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Original Article

Differences in *ex-vivo* Chemosensitivity to Anthracyclines in First Line Acute Myeloid Leukemia

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Abstract. ***Background:*** **Induction schedules in acute myeloid leukemia (AML) are based on combinations of cytarabine and anthracyclines. The choice of the anthracycline employed has been widely studied in multiple clinical trials showing similar complete remission rates.**
Materials and Methods: **Using an *ex vivo* test we have analyzed if a subset of AML patients may respond differently to cytarabine combined with idarubicin, daunorubicin or mitoxantrone. Bone marrow (BM) samples of 198 AML patients were incubated for 48 hours in 96 well plates, each well containing different drugs or drug combinations at different concentrations. *Ex vivo* drug sensitivity analysis was made using the PharmaFlow platform maintaining the BM microenvironment. Drug response was evaluated as depletion of AML blast cells in each well after incubation. Annexin V-FITC was used to quantify the ability of the drugs to induce apoptosis, and pharmacological responses were calculated using pharmacokinetic population models.**
Results: **Similar dose-respond graphs were generated for the three anthracyclines, with a slight decrease in EC_{50} with idarubicin (p=1.462E-06), whereas the interpatient variability of either drug was large. To identify those cases of selective sensitivity to anthracyclines, potency was**

compared, in terms of area under the curve. Differences in anthracycline monotherapy potency greater than 30% from 3 pairwise comparisons were identified in 28.3% of samples. Furthermore, different sensitivity was detected in 8.2% of patients comparing combinations of cytarabine and anthracyclines.
***Discussion:* A third of the patients could benefit from the use of this test in the first line induction therapy selection, although it should be confirmed in a clinical trial specifically designed.**

Keywords: Anthracycline; *ex-vivo* test; Idarubicin; Daunorubicin; Mitoxantrone; Acute myeloid leukemia; Personalized medicine.

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Introduction. Induction 1st line schedules in *de novo* acute myeloid leukemia (AML) are based in a combination of an anthracycline with cytarabine (CYT) (3+7 schedule), obtaining complete remission (CR) rates of 70-80% after 1-2 cycles.[1,2] Daunorubicin (DNR), idarubicin (IDA), mitoxantrone (MIT, an anthracenedione), and less frequently other anthracyclines have been employed in these schemes. The choice of the anthracycline employed has been widely studied in several randomized clinical trials (RCT),[3-22] showing similar CR rates, with some exceptions in which IDA reported higher CR than DNR,[4,6-8,12] finding reproduced in a Cochrane meta-analysis.[23]

Different *ex vivo* tests have been employed to select the most effective drug combination from the individualized sensitivity and resistance assays, but none of them have been recommended in clinical practice.[24] We are developing a Precision Medicine (PM) test based on an actionable native environment method (PharmaFlow platform), which showed excellent correlations with clinical responses in AML, avoiding some limitations of other *ex vivo* assays.[25]

The objective of this non-interventional study is to explore whether a significant percentage of patients AML samples may show different ex-vivo sensitivity to IDA vs DNR vs MIT combined with CYT.

Patients and Methods.

Patients and study design. A multicenter, prospective, non-interventional cohort study was carried out in 33 Spanish institutions of the PETHEMA group. The inclusion period lasted five years (2012-2017), enrolling patients aged 18 years and older with newly diagnosed AML. Diagnosis and classification of AML were performed according to the World Health Classification (WHO) criteria.[26] This study was approved by the Research Ethics Board of each participating institution and was conducted according to the Spanish law 14/2007 of biomedical research. Informed consent was provided to all patients.

Vivia's PharmaFlow PM Test.

- *Native environment whole bone marrow sample*

Ex vivo drug sensitivity analysis was made using the PharmaFlow platform (previously termed ExviTech®)[25] maintaining the bone marrow (BM) microenvironment. A minimum BM sample volume between 1 and 2 ml was collected by aspiration at AML diagnosis, before starting induction chemotherapy, and was processed by an automated method in Vivia Biotech laboratories 24 hours after extraction. Samples were incubated for 48 hours in 96 well plates, each well containing different drugs or drug combinations at different concentrations, enabling calculation of dose-response curves for every single drug (CYT, IDA, DNR, MIT) and combination used in treatments (CYT-IDA, CYT-DNR, CYT-MIT). The number of BM samples analyzed were 289 with IDA, 333 with DNR and 274 with MIT. A more detailed description of the procedure has been published elsewhere.[25] The concentrations assayed for each anthracycline were:

- Concentrations for IDA (µM): > 0.0002 ; 0.001 ; 0.002 ; 0.006 ; 0.01 ; 0.018 ; 0.02 ; 0.04 ; 0.05 ; 0.055 ; 0.08 ; 0.13 ; 0.16 ; 0.2 ; 0.26 ; 0.4 ; 0.5 ; 0.6 ; 1.5.
- Concentrations for DNR (µM): > 0.001; 0.05 ; 0.075 ; 0.093 ; 0.15 ; 0.18 ; 0.25 ; 0.3 ; 0.37 ; 0.45 ; 0.75 ; 0.85 ; 1.25 ; 1.5 ; 2.7 ; 3.

- Concentrations for MIT (μM): > 0.001 ; 0.0016 ; 0.008 ; 0.01 ; 0.04 ; 0.08 ; 0.2 ; 0.38 ; 0.6 ; 0.8 ; 1 ; 2.33 ; 3.5 ; 7.

- *Modeling of ex vivo activity of CYT, IDA, DNR, MIT.*

Evaluation of drug response was done by counting the number of live pathological cells (LPC) remaining after incubation at increasing drug concentrations. Dying cells (apoptosis) were excluded using Annexin V-FITC. Pharmacological responses were estimated using pharmacodynamic (PD) population-based models[27] which essentially perform the fitting of the dependent variable (natural log of LPC) in a non-linear mixed-effects model to derive typical population values (fixed effects) and the magnitude of inter-patient and residual variability (random effects). Model development was performed with the first-order conditional estimation method using interaction option with the software NONMEM (v7.2)[28], according to the following equation:

$$LPC = LPC_0 \times \left(1 - E_{\max} \times \frac{C^{\gamma}}{C^{\gamma} + EC_{50}^{\gamma}}\right)$$

Where LPC_0 parameter refers to the number of LPC after incubation in the absence of drug, E_{max} represents the maximum fractional decrease in LPC that the drug can elicit, EC_{50}, is the drug concentration exerting half of E_{max} , and γ is the parameter governing the steepness of the LPC vs drug concentration (C) curve. Potency (EC_{50}) and efficacy (E_{max}) are PD parameters that characterize the pharmacological response and are integrated into a single value corresponding to the measurement of the area under the dose-response curve (Area Under the Curve, AUC).

For data presentation, the survival index was computed, with the number of LPC in control wells that were not exposed to any drugs being set as 100%. The number of live cells in each drug-treated well was compared with this control value, and the survival index for each drug at each concentration was determined as the percentage of LPC at every tested concentration.

Interpatient variability (IPV) associated with all parameters was described using an exponential model of the components of variance. An additive error structure was used for the residual variability. Population PD models were built with BM samples from 227 patients that were incubated with IDA, 271 with DNR, and 212 with MIT. Bayesian estimation methods were then used to retrieve individual patient parameters based on their available exposure-response measurements in conjunction with the PD population parameters. After several trials with different modeling strategies, we could conclude that optimal approach, in terms of correlation with clinical output, was achieved by forcing typical parameters to values obtained in a different model using a dataset from samples tested at 72h. Therefore, the typical parameter value for the maximum fractional effect (E_{max}) was set to 1 for both drugs. For γ, the typical parameter value was calculated but limited to the range 0-3. IPV for both parameters could not be determined with this dataset.

For interaction analysis, a Surface Interaction model[29] was used to estimate the degree of synergy, referred as α parameter, between both drugs (R environment (v3.3.1) for statistical computing).[30] In this analysis, a value equal to 0 is an additive effect, a value > 0 indicates a synergistic effect, and a value < 0 reflects an antagonistic effect.

Study endpoints. The primary end-point was the comparison between the selective sensitivities of the different anthracyclines individually using the AUCs in the dose-response curve. For the comparisons between the combinations of anthracyclines with CYT, we employed the volume under the surface (VUS) of the dose-response curves. Besides, the differences in either drug potency or synergism *ex vivo* were also calculated according to the observed and predicted response after induction.

Results.

Patient Characteristics. Overall, 332 BM samples from patients with AML suspicion were received at the laboratory, from which 261 BM samples were completely monitored at the end of the study. Of them, 63 (24%) were not evaluable because of the following protocol issues: 1) incorrect informed consent form (32 patients), 2) no available case report form (23 patients), 3) misdiagnosis (3 patients), and 4) other unknown reasons (5 patients). Overall, clinical data from 198 patient's samples (60%) were available at the end of this study. The main baseline characteristics of these patients are displayed in **Table 1**. In summary, the median age was 61 years (range, 19 to 91), all patients were newly diagnosed AML, and 37 patients (19%) were categorized as having high-risk cytogenetics. CR rate was obtained in 93 patients (47%), whereas 65 patients obtained partial remission or were resistant to induction.

Ex vivo PharmaFlow Test characterization of IDA, DNR and MIT models. Dose-response graphs were generated for the single drugs (IDA, DNR, and MIT) using PD models (Figure 1). Most of the observations were contained within the simulation-based 95% confidence intervals of the 5-95th population percentiles proving good predictability of the selected models. Pharmacological population parameters, as well as variability and error values, are shown in **Table 2.**

The average dose-responses of the three anthracyclines were similar, with a slight decrease in EC_{50} values with IDA (p-value = 1.462E-06; **Table 2**), reproducing

Table 1. Baseline characteristics of the 198 analyzed patients.

	Median	Range
Age (years)	61	19-91
	n	**%**
18-29	7	3.5
30-39	20	10.1
40-49	31	15.7
50-59	30	15.2
>60	110	55.6
Gender	**n**	**%**
Male	113	57
Female	85	43
ECOG	**n**	**%**
0	57	29
1	73	37
2	24	12
3-4	8	4
Unknown	36	18
FAB subtype	**n**	**%**
M0	14	7
M1	46	23
M2	44	22
M4	32	16
M5	27	14
M6	2	1
Unknown	33	17
	Median	**Range**
WBC (count x 10^9/L)	18.65	0.6 - 270
	n	**%**
0-10	74	37
10-50	63	32
>50	51	26
Unknown	10	5
Cytogenetic risk profile[a]	**n**	**%**
Favorable	15	8
Intermediate	111	56
Adverse	37	19
Unknown	35	18
FLT3-ITD status	**n**	**%**
Wild	119	60
Mutant	22	11
Unknown	57	29
NPM1 status	**n**	**%**
Wild type	92	46
Mutant	50	25
Unknown	56	28
Response	**n**	**%**
CR/CRi	93	47
PR/resistance	65	33
Unknown	40	20

[a]Based on the risk groups described by Grimwade et al (2010). ECOG-PS: Eastern Cooperative Oncology Group performance status; FAB: French-American-British classification; FLT3-ITD: fms-like tyrosine kinase 3-internal tandem duplication; NPM1: Nucleophosmin 1; WBC: white blood cells; PR: partial remission.

the results of the clinical trials.[4,6-8,12] However, the interpatient variability of either drug is quite large (**Table 2**, **Figure 1**), which could explain why some patients could show very differential sensitivities to these three drugs. As an example, **Figure 2** illustrates a patient sample that is resistant to IDA and DNR (right shifted dose-response curve) but sensitive to MIT (left shifted dose-response curve).

To identify these cases of selective sensitivity to anthracyclines, we compared the potency, regarding AUC, between IDA vs. DNR, IDA vs. MIT, and DNR vs. MIT (**Figure 3**, **Table 3**). Most dots tend to line up, but red dots represent patient samples with a difference in potency between these drugs >30%. Red dots from 3 pairwise comparisons identify 28.3% of patient samples with >30% different potency among IDA-DNR-MIT (**Figure 4**).

Ex vivo PharmaFlow Test characterization of CYT-IDA, CYT-DNR, and CYT-MIT combinations and their synergism. The pairwise comparison of the combination treatments CYT-IDA, CYT-DNR, and CYT-MIT obtained differential sensitivity to these anthracyclines (red dots of **Figure 5**). In this case, the red dots represent patient samples with a difference in CYT + anthracyclines synergy differences >30%, and

Table 2. Estimates of the ex vivo population pharmacodynamic parameters. Parameters typical and random (variability and residual error percentage) are shown together with the corresponding relative standard error calculated as the ratio between the standard error provided by NONMEM and the estimate. Estimates of inter-patient variability (IPV) are expressed as coefficient of variation (%).

Parameter (units)	Mitoxantrone	Idarubicin	Daunorubicin
LPC_0 (cells)	7443 (10.04)	8384 (14.18)	7926 (10.21)
E_{max} (unitless)	1 (-)	1 (-)	1 (-)
EC_{50} (μM)	0.329* (16.84)	0.07* (14.58)	0.458*(12.08)
γ (unitless)	0.77 (-)	1.04 (-)	1.13 (-)
Residual Error (log(μM))	845 (10.07)	1027 (15.61)	924 (11.79)
Inter-patient variability (IPV)			
LPC_0	86.4 (6.56)	107.3 (6.83)	92.9 (5.76)
Emax	N/D	N/D	N/D
EC_{50}	224.2 (6)	181.8 (5.46)	168.6 (4.6)
γ	N/D	N/D	N/D
Residual Error	83.1 (7.63)	107.4 (7.21)	97.4 (6.38)

E_{max}: maximum fractional decrease in live pathological cells that the drug can elicit; EC_{50}: drug concentration exerting half of E_{max}; LPC_0: Starting live pathological cells in the absence of drug; N/D: not determined; γ: parameter governing the steepness of the LPC vs drug concentration curve.
* p value = 1.462E-06

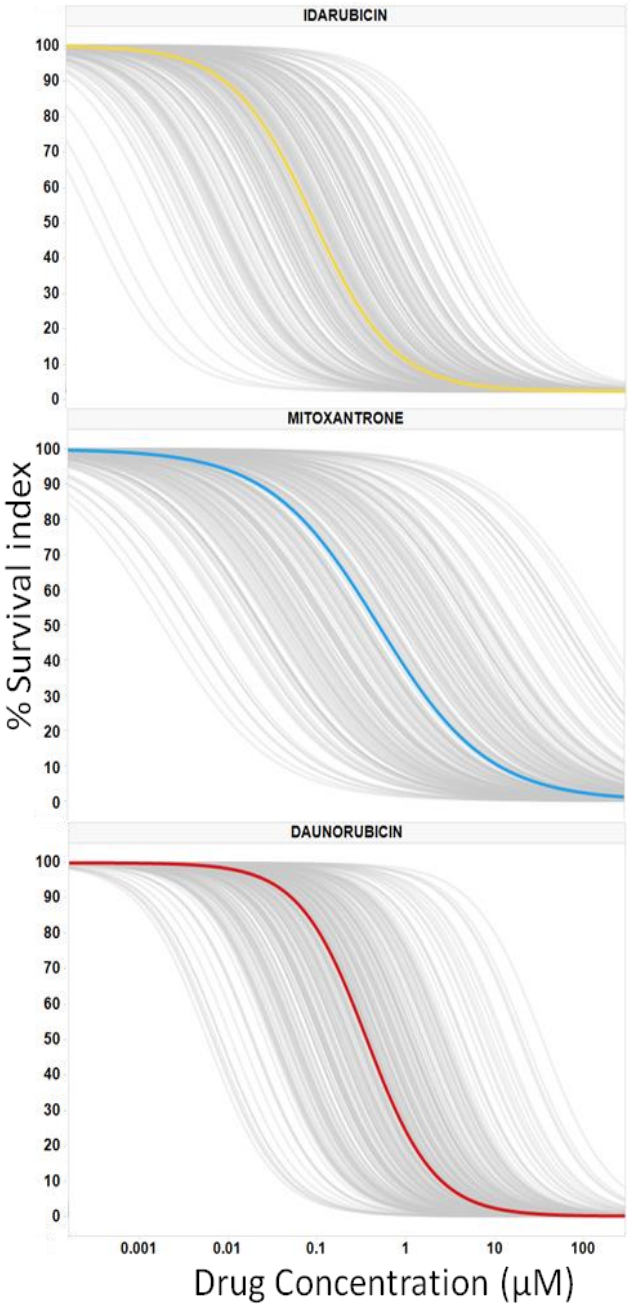

Figure 1. Average and Individual Dose Responses ex vivo for AML Drugs. Dose-Response Analysis was Completed for 3 Anthracyclines in Bone Marrow Samples From Patients With Acute Myeloid Leukemia; 227 with Idarubicin, 212 with Mitoxantrone and 271 with Daunorubicin. The Survival Index (y-Axis) Ranges From 100% to 0%, Displaying the Selective Acute Myeloid Leukemia Cell Depletion Calculated With Population Models. The Gray Lines Display Each Individual Response, With the Median Response Shown in yellow for Idarubicin, Panel (A); in blue for Mitoxantrone, Panel (B); and in red for Daunorubicin, Panel (C).

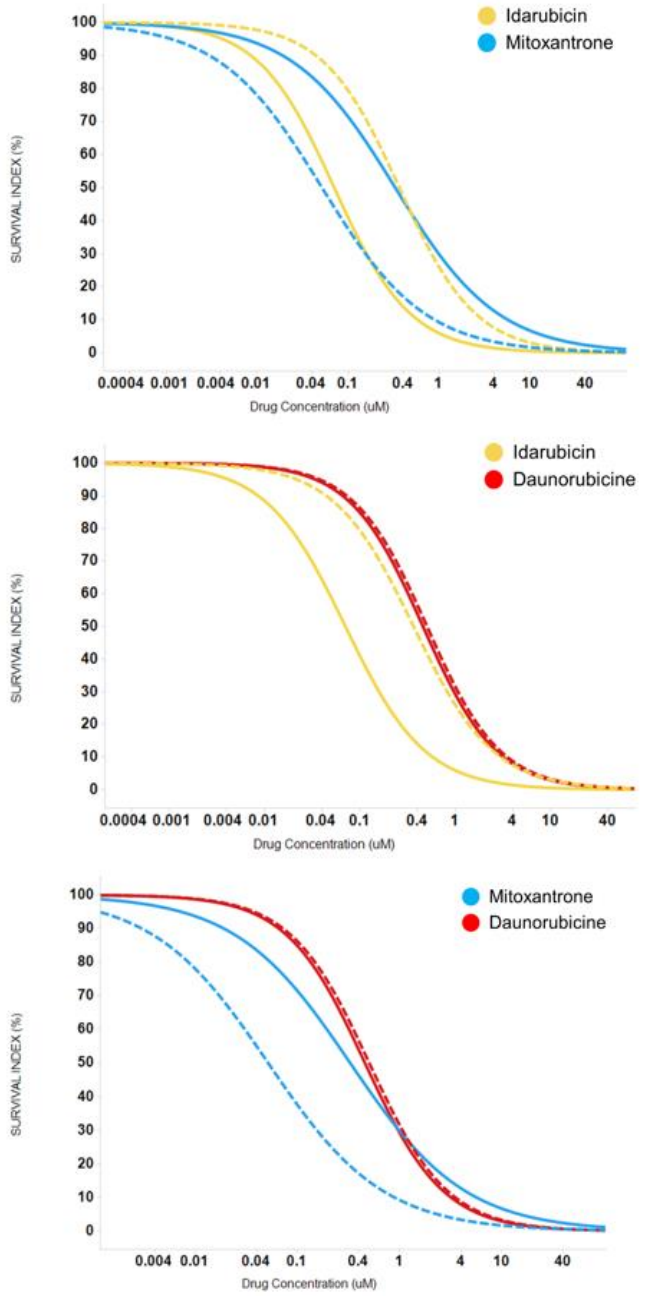

Figure 2. Example of differential individual sensitivities to anthracyclines. Dotted lines represented individual response to each drug and cotinuous lines the median response to each drug. Panel (A) shows an example of a patient resistant to Idarubicin (right shifted dose response curve) but sensitive to Mitoxantrone (left shifted dose response curve). Panel (B) shows an example of a patient resistant to Idarubicin and Daunorubicin (right shifted dose response curve). Panel (C) shows an example of a patient resistant to Daunorubicin (right shifted dose response curve) but sensitive to Mitoxantrone (left shifted dose response curve).

Table 3. Differences in Area Under the Dose-Response Curve between anthracyclines.

	AUC			
	Over30%	**Normal**	**Total**	**%**
DNR_IDA	15	102	117	12.82
DNR_MIT	32	172	204	15.69
IDA_MIT	17	100	117	14.53

AUC: area under the curve; DNR: daunorubicin; IDA: idarubicin; MIT: mitoxantrone.

Table 4. Differences in Volume Under the Surface (VUS) between the combinations of cytarabine and different anthracyclines.

	VUS			
	Over30%	**Normal**	**Total**	**%**
CYT+DNR_CYT+IDA	2	125	127	1.57
CYT+MIT_CYT+IDA	6	81	87	6.90
CYT+DNR_CYT+MIT	9	153	162	5.56

CYT: cytarabine; DNR: daunorubicin; IDA: idarubicin; MIT: mitoxantrone; VUS: volume under the surface.

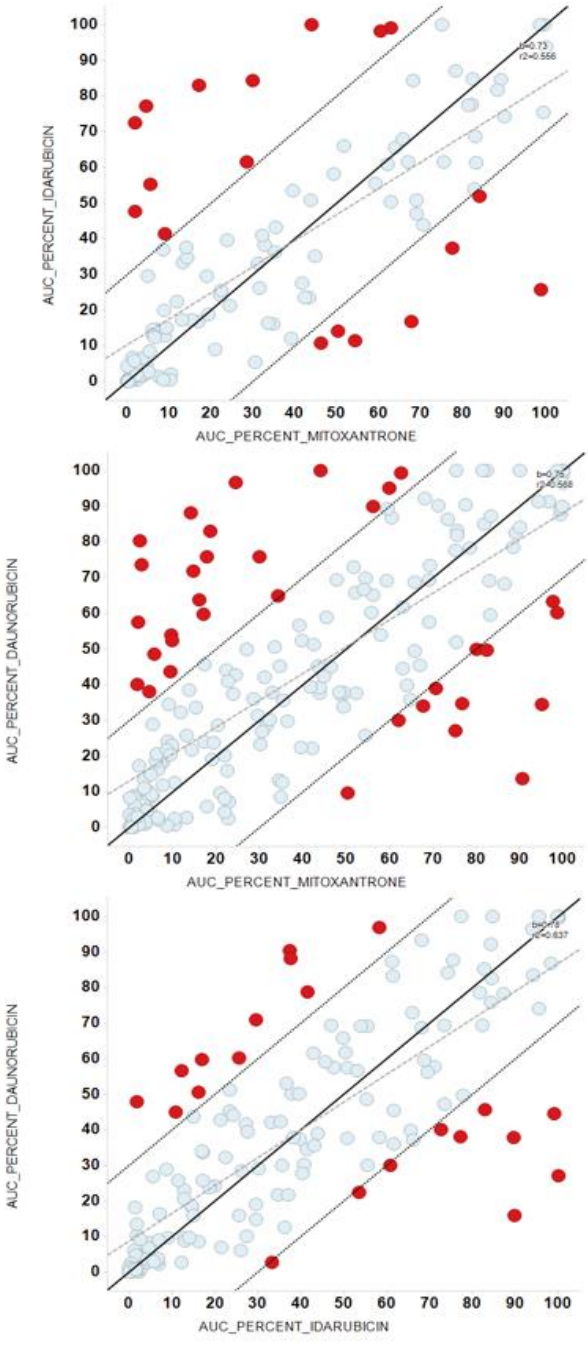

Figure 3. Comparison of the potency between anthracyclines. Panels A-C represented the pairwise comparisons between Area Under (AUC) the Dose-Response Curve of the anthracyclines, with their bisectors, linear regression lines and R2 values. Red dots represent patient samples with a difference in potency between these drugs greater than 30%. Panel (A) comparison between AUCs of Idarubicin and Mitoxantrone; Panel (B) comparison between AUCs of Daunorubicin and Mitoxantrone; Panel (C) comparison between AUCs of Daunorubicin and Idarubicin.

red dots from 3 pairwise comparisons identified an 8.2% of patient samples (**Figure 6, Table 4**).

Furthermore, the values for the alpha parameters of the interaction models of CYT-IDA, CYT-MIT, CYT-DNR were 0.72, 0.59 and 0.25, indicating synergistic response in the *ex vivo* combination experiments.

Discussion. The findings of this study show that PharmaFlow PM test seems able to identify a subset of AML patients who have a significantly different *ex vivo* pharmacological response to anthracycline drugs. We can hypothesize that if these selective anthracycline *ex vivo* responses were translated to *in vivo* responses, a fraction of this 28.3% subpopulation could benefit significantly from receiving a specific anthracycline-based on the *ex vivo* test sensitivity results. Furthermore, an 8.2% of patients showed a significant difference in the synergy between CYT and anthracyclines, in which the choice of the anthracycline could be crucial.

The first line induction therapy recommended by ELN[1] and NCCN[2] clinical guidelines includes seven days of a standard dose of CYT plus three days of an anthracycline, especially IDA (12 mg/m^2) or DNR (60-90 mg/m^2). The combination of CYT-MIT was not considered standard therapy, although it has been widely employed.

The influence of the anthracycline´s selection in the efficacy of induction therapy was analyzed in some RCTs.[3-22] The comparison between CYT-DNR and CYT-IDA has been studied in 13 different trials,[3-15] but only five studies reported differences in CR rates in favor of CYT-IDA.[4,6-8,12] A meta-analysis confirmed the superiority of CYT-IDA against CYT-DNR, obtaining higher overall survival (OS), disease-free survival (DFS), CR, lower relapse rate, although this scheme increased induction death and mucositis.[23] Regarding the employment of CYT-DNR or CYT-MIT, a clinical trial reported similar CR, length of duration of CR, OS, and toxicity.[16] No evidence of differences between CYT-IDA and CYT-MIT in CR, survival rates, and toxicity was observed in 6 RCTs[9,11,17-20] and one meta-analysis.[23] Combinations of CYT-doxorubicin showed worse outcomes than CYT-DNR[21] and CYT-IDA.[22] According to clinical trials, in our study the average dose-responses of IDA, DNR, and MIT were similar, with a slight decrease in EC_{50}

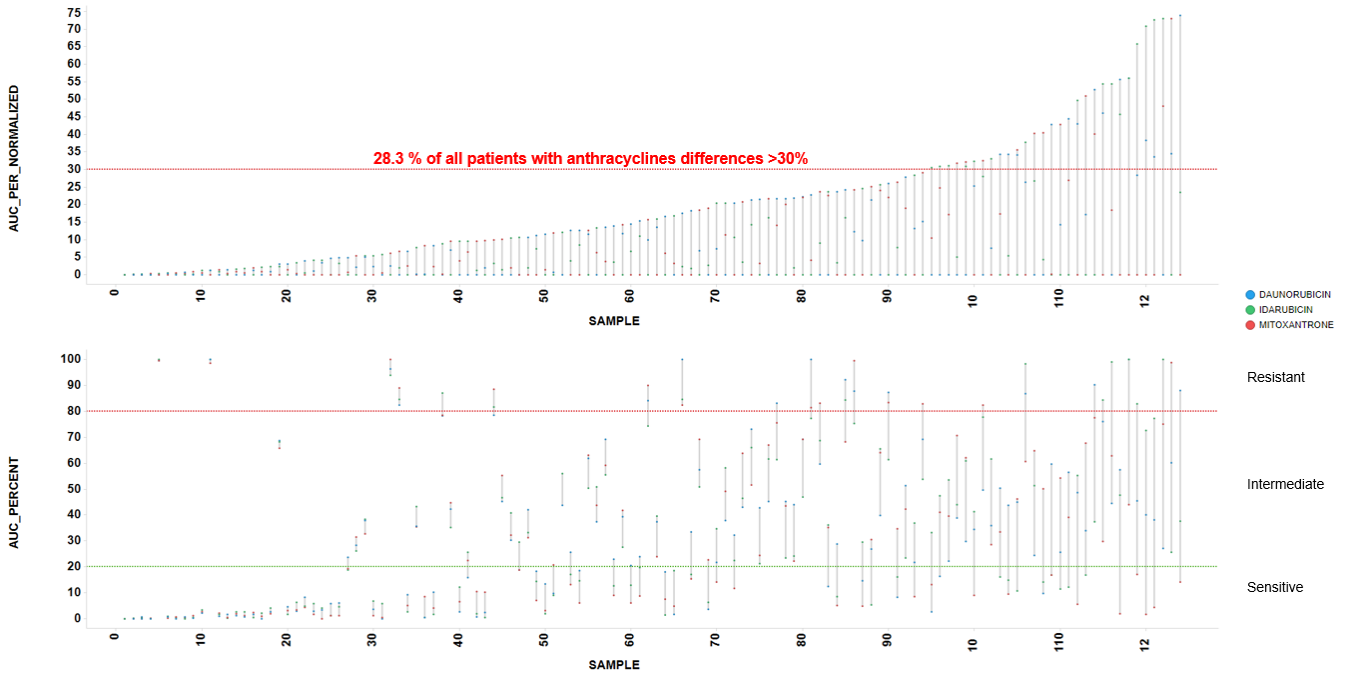

Figure 4. Differences in Area Under the Dose-Response Curve between anthracyclines. A 28.3% of patients samples showed >30% different potency among Idarubicin-Daunorubicin-Mitoxantrone Area Under the Dose-Response Curve (AUC).

with IDA, indicating a probable higher potency with IDA than DNR and MIT. However, the anthracycline dosage of induction protocols assumed a cumulative doses proportion of 4:1 for DNR: IDA and DNR: MIT,[31] but these proportions are not based in well-designed trials. In our cohort, according to this proportion and EC_{50} of DNR (0.458), the estimated EC_{50} of IDA and MIT was 0.115, a proportion 1.6 fold higher than IDA EC_{50} and three fold lower than MIT EC_{50} measured with *ex vivo* test.

Other studies analyzed the role of different anthracyclines in the AML induction with CYT and a third component, but CR and survival rates were similar for DNR, MIT, and aclarubicin.[32,33] Besides the selection of the anthracycline, the dose intensity is crucial in the therapy success. An RCT[34] reported significant improvements in CR, OS and event-free survival (EFS) using DNR doses of 90 mg/m^2 compared to doses of 45 mg/m^2. The response-oriented individualized induction therapy is another approach tested with IDA+CYT scheme without any advantage over the standard scheme.[35] In addition, some specific AML characteristics could modify the anthracycline response, such as FLT3-ITD mutated patients which showed higher CR and survival with high-dose DNR compared to standard-dose DNR or IDA.[36,37] These findings were reproduced *in vitro* in FLT3-ITD-mutated cell lines.[37] Unfortunately, we have not enough data to analyze the impact of this mutation in our cohort.

Despite the previous experiences of *ex vivo* drug testing with limited sensitivity[38-44], the PharmaFlow PM test aims to solve technical limitations including some novelties[25]:

a) the use of whole BM sample, maintaining the native environment, which has been hypothesized that it can influence the emergence of resistance;[45-48]

b) the increase of the accuracy obtained modeling *ex vivo* activity with PD population models in one single step;[49]

c) the improvements in the measures performed by automated flow cytometry platform (PharmaFlow).

The correlation between *in vitro* and *in vivo* therapy sensitivity of PharmaFlow PM test has been recently demonstrated in a cohort of 123 AML patients after induction therapy with CYT-IDA (most of these patients were also included in this study).[50] This study achieved an 81% of overall accuracy in the correlations between test predictions and hematological response, identifying with success responders (CR/CR with incomplete recovery) in 93% of cases and non-responders (partial remission/resistance) in 60% of cases. The present study generates a theoretical role of PM tests in individual anthracycline selection but does not provide enough data and critical analyses to allow to translate their use in the routine clinical practice.

Regarding the synergism between anthracyclines and CYT, we observed a synergistic response with the three combinations, especially with CYT-IDA and CYT-MIT. In a previous study, we also reported a higher synergy with CYT-IDA and CYT-MIT combination and a trend to an additive effect with CYT-DAU.[25] Curiously, a novel approach in AML therapy is the use of the liposomal formulation of CYT and DNR in a molar ratio concentration of 5:1, based

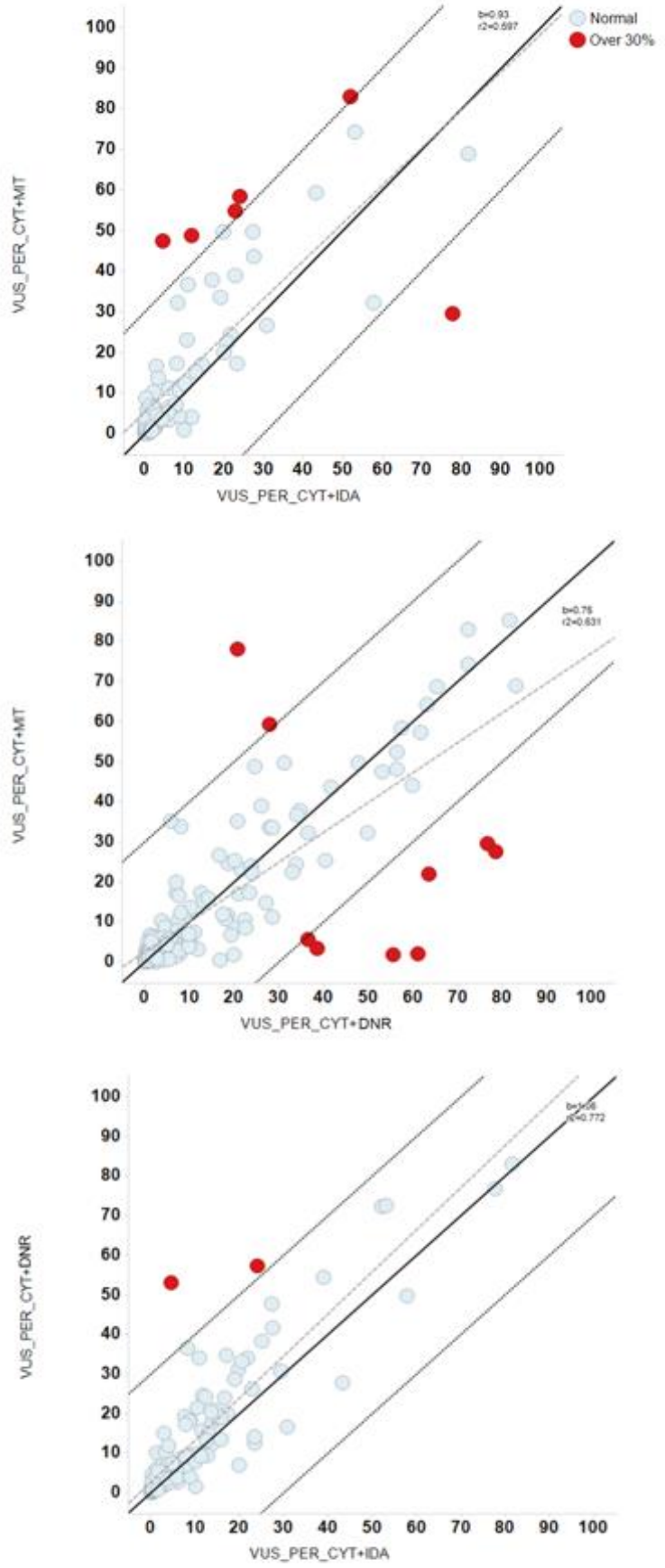

Figure 5. Comparison of the potency between combinations of cytarabine and anthracyclines. Panels A-C represented the pairwise comparisons between Volume Under the Surface (VUS) of the combinations of cytarabine (CYT) with anthracyclines, with their bisectors, linear regression lines and R2 values. Red dots represent patient samples with a difference in potency between these drugs greater than 30%. Panel (A) comparison between VUS of Cytarabine + Mitoxantrone (CYT+MIT) and Cytarabine + Idarubicin (CYT+IDA); Panel (B) comparison between VUS of CYT+MIT and Cytarabine + Daunorubicin (CYT+DNR); Panel (C) comparison between VUS of CYT+DNR and CYT+IDA.

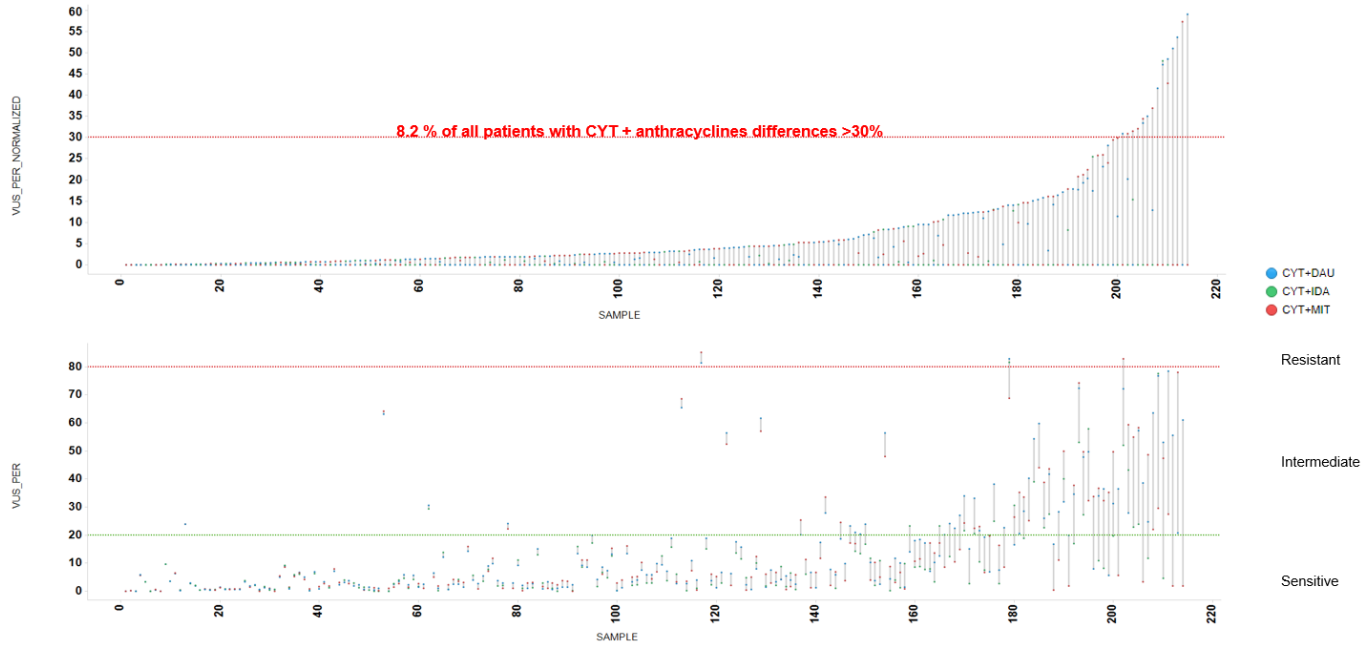

Figure 6. Differences in Volume Under the Surface between combinations of cytarabine and different anthracyclines. An 8.2 % of patients samples obtained >30% of different sensitivity in Volume Under the Surface (VUS) of Cytarabine + Idarubicin (CYT+IDA), Cytarabine + Daunorubicin (CYT+DNR) and Cytarabine + Mitoxantrone (CYT+MIT).

on a probable higher synergistic effect.[51,52] Furthermore, the pairwise comparisons between combinations of CYT-IDA, CYT-DNR, and CYT-MIT found in an 8.2% of patients synergy differences >30%, probably associated to the interpatient variability in drug sensibility observed in dose-response graphs.

Some limitations should be addressed in this study. First, this study analyzes the differences between *ex vivo* sensitivities to three different anthracyclines combined with CYT in BM samples of AML patients at diagnosis, but the correlation between *ex vivo* responses and clinical response was not analyzed. Second, although the incubation time was relatively short, additional transportation and processing time could lead, in several patients, to a non-affordable delay to start induction chemotherapy while receiving the test report. Third, associations of the different in vitro response of each anthracycline and specific characteristics of AML (age, WBC, cytogenetic risk, FLT3-ITD, and NPM1 status, etc.) were not analyzed. Finally, the findings reported are not yet validated in an independent cohort.

Conclusions. The *ex vivo* PharmaFlow PM test obtained in a 28.3% of the BM samples analyzed overall differences in sensitivity to anthracyclines in monotherapy. This test could allow designing a trial to explore a personalized selection of anthracycline therapy in AML patients. A similar approach is being tested in a clinical trial by PETHEMA group in relapsed or refractory AML patients to select the salvage therapy based on the *ex vivo* sensitivity to conventional chemotherapy agents. The role an adequate selection in this subset of AML patients is critical because none of the salvage regimens[53] has achieved outstanding CR rates, long-lasting remissions, and acceptable OS.

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