.*, 2013b). Moreover, the risk score was developed considering survival time, a hard clinical endpoint that was pinpointed as one of the standards against which *ex vivo* chemosensitivity tests had not been validated previously (Staib *et al.*, 2005). The risk score outperformed risk classification done exclusively on molecular grounds as well as the established ELN risk classification when applied in this same set of patients.

Regarding the prediction capacity of resistance to induction treatments evaluating several tests at once, we identified the multiresistant *ex vivo* pattern for the first time as a powerful drug resistance marker (predicts 68·8%) with greater specificity than the PharmaFlow test when a single drug was considered (81·5% vs. 68·4%). We also observed that mutational status was correlated with the clustered drug response groups: the multisensitive *ex vivo* group showed no mutations in *EPOR*, *EZH2*, *RUNX1* or *U2AF1*, and was the only group that presented mutated *KMT2A*. Nevertheless, mutations in *FLT3* (*ITD* and/or *SNV) NPM1*, *DNMT3A*, *IDH1/2, KIT, NRAS, TET2* and *TP53* were found in all three groups equally. Of note, *NPM1* mutations were included in the multiresistant *ex vivo* group, and these patients had partial responses to induction treatment. Multiple PharmaFlow assays should be routinely considered in the analysis of drug resistance, in the same way that multigene markers are recommended in the analysis of pharmacogenomic data to identify drug-sensitive tumours (Naulaerts, Dang & Ballester, 2017). A better accuracy in resistance detection is a priority to optimise treatments and improve outcomes.

Regarding the prediction of survival, an unusual number of patients resistant to induction therapy was detected (43%) in our cohort, which could be due to the inclusion of 24% of patients receiving low-intensity treatment (FLUGA). Patients with a multiresistant profile in the *ex vivo* test present worse survival than patients with neutral/sensitive profile (HR 2·58 vs. 1·91). The high-risk mutational profile confers great weight in the prediction of cases with worse survival (HR 4·19) and is slightly improved with the inclusion of the *ex vivo* multiresistant pattern (HR 4·82).

Drug resistance is considered to underlie most chemotherapy failures in AML. However, since the drugs are usually given in combination, it is difficult to identify the individual drug responsible for therapeutic failure. Accordingly, an efficient *ex vivo* chemosensitivity test could be of enormous benefit in such an identification, and would also help in selecting alternative drugs that could potentially improve the response rate and minimise toxicity. Despite the great efforts in developing chemosensitivity tests for many years and the promising results obtained in tailored trials to validate their use in clinical practice, there remains a lack of actionable, robust tests that can be readily used to guide therapeutic decisions in routine practice (Jun *et al.*, 2007). In this way, we detected patients that despite having multiresistance show sensitivity to other drugs such as clofarabine (Clo) and fludarabine (Flu), as alternative therapies.

The principal limitation of the present study is the lack of *ex vivo* chemosensitivity data from the remaining 116 patients. This reduced the statistical power to detect candidate genes in the Cox model used to produce the risk score. While mutational profiling is common in the workup of AML, the implementation of *ex vivo* sensitivity drugs is difficult and their application to clinical practice is not easy. Future studies should address the relationships between drug resistance and specific genetic biomarkers within clinical trials, which would allow the establishment of individualised risk profiles.

We believe that the combined test could guide therapeutic options in an effective manner, and patients could be prevented from ineffective treatment lines or have more opportunity to start more sensitive treatments a priori. Regarding the economic aspects, the cost of the complete test is equivalent to that of any flow cytometry test and NGS test. We estimated the cost of NGS sequencing for targetted gene panels at €500 and the cost of multiparametric flow cytometry at €1000. In addition, the current recommendations attempt to wait for the result to properly guide treatment. This study does not require more time than is necessary for the current recommendations. The results obtained show that the combined test could guide treatments in an effective manner, especially in the case of resistance detection.

Conclusion

We have developed a new score based on personalised testing to identify individual drug resistance and also to improve current early risk stratification and predictive outcomes for patients with AML. Our score combines innate resistance to chemotherapy *ex vivo* with known mutational analysis. *Ex vivo* drug sensitivity testing can capture some information regarding mutations with unknown significance not otherwise captured in NGS, but that may be playing an important role in the disease. However, it would be necessary to validate these results in a prospective study before including this score in decision-making in clinical practice.

Author contributions

EO collected samples, performed the experiments, analysed and interpreted data, and wrote the manuscript. YR analysed and interpreted data and wrote the manuscript. IR and ML analysed and interpreted data. DM-C, EB, JP-O, PM, EM, PH, BB and CS collected samples and clinical data. JG, JR and JV analysed and interpreted data experiments and JB and PM supervised the research, analysed and interpreted data, and wrote the manuscript. RA and JML collected samples and clinical data, designed and supervised research and experiments, analysed and interpreted data, and wrote the manuscript. All authors prepared the report and approved the final version.

Funding information

This study was supported by the Subdirección General de Investigación Sanitaria (Instituto de Salud Carlos III, Spain) grants PI13/02387 and PI16/01530, and the CRIS against Cancer foundation grant 2014/0120. ML holds a postdoctoral fellowship ffrom the Spanish Ministry of Economy and Competitiveness (FPDI-2013-16409). YR holds a predoctoral fellowship from the Spanish of Instituto de salud Carlos III: Contratos Predoctorales de Formación en Investigación en Salud i-PFIS (IFI 14/ 00008).

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was reviewed and approved by the institutional review board/independent ethics committee of the participating centres.

Consent for publication

Not applicable.

Conflicts of interest

YR-H, JR, JG, and JB are employees of Vivia Biotech, Tres Cantos, Madrid, Spain. The other authors declare that they have no conflicts of interest.

Data availability statement

The datasets used and/or analysed in the current study are available from the corresponding author on reasonable request.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Population frequency.

Fig S2. Kaplan–Meier overall survival curves in AML patients classified by global mutational profile.

Fig S3. Kaplan–Meier overall survival curves in AML patients classified by global mutational profile.

Table S1. NGS custom panel**.**

Table S2. *Ex vivo* chemosensitivity tests.

Data S1. Supplemental data.

References

Adzhubei, I., Jordan, D.M. & Sunyaev, S.R. (2013) 'Predicting functional effect of human missense mutations using PolyPhen-2', *Curr Protoc Hum Genet*, Chapter 7, pp. Unit 7 20.

Bennett, T.A., Montesinos, P., Moscardo, F., Martinez-Cuadron, D., Martinez, J., Sierra, J., Garcia, R., de Oteyza, J.P., Fernandez, P., Serrano, J., Fernandez, A., Herrera, P., Gonzalez, A., Bethancourt, C., Rodriguez-Macias, G., Alonso, A., Vera, J.A., Navas, B., Lavilla, E., Lopez, J.A., Jimenez, S., Simiele, A., Vidriales, B., Gonzalez, B.J., Burgaleta, C., Hernandez Rivas, J.A., Mascunano, R.C., Bautista, G., Perez Simon, J.A., Fuente Ade, L., Rayon, C., Troconiz, I.F., Janda, A., Bosanquet, A.G., Hernandez-Campo, P., Primo, D., Lopez, R., Liebana, B., Rojas, J.L., Gorrochategui, J., Sanz, M.A. & Ballesteros, J. (2014) Pharmacological profiles of acute myeloid leukemia treatments in patient samples by automated flow cytometry: a bridge to individualized medicine. *Clinical Lymphoma Myeloma and Leukemia*, **14**, 305–318.

Burge, C. & Karlin, S. (1997) Prediction of complete gene structures in human genomic DNA. *Journal of Molecular Biology*, **268**, 78–94.

Cedena, M.T., Rapado, I., Santos-Lozano, A., Ayala, R., Onecha, E., Abaigar, M., Such, E., Ramos, F., Cervera, J., Diez-Campelo, M., Sanz, G., Rivas, J.H., Lucia, A. & Martinez-Lopez, J. (2017) Mutations in the DNA methylation pathway and number of driver mutations predict response to azacitidine in myelodysplastic syndromes. *Oncotarget*, **8**, 106948–106961.

Dohner, H., Estey, E., Grimwade, D., Amadori, S., Appelbaum, F.R., Buchner, T., Dombret, H., Ebert, B.L., Fenaux, P., Larson, R.A., Levine, R.L., Lo-Coco, F., Naoe, T., Niederwieser, D., Ossenkoppele, G.J., Sanz, M., Sierra, J., Tallman, M.S., Tien, H.F., Wei, A.H., Lowenberg, B. & Bloomfield, C.D. (2017) Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*, **129**, 424–447.

van Dongen, J.J., Lhermitte, L., Bottcher, S., Almeida, J., van der Velden, V.H., Flores-Montero, J., Rawstron, A., Asnafi, V., Lecrevisse, Q., Lucio, P., Mejstrikova, E., Szczepanski, T., Kalina, T., de Tute, R., Bruggemann, M., Sedek, L., Cullen, M., Langerak, A.W., Mendonca, A., Macintyre, E., Martin-Ayuso, M., Hrusak, O., Vidriales, M.B., Orfao, A. & EuroFlow, C. (2012) EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. *Leukemia*, **26**, 1908–1975.

Gale, R.E., Green, C., Allen, C., Mead, A.J., Burnett, A.K., Hills, R.K. & Linch, D.C., and Medical Research Council Adult Leukaemia Working, P (2008) The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. *Blood*, **111**, 2776–2784.

Gorello, P., Cazzaniga, G., Alberti, F., Dell'Oro, M.G., Gottardi, E., Specchia, G., Roti, G., Rosati, R., Martelli, M.F., Diverio, D., Lo Coco, F., Biondi, A., Saglio, G., Mecucci, C. & Falini, B. (2006) Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. *Leukemia*, **20**, 1103–1108.

Hernandez, P., Gorrochategui, J., Primo, D., Robles, A., Rojas, J.L., Espinosa, A.B., Gomez, C., Martinez-Lopez, J., Bennett, T.A. & Ballesteros, J. (2017) Drug discovery testing compounds in patient samples by automated flow cytometry. *SLAS Technol*, **22**, 325–337.

Itzykson, R., Kosmider, O., Cluzeau, T., Mansat-De Mas, V., Dreyfus, F., Beyne-Rauzy, O., Quesnel, B., Vey, N., Gelsi-Boyer, V., Raynaud, S., Preudhomme, C., Ades, L., Fenaux, P. & Fontenay, M., and Groupe Francophone des, M (2011) Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. *Leukemia*, **25**, 1147–1152.

Jun, K.R., Jang, S., Chi, H.S., Lee, K.H., Lee, J.H., Choi, S.J., Seo, J.J., Moon, H.N., Im, H.J. & Park, C.J. (2007) Relationship between in vitro chemosensitivity assessed with MTT assay and clinical outcomes in 103 patients with acute leukemia. *Korean Journal of Laboratory Medicine*, **27**, 89–95.

Kalina, T., Flores-Montero, J., van der Velden, V.H., Martin-Ayuso, M., Bottcher, S., Ritgen, M., Almeida, J., Lhermitte, L., Asnafi, V., Mendonca, A., de Tute, R., Cullen, M., Sedek, L., Vidriales, M.B., Perez, J.J., te Marvelde, J.G., Mejstrikova, E., Hrusak, O., Szczepanski, T., van Dongen, J.J., Orfao, A. & EuroFlow, C. (2012) EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia*, **26**, 1986–2010.

Klaeger, S., Heinzlmeir, S., Wilhelm, M., Polzer, H., Vick, B., Koenig, P.A., Reinecke, M., Ruprecht,

B., Petzoldt, S., Meng, C., Zecha, J., Reiter, K., Qiao, H., Helm, D., Koch, H., Schoof, M., Canevari, G., Casale, E., Depaolini, S.R., Feuchtinger, A., Wu, Z., Schmidt, T., Rueckert, L., Becker, W., Huenges, J., Garz, A.K., Gohlke, B.O., Zolg, D.P., Kayser, G., Vooder, T., Preissner, R., Hahne, H., Tonisson, N., Kramer, K., Gotze, K., Bassermann, F., Schlegl, J., Ehrlich, H.C., Aiche, S., Walch, A., Greif, P.A., Schneider, S., Felder, E.R., Ruland, J., Medard, G., Jeremias, I., Spiekermann, K. & Kuster, B. (2017) The target landscape of clinical kinase drugs. *Science*, **358**, https://doi.org/10.1126/science.aan4368

Martinez-Cuadron, D., Boluda, B., Martinez, P., Bergua, J., Rodriguez-Veiga, R., Esteve, J., Vives, S., Serrano, J., Vidriales, B., Salamero, O., Cordon, L., Sempere, A., Jimenez-Ubieto, A., Prieto-Delgado, J., Diaz-Beya, M., Garrido, A., Benavente, C., Perez-Simon, J.A., Moscardo, F., Sanz, M.A., Montesinos, P. & Cetlam and groups, P (2018) Correction to: A phase I-II study of plerixafor in combination with fludarabine, idarubicin, cytarabine, and G-CSF (PLERIFLAG regimen) for the treatment of patients with the first early-relapsed or refractory acute myeloid leukemia. *Annals of Hematology*, **97**, 923.

Martinez-Cuadron, D., Gil, C., Serrano, J., Rodriguez, G., Perez-Oteyza, J., Garcia-Boyero, R., Jimenez-Bravo, S., Vives, S., Vidriales, M.B., Lavilla, E., Perez-Simon, J.A., Tormo, M., Colorado, M., Bergua, J., Lopez, J.A., Herrera, P., Hernandez-Campo, P., Gorrochategui, J., Primo, D., Rojas, J.L., Villoria, J., Moscardo, F., Troconiz, I., Linares Gomez, M., Martinez-Lopez, J., Ballesteros, J., Sanz, M., Montesinos, P., Spanish, P. g (2019) A precision medicine test predicts clinical response after idarubicin and cytarabine induction therapy in AML patients. *Leukemia Research*, **76**, 1–10.

Mollgard, L., Deneberg, S., Nahi, H., Bengtzen, S., Jonsson-Videsater, K., Fioretos, T., Andersson, A., Paul, C. & Lehmann, S. (2008) The FLT3 inhibitor PKC412 in combination with cytostatic drugs in vitro in acute myeloid leukemia. *Cancer Chemotherapy and Pharmacology*, **62**, 439–448.

Naulaerts, S., Dang, C.C. & Ballester, P.J. (2017) Precision and recall oncology: combining multiple gene mutations for improved identification of drug-sensitive tumours. *Oncotarget*, **8**, 97025–97040.

Ng, P.C. & Henikoff, S. (2003) SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Research*, **31**, 3812–3814.

Onecha, E., Linares, M., Rapado, I., Ruiz-Heredia, Y., Martinez-Sanchez, P., Cedena, T., Pratcorona, M., Perez Oteyza, J., Herrera, P., Barragan, E., Montesinos, P., Garcia Vela, J.A., Magro, E., Anguita, E., Figuera, A., Riaza, R., Martinez-Barranco, P., Sanchez-Vega, B., Nomdedeu, J., Gallardo, M., Martinez-Lopez, J. & Ayala, R. (2018) Novel deep targeted sequencing method for minimal residual disease monitoring in acute myeloid leukemia. *Haematologica*, **104**, 288–296.

Papaemmanuil, E., Gerstung, M., Bullinger, L., Gaidzik, V.I., Paschka, P., Roberts, N.D., Potter, N.E., Heuser, M., Thol, F., Bolli, N., Gundem, G., Van Loo, P., Martincorena, I., Ganly, P., Mudie, L., McLaren, S., O'Meara, S., Raine, K., Jones, D.R., Teague, J.W., Butler, A.P., Greaves, M.F., Ganser, A., Dohner, K., Schlenk, R.F., Dohner, H. & Campbell, P.J. (2016) Genomic classification and prognosis in acute myeloid leukemia. *New England Journal of Medicine*, **374**, 2209–2221.

Patel, J.P., Gonen, M., Figueroa, M.E., Fernandez, H., Sun, Z., Racevskis, J., Van Vlierberghe, P., Dolgalev, I., Thomas, S., Aminova, O., Huberman, K., Cheng, J., Viale, A., Socci, N.D., Heguy, A., Cherry, A., Vance, G., Higgins, R.R., Ketterling, R.P., Gallagher, R.E., Litzow, M., van den Brink, M.R., Lazarus, H.M., Rowe, J.M., Luger, S., Ferrando, A., Paietta, E., Tallman, M.S., Melnick, A., Abdel-Wahab, O. & Levine, R.L. (2012) Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. *New England Journal of Medicine*, **366**, 1079–1089.

Pemovska, T., Kontro, M., Yadav, B., Edgren, H., Eldfors, S., Szwajda, A., Almusa, H., Bespalov, M.M., Ellonen, P., Elonen, E., Gjertsen, B.T., Karjalainen, R., Kulesskiy, E., Lagstrom, S., Lehto, A., Lepisto, M., Lundan, T., Majumder, M.M., Marti, J.M., Mattila, P., Murumagi, A., Mustjoki, S., Palva, A., Parsons, A., Pirttinen, T., Ramet, M.E., Suvela, M., Turunen, L., Vastrik, I., Wolf, M., Knowles, J., Aittokallio, T., Heckman, C.A., Porkka, K., Kallioniemi, O. & Wennerberg, K. (2013a) 'c', Cancer Discov, **3**, 1416–1429.

Pemovska, T., Kontro, M., Yadav, B., Edgren, H., Eldfors, S., Szwajda, A., Almusa, H., Bespalov, M.M., Ellonen, P., Elonen, E., Gjertsen, B.T., Karjalainen, R., Kulesskiy, E., Lagstrom, S., Lehto, A., Lepisto, M., Lundan, T., Majumder, M.M., Marti, J.M., Mattila, P., Murumagi, A., Mustjoki, S., Palva, A., Parsons, A., Pirttinen, T., Ramet, M.E., Suvela, M., Turunen, L., Vastrik, I., Wolf, M., Knowles, J., Aittokallio, T., Heckman, C.A., Porkka, K., Kallioniemi, O. & Wennerberg, K. (2013b) Individualized systems medicine strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. *Cancer Discovery*, **3**, 1416–1429.

Rowe, J.M. & Tallman, M.S. (2010) How I treat acute myeloid leukemia. *Blood*, **116**, 3147–3156.

Sato, T., Yang, X., Knapper, S., White, P., Smith, B.D., Galkin, S., Small, D., Burnett, A. & Levis, M. (2011) FLT3 ligand impedes the efficacy of FLT3 inhibitors in vitro and in vivo. *Blood*, **117**, 3286–3293.

Staib, P., Staltmeier, E., Neurohr, K., Cornely, O., Reiser, M. & Schinkothe, T. (2005) Prediction of individual response to chemotherapy in patients with acute myeloid leukaemia using the chemosensitivity index Ci. *British Journal of Haematology*, **128**, 783–791.

Swords, R.T., Azzam, D., Al-Ali, H., Lohse, I., Volmar, C.H., Watts, J.M., Perez, A., Rodriguez, A., Vargas, F., Elias, R., Vega, F., Zelent, A., Brothers, S.P., Abbasi, T., Trent, J., Rangwala, S., Deutsch, Y., Conneally, E., Drusbosky, L., Cogle, C.R. & Wahlestedt, C. (2018) Ex-vivo sensitivity profiling to guide clinical decision making in acute myeloid leukemia: A pilot study. *Leukemia Research*, **64**, 34–41.

Tate, J.G., Bamford, S., Jubb, H.C., Sondka, Z., Beare, D.M., Bindal, N., Boutselakis, H., Cole, C.G., Creatore, C., Dawson, E., Fish, P., Harsha, B., Hathaway, C., Jupe, S.C., Kok, C.Y., Noble, K., Ponting, L., Ramshaw, C.C., Rye, C.E., Speedy, H.E., Stefancsik, R., Thompson, S.L., Wang, S., Ward, S., Campbell, P.J. & Forbes, S.A. (2019) COSMIC: the Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Research*, **47**, D941–D947.

Tyner, J.W., Tognon, C.E., Bottomly, D., Wilmot, B., Kurtz, S.E., Savage, S.L., Long, N., Schultz, A.R., Traer, E., Abel, M., Agarwal, A., Blucher, A., Borate, U., Bryant, J., Burke, R., Carlos, A., Carpenter, R., Carroll, J., Chang, B.H., Coblentz, C., d'Almeida, A., Cook, R., Danilov, A., Dao, K.T., Degnin, M., Devine, D., Dibb, J., Edwards, D.K.t., Eide, C.A., English, I., Glover, J., Henson, R., Ho, H., Jemal, A., Johnson, K., Johnson, R., Junio, B., Kaempf, A., Leonard, J., Lin, C., Liu, S.Q., Lo, P., Loriaux, M.M., Luty, S., Macey, T., MacManiman, J., Martinez, J., Mori, M., Nelson, D., Nichols, C., Peters, J., Ramsdill, J., Rofelty, A., Schuff, R., Searles, R., Segerdell, E., Smith, R.L., Spurgeon, S.E., Sweeney, T., Thapa, A., Visser, C., Wagner, J., Watanabe-Smith, K., Werth, K., Wolf, J., White, L., Yates, A., Zhang, H., Cogle, C.R., Collins, R.H., Connolly, D.C., Deininger, M.W., Drusbosky, L., Hourigan, C.S., Jordan, C.T., Kropf, P., Lin, T.L., Martinez, M.E., Medeiros, B.C., Pallapati, R.R., Pollyea, D.A., Swords, R.T., Watts, J.M., Weir, S.J., Wiest, D.L., Winters, R.M., McWeeney, S.K. & Druker, B.J. (2018) Functional genomic landscape of acute myeloid leukaemia. *Nature*, **562**, 526–531.

Walter, R.B., Othus, M., Burnett, A.K., Lowenberg, B., Kantarjian, H.M., Ossenkoppele, G.J., Hills, R.K., Ravandi, F., Pabst, T., Evans, A., Pierce, S.R., Vekemans, M.C., Appelbaum, F.R. & Estey, E.H. (2015) Resistance prediction in AML: analysis of 4601 patients from MRC/NCRI, HOVON/SAKK, SWOG and MD Anderson Cancer Center. *Leukemia*, **29**, 312–320.

Welch, J.S., Petti, A.A. & Ley, T.J. (2017) Decitabine in TP53-Mutated AML. *New England Journal of Medicine*, **376**, 797–798.