Research Article

BIOMEDICAL Journal of Scientific & Technical Research

ISSN: 2574 -1241

DOI: 10.26717/BJSTR.2023.53.008401

Clinical Utility of Individualized Follow-Up in Acute Myeloid Leukemia (AML) Patients Using a Myeloid NGS Panel

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ARTICLE INFO

Received: September 07, 2023

Published: October 25, 2023

Citation: María José Larráyoz, Almudena Aguilera Díaz, Zuriñe Blasco Iturri, Iria Vázquez, Beñat Ariceta, Amagoia Mañú, Paula Aguirre Ruiz, Maria Cruz Viguria, María Teresa Zudaire, Eva Bandrés, María del Carmen Mateos, José Rifón Roca, Ana Alfonso Piérola, Felipe Prósper, Marta Fernández Mercado and María José Calasanz. Clinical Utility of Individualized Follow-Up in Acute Myeloid Leukemia (AML) Patients Using a Myeloid NGS Panel. Biomed J Sci & Tech Res 53(3)-2023. BJSTR. MS.ID.008401.

ABSTRACT

The current standard to determine complete remission in acute myeloid leukemia (AML) includes morphologic criteria, cytogenetics, classical molecular analysis -such as polymerase chain reaction (PCR) and quantitative reverse-transcription PCR (RT-qPCR), and flow cytometry. Recently, Next-Generation Sequencing (NGS) is being a useful tool to gain insights into the molecular alterations in patients diagnosed with AML. We hypothesized that deeper knowledge of the clonal dynamics of AML could potentially be of clinical utility. We studied with our custom Pan-Myeloid NGS Panel the genomic alterations of 71 samples, corresponding to 20 AML cases during disease follow-up. Sequencing data identified genomic clonal markers with clinical utility in 90% of cases.

In patients not receiving hematopoietic stem cell transplant (HSCT) (n=6), NGS classified patients in two genetic profiles:

(i) Those with negative measurable residual disease (MRD).

(ii) Those not responding to treatment and undergoing disease progression (these, with pathogenic variants in *DDX41, DNMT3A, IDH1, JAK2, NRAS, SRSF2, U2AF1* genes).

In patients receiving HSCT (n=14), NGS was useful to classify them into three groups:

(i) Patients not presenting clinically relevant variants.

(ii) Patients clearing pathogenic variants upon HSCT.

(iii) Patients with persistent variants after HSCT. Interestingly, NGS data detected clones harboring pathogenic variants in two patients with negative MRD by flow cytometry, indicating that NGS could complement the current gold standard follow-up method in some instances.

Keywords: AML; NGS; MRD; Variants; Follow-Up

Abbreviations: AML: Acute Myeloid Leukemia; PCR: Polymerase Chain Reaction; NGS: Next-Generation Sequencing; HSCT: Hematopoietic Stem Cell Transplant; MRD: Measurable Residual Disease; CR: Complete Remission; WES: Whole Genome Sequencing; PMP: Pan-Myeloid Panel; UN: University of Navarra; ACMG: American College of Medical Genetics and Genomics; IGV: Integrative Genomics Viewer; VUS: Variant of Uncertain Significance

Introduction

Acute Myeloid Leukemia (AML) is a genetically heterogeneous neoplasm characterized by the accumulation of blasts due to genetic alterations in hematopoietic stem and/or progenitor cells [1,2]. These malignancies are one of the most common in adults and tend to be more aggressive than other leukemias [3]. Approximately 70% of AML patients achieve morphologic complete remission (CR) after chemotherapy; unfortunately, 50% of these patients eventually relapse [4-6]. Therefore, the course of these patients needs to be followed up in deeper detail, in order to tailor the therapeutic strategies to the specific clonal evolution of each case. The diagnosis of AML is a multidisciplinary process that integrates the results of different techniques including cytomorphology, flow cytometry, cytogenetics, and molecular biology. Specifically, cytogenetic and molecular studies are essential to determine risk groups, guide treatment, and define markers of follow-up to detect measurable residual disease (MRD) [7-9].

The current availability of immunophenotyping and/or molecular markers allows determining the kinetics of the disappearance of the disease, design of individualized post- remission treatment strategies, and early detection of relapse. Molecular detection MRD is classically carried out by reverse transcription quantitative polymerase chain reaction (RT-qPCR), due to the high sensitivity of this technique (10^{-4}-10^{-6}) [10]. In the last few years, Next Generation Sequencing (NGS) is being increasingly used for the genomic characterization of clinical samples. Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS) studies have shown that 70% of AML patients present somatic mutations [1,2,11]; moreover, mounting evidence shows that clinically relevant mutations are associated with an increased risk of relapse and reduced overall survival [6,9,12]. For example, internal tandem duplications in *FLT3* gene (*FLT3*-ITD), partial tandem duplications in *KMT2A* (*MLL*) gene (*MLL*-PTD), and mutations in *ASXL1, RUNX1*, and *TP53* genes are associated with shortened overall survival [7,13], to name a few. The number of clinically relevant genes in AML is already more than 30, and these numbers, as well as our understanding of this pathology, are growing mostly thanks to NGS technologies [14].

In particular, NGS gene panels, due to their focus on specific genes related to the pathology in study, have been shown to achieve a high sequencing depth (1000-5000x), which greatly improves sensitivity over traditional Sanger Sequencing [15] and is more sensitive than other NGS techniques such as WES [16]. This, together with the fact that NGS costing is lower and the turnaround time is shorter than individual Sanger Sequencing testing, makes NGS panels ideal tools for molecular monitoring of AML patients [2,17-19]. The present study aims to evaluate the performance of a custom Pan-Myeloid Panel (PMP) (48 genes, SOPHiA GENETICS), in the monitoring of 20 patients diagnosed as or progressing to AML during the course of the disease, the majority of them receiving hematopoietic stem cell transplant (HSCT, n=14). The characterization of the dynamics of the molecular architecture of these patients over time was then correlated to treatment efficacy in order to evaluate the clinical utility of this technique.

Materials and Methods

Sample Collection

We collected 71 bone marrow samples at different stages of the disease (diagnosis, post-treatment, post-HSCT) from 20 patients diagnosed with AML (13 *de novo,* and 7 secondaries to a preexisting myeloid neoplasm) during disease follow-up; 14 of these patients received HSCT during the study (Table 1). The majority (n=17) of the first samples used in this cohort of patients were collected at the time of AML diagnosis. The remaining three samples were collected after relapse (Unique Patient Number 1, UPN1, and UPN13) and post-treatment (UPN9). All patients signed a written informed consent form for genetic testing, research, and tissue banking provided by the Biobank of the University of Navarra (UN) and were processed following standard operating procedures approved by the CEI (Comité de Ética de la Investigación) of UN. Patient data were fully anonymized, and all patients provided informed written consent to have data from their medical records such as age, gender, and diagnosis to be used for research purposes.

Table 1: Patient Cohort Description.

UPN	Age at first sampling	Gender	Type AML	Karyotype at Dx	HSCT
UPN1	6	Female	*de novo*	46,XX[30]	Yes
UPN2	23	Male	*de novo*	46,XY[50]	Yes
UPN3	62	Male	Secondary	46,XY[30]	ND
UPN4	58	Male	*de novo*	42,XY,-2,del(5)(q13q33),-8,der(10)(q?),-11,add(12)(q12),-13,-16, -17, add(21)(p13),add(22)(p13),+2mar[25]/82<4n>,XXYY,-2,-2,-4, -4,del(5)(q13q33),-8,-8,-9,-9,der(10)(q?)x2,-11,-11,-12,-12,-13,-13, -16,-16,-17,-17,add(21)(p13)x2,add(22)(p13)x2, +8mar[23]/46,XY[2]	Yes
UPN5	40	Female	*de novo*	ND	Yes
UPN6	60	Male	*de novo*	46,XY,del(5)(q21q31)[1]/46,XY,del(5)(q21q31),t(9;22)(q34;q11.2), der(11) t(11;13)(q23;q13)[4]/46,XY[95]	Yes
UPN7	57	Female	*de novo*	47,XX,+22[12]/46,XX[38]	Yes
UPN8	69	Male	*de novo*	46,XYqh+[30]	ND
UPN9	69	Male	Secondary	46,XY,add(2)(p25)[5]/46,XY[25]	Yes
UPN10	56	Female	Secondary	47,XX,+8[12]/46,XX[8]	Yes
UPN11	50	Male	*de novo*	46,XY[30]	ND
UPN12	47	Male	*de novo*	46,XY[20]	ND
UPN13	75	Male	*de novo*	46,XY[30]	Yes
UPN14	72	Female	*de novo*	46,XX,del(1)(p34),del(5)(q13q33),del(11)(q14),add(19)(q13)[28]/ 46,XX[2]	Yes
UPN15	68	Male	Secondary	46,XY[30]	Yes
UPN16	17	Male	Secondary	ND	Yes
UPN17	68	Male	Secondary	46,XY[30]	Yes
UPN18	60	Female	*de novo*	45,XX,-7[15]/46,XX[15]	Yes
UPN19	75	Female	*de novo*	46,XX,del(20)(q12)[15]/46,XX[15]	ND
UPN20	60	Male	Secondary	46,XY[30]	ND

Note: UPN=Unique Patient Number; AML= Acute Myeloid Leukemia; Dx=Diagnosis; HSCT= Hematopoietic Stem Cell Transplant; ND=not done.

Sample Preparation

Genomic DNA from each sample was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), quantified using Qubit dsDNA BR Assay Kit on a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and DNA quality was assessed by DNA genomic kit on a Tape Station 4100 (Agilent Technologies, Santa Clara, CA, USA).

Pan-Myeloid Panel (PMP), Alignment, and Variant Calling

Our custom Pan-Myeloid Panel (PMP) is a hybridization capture-based panel that counts on a total genomic footprint of 114 kb, targeting 63 genes. For the detection of Single Nucleotide Variants (SNV), insertions and deletions (indels) we targeted 48 genes: full CDS of 22 genes, and exonic hotspots of 26 additional genes [20]. NGS libraries were prepared following manufacturer's instructions (SOPHiA GENETICS, Saint Sulpice, Switzerland). Final NGS libraries were quantified using Qubit dsDNA HS Assay Kit in a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and quality was assessed using DNA D1000 kit and visualized on Agilent 4100 Tape Station (Agilent Technologies, Santa Clara, CA, USA). A total of 10.5 pM of 8 pooled libraries was pair-end sequenced on a MiSeq (Illumina, San Diego, CA, USA) with 251x2 cycles using the Reagent Kit V3 600 cycles cartridge, according to the manufacturer's instructions. FastQ files were directly obtained from the MiSeq and uploaded onto SOPHiA GENETICS DDM software (SOPHiA GENETICS, Saint Sulpice, Switzerland), where alignment, variant calling of SNV/indels, and annotation were performed.

Variant Data Analysis

The list of annotated variants was filtered to exclude intronic, intergenic, and synonymous ones. Two geneticists with expertise in hematological malignancies categorized variants according to current

guidelines from the Spanish Group of Myelodysplastic Syndromes [21] and from the American College of Medical Genetics and Genomics (ACMG) [22]. Aligned reads were manually curated for confirmation of the presence of the filtered-in variants within the Integrative Genomics Viewer (IGV) software (Broad Institute) [23].

Results

Overall, the results showed that 18 out of 20 patients presented at least one clinically relevant mutation (meaning pathogenic and likely pathogenic variants) throughout the time course of the disease. The most recurrent mutated genes were *DNMT3A* (35% of cases, 7 patients), *FLT3* (30% of cases, 6 patients) *ASXL1* (20%, of cases, 4 patients), *SRSF2* (20% of cases, 4 patients), and *NPM1* (20% of cases, 5 patients). We indeed found that in 4 cases mutations in *NPM1* gene were concomitant with mutations in *FLT3* gene (except UPN3), as it has extensively been described in the literature in AML [24]. *NRAS* and *CBL* genes were mutated in 15% of the patients. *IDH2, CUX1, JAK2, RUNX1, WT1* and *ZRSR2* genes were mutated in 2 cases each (10%); and finally, *BCORL1, IDH1, DDX41, TET2, TP53, U2AF1, ETNK1*, and *PTPN11* genes were mutated in 1 case each (5%).

Variants Detected in Patients that did not Undergo HSCT

A total of 6 patients out of the 20 cases included in our study did not get HSCT, and all of them presented at least 1 pathogenic mutation (Table 2).

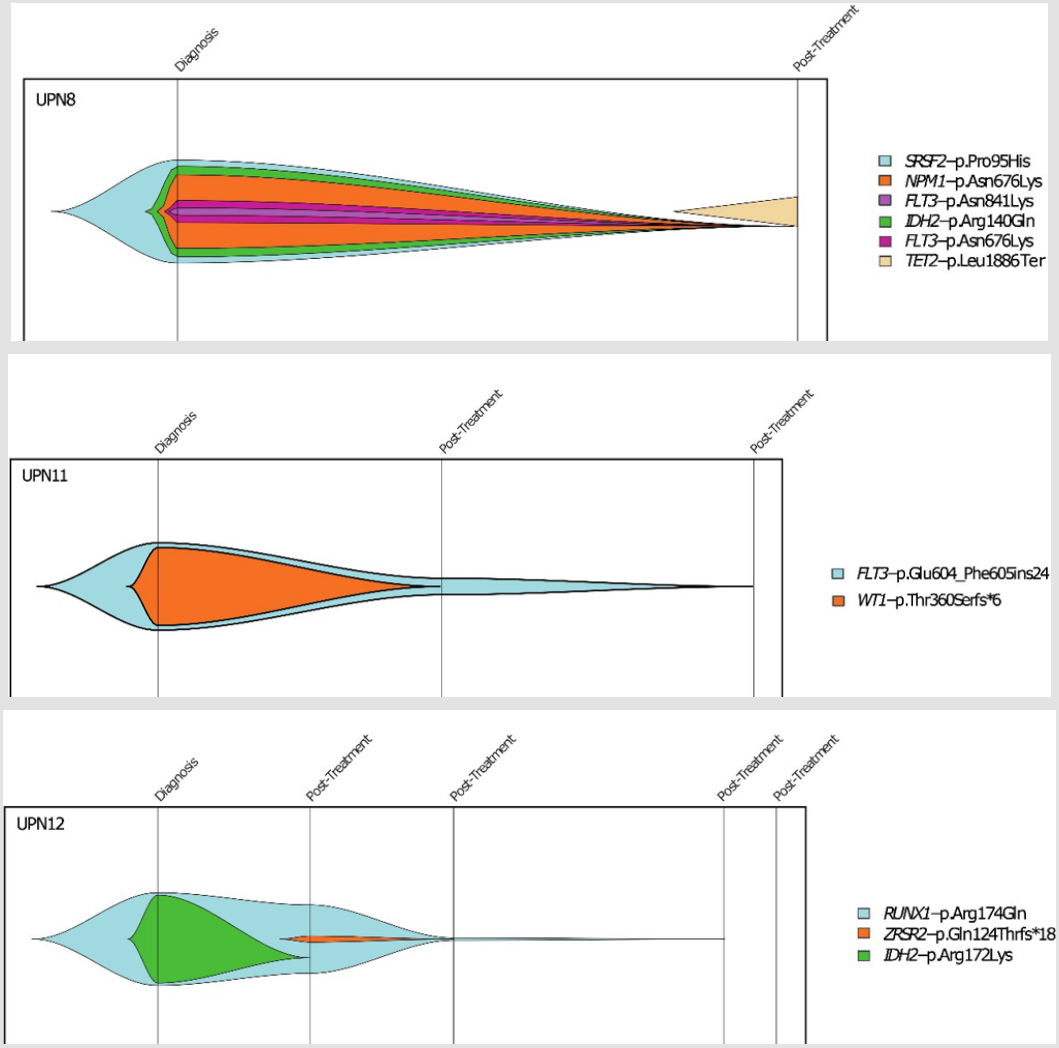
Table 2: Mutational Profile of patients that did not have HSCT.

UPN	Gene	Chr	Position	Protein	Variant Class.	VAF (%)					
						Dx	Check- up	Post- Tx	Post- Tx	Post- Tx	Post- Tx
3	*CBL*	11	119148991	p.Cys404Tyr	Pathogenic	86%	-	93%	-	-	-
	ASXL1	20	31023717	p.Arg1068Ter	Pathogenic	46%	-	48%	-	-	-
	JAK2	9	5073770	p.Val617Phe	Pathogenic	5%	-	3%	-	-	-
	NPM1	5	170837547	p.Trp288Cysfs*12	Pathogenic	0%	-	12%	-	-	-
	TET2	4	106180815	p.Ala1283Cysfs*17	Lik. Path.	46%	-	45%	-	-	-
8	*SRSF2*	17	74732959	p.Pro95His	Pathogenic	42%	-	0%	-	-	-
	IDH2	15	90631934	p.Arg140Gln	Pathogenic	37%	-	0%	-	-	-
	NPM1	5	70837546	p.Trp288Cysfs*12	Pathogenic	30%	-	0%	-	-	-
	FLT3	13	28602340	p.Asn676Lys	Pathogenic	9%	-	0%	-	-	-
	FLT3	13	28592622	p.Asn841Lys	Pathogenic	3%	-	0%	-	-	-
	TET2	4	106197321	p.Leu1886Ter	Lik. Path.	0%	-	12%	-	-	-
11	*FLT3*	13	28608241	p.Glu604_Phe605ins24	Pathogenic	33%	-	0%	0%	-	-
	WT1	11	32417917	p.Thr360Serfs*6	Lik. Path.	37%	-	7%	0%	-	-
12	*RUNX1*	21	36231782	p.Arg174Gln	Pathogenic	38%	-	28%	1%	0%	0%
	IDH2	15	90631838	p.Arg172Lys	Pathogenic	36%	-	0%	0%	0%	0%
	ZRSR2	X	15822289	p.Gln124Thrfs*18	Lik. Path.	0%	-	5%	0%	0%	0%
19	*JAK2*	9	5073770	pVal617Phe	Pathogenic	12%	48%	4%	12%	-	-
	DNMT3A	2	25459830	p.Cys818Tyr	Lik. Path.	7%	25%	5%	9%	-	-
	DDX41	5	176938849	p.Gln604Hisfs*38	Lik. Path.	53%	52%	49%	49%	-	-
	DDX41	5	176939370	p.Arg525His	Pathogenic	1%	23%	0%	4%	-	-
	ASXL1	20	31022277	p.Gln588Ter	Pathogenic	0%	0%	0%	1%	-	-

20	*DNMT3A*	2	25463170	p.? (*splice site*)	Pathogenic	40%	-	49%	43%	-	-
	U2AF1	21	44524456	pSer34Phe	Pathogenic	38%	-	48%	36%	-	-
	KRAS	12	25398284	p.Gly12Val	Pathogenic	0%	-	17%	36%	-	-
	BCOR	X	39921609	p.Ser1370Ilefs*80	VUS	0%	-	83%	72%	-	-
	TET2	4	106158378	p.Arg1095Glufs*11	VUS	39%	-	47%	39%	-	-
	TET2	4	106164934	p.Glu1268Ter	VUS	21%	-	46%	40%	-	-
	TET2	4	106164894	p.Tyr1255Thrfs*11	VUS	10%	-	3%	1%	-	-

Note: UPN=Unique Patient Number; Chr=Chromosome; Class=Classification; VAF=Variant Allele Frequency; Dx=Diagnosis; Tx=Treatment; HSCT= Hematopoietic Stem Cell Transplant; Lik. Path.= Likely Pathogenic; VUS=Variant of Uncertain Significance.

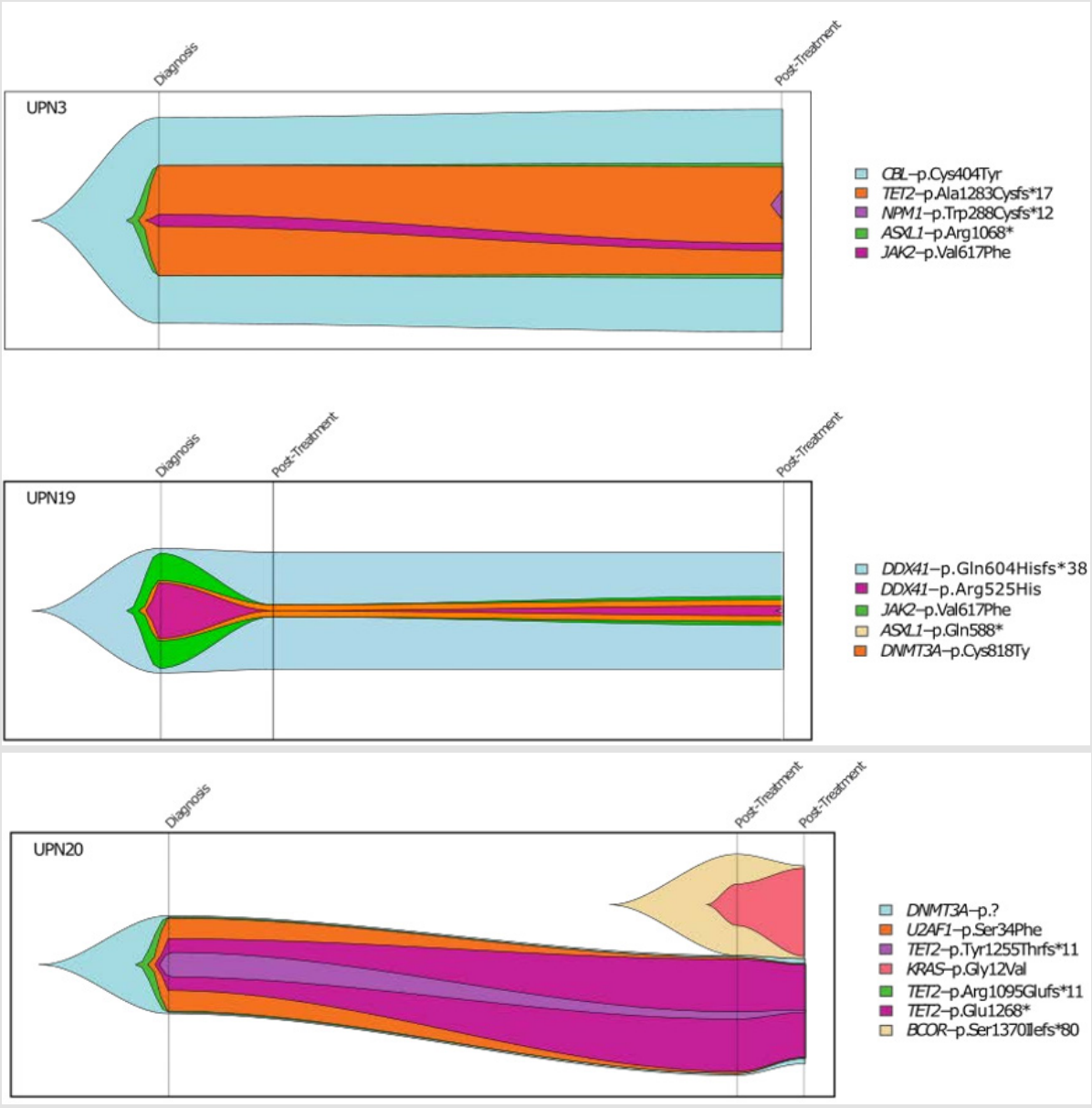
Patients Presenting Clinically Relevant Variant Clearing After Treatment: Amongst patients not receiving HSCT, 3 of them cleared all clinically relevant variants after treatment (UPN8, UPN11, and UPN12) (Figure 1). Of note, UPN8 did show persistent clonality after treatment as revealed by the presence of a Likely Pathogenic variant.



Note: UPN= Unique Patient Number.

Figure 1: Cases presenting clinically relevant variant clearing after treatment. UPN8, UPN11 and UPN12 showed clearance of the clinically relevant variants after treatment; however, UPN8 showed persistent clonality after treatment, as shown by the presence of a new Likely Pathogenic variant.

Patients With Positive MRD After Treatment: Out of the 6 patients that had been treated with the standard chemotherapy scheme, 3 of them presented positive MRD (Figure 2). Moreover, pathogenic mutations in *NPM1* (UPN3) and *KRAS* (UPN20) genes appeared for the first time after treatment. In addition, we found that UPN19 presented two pathogenic mutations in *DDX41* gene, having been this mutational pattern described as an indicative of a germline predisposition [25,26].



Note: UPN= Unique Patient Number.
Figure 2: Cases with positive MRD after treatment. UPN3, UPN19 and UPN20 were found not clearing their gene variants upon having been treated with the standard chemotherapy scheme.

Variants Detected in Patients Who Underwent HSCT

A total of 14 out of the 20 patients included in our study underwent HSCT, and 11 of them (79%) presented at least 1 pathogenic mutation (Table 3). The first sample analyzed on the majority of the cases was collected at the tie of diagnosis, except in three cases: UPN1 and UPN13 were firstly analyzed at relapse, and UPN9 was analyzed for the first time after treatment.

 DOI: 10.26717/BJSTR.2023.53.008401

Table 3: Mutational profile of the patients that underwent HSCT.

UPN	Gene	Chr	Position	Protein	Variant Class.	VAF (%)						
						First Sample	Post-Tx	Post-Tx	Post-HSCT	Post-HSCT	Post-HSCT	Post-2 HSCT
1	*RAD21*	8	117868512	p.Ser277Ile	VUS	3%	-	-	4%	4%	13%	2%
	IKZF1	7	50459531	p.Arg232Cys	VUS	0%	-	-	0%	0%	17%	0%
2	*BCORL1*	X	129148510	p.Glu588Lys	VUS	93%	-	-	0%	0%	-	-
4	*TP53*	7	7577539	p.Arg248Trp	Pathogenic	76%	1%	-	0%	2%	65%	-
	NRAS	1	115256530	p.Gln61Lys	Pathogenic	0%	0%	-	0%	0%	6%	-
5	*FLT3*	13	28608261	p.Asp586_Glu598dup	Pathogenic	47%	-	-	0%	-	-	-
	NPM1	5	170837544	p.Trp288Cysfs*12	Pathogenic	39%	-	-	0%	-	-	-
	CUX1	7	101758502	p.Arg219Gln	Lik. Path.	49%	-	-	0%	-	-	-
	GATA2	3	128205011	p.Ala144Thr	VUS	46%	-	-	0%	-	-	-
	GATA2	3	128205042	p.Gly135Trpfs*50	VUS	44%	-	-	0%	-	-	-
6	*RUNX1*	21	36259172	p.Arg80Cys	Pathogenic	10%	0%	0%	0%	-	-	-
	ASXL1	20	31022402	p.Glu635Argfs*15	Pathogenic	5%	0%	0%	0%	-	-	-
7	*FLT3*	13	28602340	p.Asn676Lys	Pathogenic	3%	3%	-	0%	-	-	-
	PTPN11	12	12888202	p.Thr73Ile	Pathogenic	1%	3%	-	0%	-	-	-
	BCORL1	X	129150130	p.Glu1128Glyfs*96	VUS	12%	11%	-	0%	-	-	-
9	*SRSF2*	17	74732959	p.Pro95His	Pathogenic	30%	3%	-	0%	-	-	-
	JAK2	9	5070033	p.Asn542_Glu543del	Pathogenic	0%	2%	-	0%	-	-	-
	ETNK1	12	22811995	p.Asn244Ser	Pathogenic	0%	1%	-	0%	-	-	-
	STAG2	X	123181356	p.? (*splice site*)	VUS	58%	4%	-	0%	-	-	-
10	*FLT3-ITD*	13	28608214	p.?	Pathogenic	42%	-	-	0%	0%	0%	-
	NPM1	5	170837545	p.Trp288Cysfs*12	Pathogenic	39%	-	-	0%	0%	0%	-
	DNMT3A	2	25457243	p.Arg882Ser	Pathogenic	42%	-	-	0%	0%	0%	-
	NRAS	1	115258747	p.Gly12Val	Pathogenic	8%	-	-	0%	0%	0%	-
	CUX1	7	101713691	p.Val99Ile	Lik. Path.	50%	-	-	0%	0%	0%	-
13	*SRSF2*	17	74732960	p.Pro95Thr	Pathogenic	25%	-	-	0%	1%	-	-
	NPM1	5	170837546	p.Trp288Cysfs*12	Pathogenic	22%	-	-	0%	0%	-	-
	NRAS	1	115258747	p.Gly12Asp	Pathogenic	14%	-	-	0%	0%	-	-
	FLT3	13	28592642	p.Asp835Tyr	Pathogenic	4%	-	-	0%	0%	-	-
	WT1	11	32417911	p.Ser364Alafs*73	Lik. Path.	25%	-	-	0%	0%	-	-
	DNMT3A	2	25463170	p.? (*splice site*)	Lik. Path.	0%	-	-	6%	6%	-	-
14	*CBL*	11	119148919	p.Leu380Pro	Pathogenic	5%	0%	-	0%	0%	-	-
	CBL	11	119148879	p.Gln367Lys	Lik. Path.	15%	0%	-	0%	0%	-	-
	DNMT3A	2	25463224	p.Asn757Tyr	Pathogenic	35%	0%	-	0%	0%	-	-
	ASXL1	20	31022738	p.Gly742Serfs*5	VUS	25%	0%	-	0%	0%	-	-
	DNMT3A	2	25463478	p.? (*splice site*)	VUS	6%	16%	-	24%	0%	-	-
	CBL	11	119149251	p.Arg420Gln	Pathogenic	1%	0%	-	0%	0%	-	-
	DNMT3A	2	25457243	p.Arg882Ser	Pathogenic	0%	0%	-	0%	1%	-	-

15	*CBL*	11	119148931	p.Cys384Tyr	Pathogenic	0%	14%	49%	4%	-	-	-
	SRSF2	17	74732959	p.Pro95His	Pathogenic	38%	40%	46%	9%	-	-	-
	TET2	4	106193892	p.Arg1452Ter	Pathogenic	31%	40%	45%	9%	-	-	-
	CBL	11	119148891	p.Tyr371His	Pathogenic	0%	0%	21%	0%	-	-	-
	JAK2	9	5073770	p.Val617Phe	Pathogenic	0%	7%	5%	1%	-	-	-
	NRAS	1	115258747	p.Gly12Asp	Pathogenic	0%	2%	4%	1%	-	-	-
	KRAS	12	25398284	p.Gly12Asp	Pathogenic	0%	1%	1%	0%	-	-	-
	BCORL1	X	129162789	p.Arg1420Ter	Lik. Path.	77%	82%	94%	9%	-	-	-
	DNMT3A	2	25463284	p.Leu737Phe	Lik. Path.	41%	45%	50%	9%	-	-	-
	DNMT3A	2	25470464	p.Ser337Leu	Lik. Path.	40%	41%	47%	9%	-	-	-
	TET2	4	106164929	p.Asn1266Ser	VUS	34%	40%	46%	9%	-	-	-
	CBL	11	119149251	p.Arg420Gln	Pathogenic	0%	18%	1%	3%	-	-	-
16	*NRAS*	1	115256530	p.Gln61Lys	Pathogenic	45%	1%	-	0%	16%	39%	-
	WT1	11	32417914	p.Arg363Thrfs*5	VUS	0%	0%	-	0%	18%	42%	-
	WT1	11	32417910	p.Ser364Ter	VUS	0%	0%	-	0%	15%	40%	-
17	*ASXL1*	20	31022288	p.Tyr591Ter	Pathogenic	9%	42%	38%	1%	-	-	-
	KIT	4	55599321	p.Asp816Val	Lik. Path.	0%	4%	0%	0%	-	-	-
	ZRSR2	15	15809121	p.Arg36Ter	Pathogenic	57%	94%	90%	1%	-	-	-
	TET2	4	106156139	p.Ala347Valfs*3	VUS	50%	91%	81%	1%	-	-	-
18	*DNMT3A*	2	25457242	p.Arg882His	Pathogenic	40%	40%	-	0%	-	-	-
	IDH1	2	209113113	p.Arg132Cys	Pathogenic	36%	18%	-	1%	-	-	-
	DNMT3A	2	25457284	p.Phe868Ser	VUS	30%	4%	-	0%	-	-	-
	CUX1	7	101877457	p..Leu1198Met	VUS	20%	0%	-	0%	-	-	-
	GATA2	3	128202765	p.Cys319Arg	VUS	15%	0%	-	0%	-	-	-

Note: UPN=Unique Patient Number; Chr= Chromosome; Class= Classification; VAF= Variant Allele Frequency; Tx=Treatment; HSCT= Hematopoietic Stem Cell Transplant; VUS= Variant of Uncertain Significance; Lik. Path.= Likely Pathogenic.

Patients No Presenting Clinically Relevant Variants: The two patients who did not show any pathogenic variant were UPN1 and UPN2. UPN1 was a 6-year-old girl diagnosed with AML, who showed two variant of uncertain significance (VUS), one in *RAD21* gene present since the time of relapse, and one in *IKZF1* gene found only after HSCT relapse. UPN2 was a 23-year-old man, who only presented a VUS in *BCORL1* gene at diagnosis, which was found to be cleared after HSCT (Figure 3). Although at this moment there is no clinical significance associated with these variants, we included them because it is possible that the continuous updating of the relevant databases might confer them a clinical meaning in the near future. Moreover, the presence of these variants is indicative of clonality, which might be useful for disease monitoring.

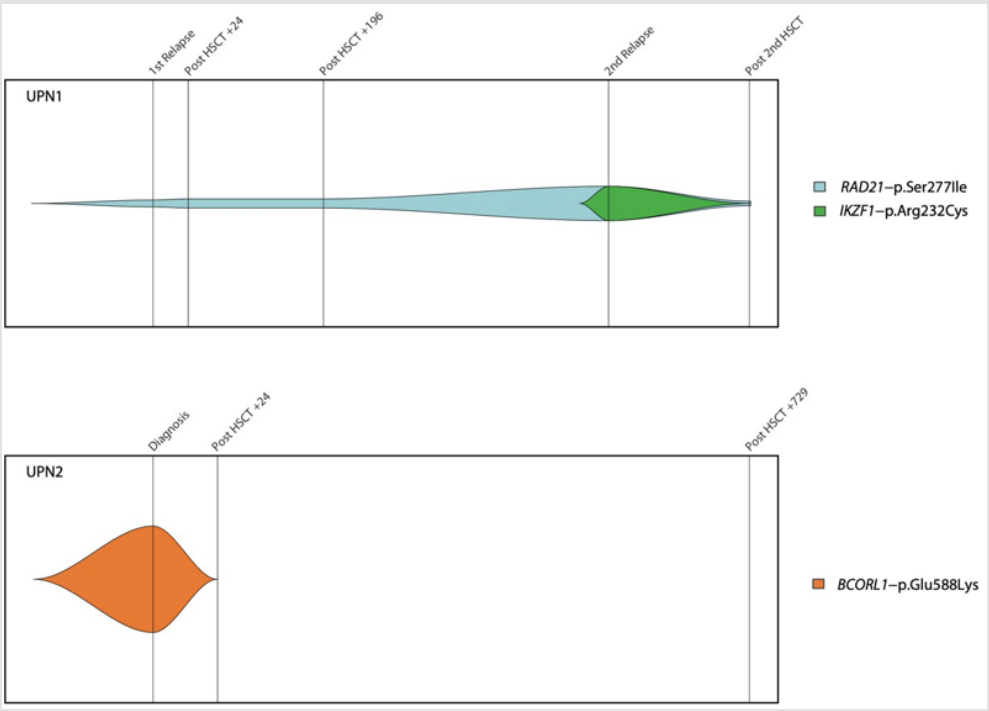


Figure 3: Cases which did not present any clinically relevant variant. UPN1 showed two VUS variants, firstly in *RAD21* gene, and afterwards in *IKZF1* gene (at relapse). UPN2 presented a VUS variant in *BCORL1* gene at diagnosis that cleared after HSCT.

Note: UPN= Unique Patient Number; VUS= Variant of Uncertain Significance; HSCT= Hematopoietic Stem Cell Transplant.

Patients Presenting Clinically Relevant Variant Clearing After Treatment: Five patients presented clearance of all variants after treatment (UPN5, UPN6, UPN7, UPN9 and UPN10). Four of them were perfect examples of successful HSCT, showing complete disappearance of all variants, both pathogenic and VUS, upon HSCT (Figure 4). UPN6 was found with negative MRD after treatment, but still needed to be transplanted because of his complex karyotype (Table 1).

Patients with Positive MRD: A total of 7 patients showed a MRD upon HSCT, (UPN4, UPN13, UPN14, UPN15, UPN16, UPN17 and UPN18) (Figures 5 & 6). From these, in 5 cases the unsuccessful transplant had already been confirmed by positive MRD, as measured by flow cytometry. Interestingly, the remaining 2 cases (UPN4, UPN14) were found to present a negative MRD by flow cytometry, while NGS showed evidence of the presence of malignant clones (Figure 6). Remarkably, 3 of those 7 cases not only presented pathogenic mutations from the time of diagnosis that did not clear after treatment, but they also developed additional pathogenic variants for the first time after HSCT (UPN4, UPN13, UPN14).

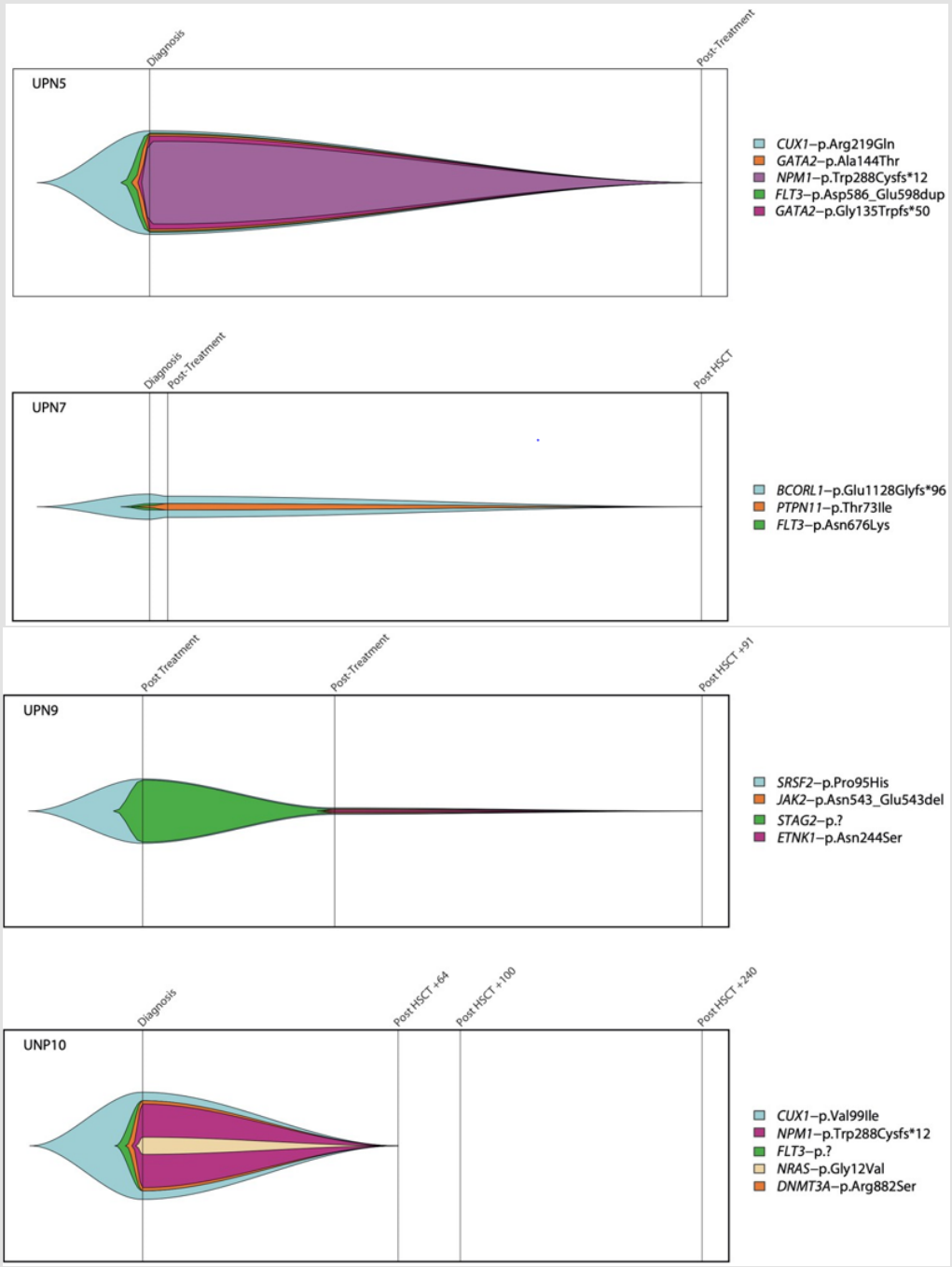


Figure 4: Cases presenting clinically relevant variant clearance upon HSCT. UPN5, UPN7, UPN9 and UPN10 were found with complete disappearance of all variants after HSCT.

Note: UPN= Unique Patient Number; HSCT= Hematopoietic Stem Cell Transplant.

 DOI: 10.26717/BJSTR.2023.53.008401

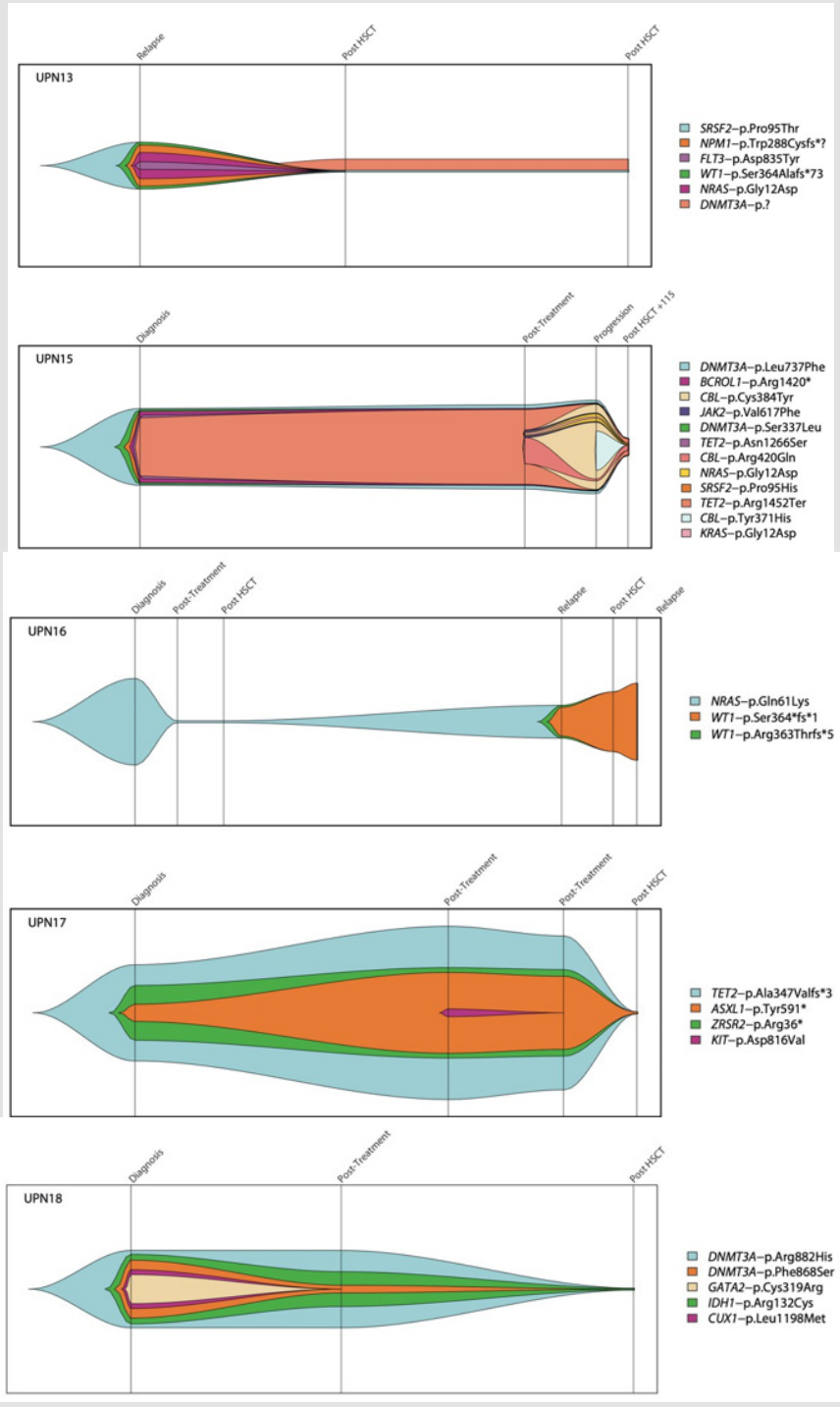


Figure 5: Cases presenting clinically relevant variants upon HSCT. UPN13, UPN15, UPN16, UPN17, and UPN18 showed clinically relevant variants after HSCT.

Note: UPN= Unique Patient Number; HSCT= Hematopoietic Stem Cell Transplant.

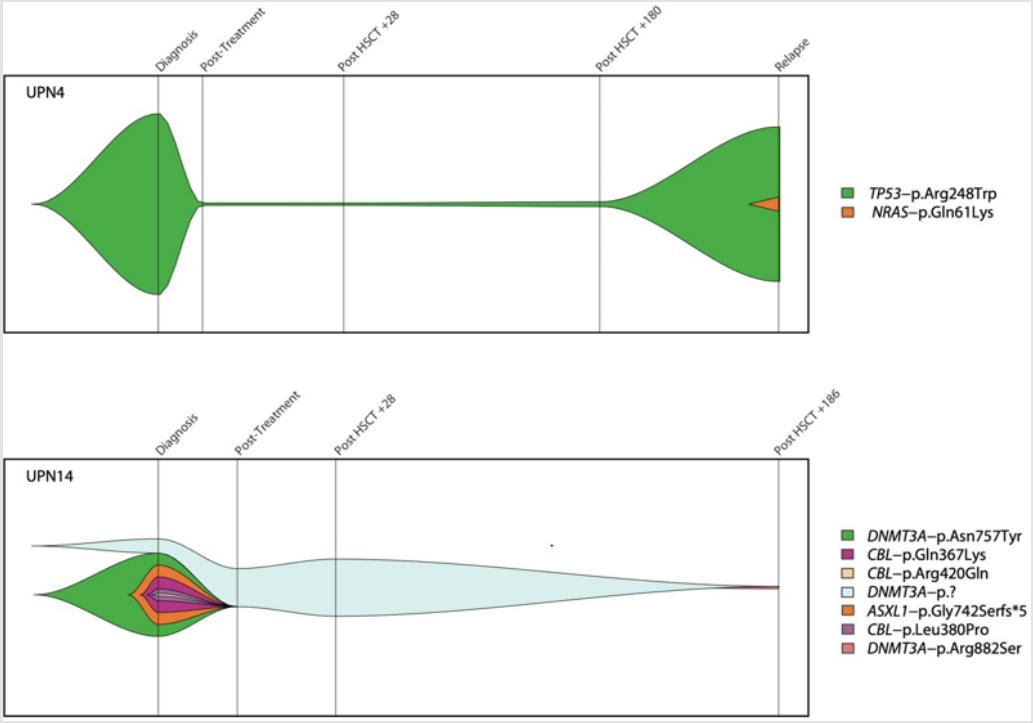


Figure 6: Cases that were found to be negative MRD by flow cytometry upon HSCT, but were found to harbour clonal mutations by NGS. UPN4 and UPN14 had been declared negative MRD (i.e. disease-free); however, NGS revealed clonality, ergo persistence of the disease.

Note: UPN= Unique Patient Number; HSCT= Hematopoietic Stem Cell Transplant; MRD= Measurable Residual Disease.

Discussion

Our sequencing data on 71 samples from 20 AML patients diagnosed as or progressing to AML identified genomic clonal markers with clinical utility in 90% of cases. Eighteen out of 20 patients presented at least one clinically relevant mutation (average 2.6 mutations per patient); these, together with the mutation frequency observed in the present study, are in agree to previously published data in AML [14,27,28]. Identification of the pathogenic clones in these AML patients is crucial, due to the fact that a number of the detected mutated genes possess diagnostic, prognostic, and predictive value. For example, there is available targeted therapy for *FLT3*- ITD, *IDH1*, and *IDH2* gene mutations, like Midostaurin or Gilteritinib, and Ivosidenib, and Enasidenib, respectively [9,29-31], that improve results associated with conventional therapy. Also, while mutations in *NRAS* genes have been related to progression of the disease [32], *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, TP53, U2AF1* and *ZRSR2* have an adverse prognostic value [7,14].

Therefore, the genomic characterization of AML patients is indeed useful for therapeutic decisions and clinical management of patients, such as new cycles of consolidation chemotherapy, or different therapeutic interventions after HCST. Importantly, in this study NGS data in these 18 cases showed additional value when examined during the time course of the disease. We observed two different clonal genetic dynamic patterns: some patients presented persistent clinically relevant mutations after treatment (n=10), whereas some others showed pathogenic variants clearance after treatment (n=8). On the one hand, we have observed that 5 out of the 10 patients with persistent mutations, were found with new clones harboring additional pathogenic mutations after treatment on top of the founder pathogenic clone present from the time of the first diagnosis. On the other hand, in the remaining 5 cases all detected pathogenic clones were present from the time of diagnosis. In the first group, the mutations that sprang upon treatment failure affected *NRAS, DNMT3A* and *KRAS* genes.

In the second group, in 4 of the cases all the founding clones reduced their size after treatment, although the mutations were not totally cleared even after HSCT, which might be a sign of incipient early relapse, like indeed happened with UPN16 (Figure 5 & Table 3). The persistent pathogenic variants in these cases were located in *BCORL1, DNMT3A, SRSF2, TET2* and *DNMT3A* genes in UPN15; *ASXL1* and *ZRSF2* genes in UPN17, and *IDH1* in UPN18 (Table 3). For UPN19 there were variants that persisted during the time course of the disease with similar frequencies of those detected at the time of diagnosis. *DDX41* gene presented 2 variants; *DDX41* p.Gln604Hisfs*38 showed a stable VAF of ~50% in all samples, while *DDX41* p.Arg525His presented a lower VAF that varied across the analyzed samples (Table 2). We suspected that the first variant could be germline since the VAF dynamic fits the double hit mutation pattern described by several studies in the *DDX41* gene [25,26,33,34].

Indeed, specifically *DDX41* p.Gln604Hisfs*38 variant has already been described to be of germline nature [26], although for this particular case it would be required to sequence a non-myeloid tissue (e.g. skin fibroblasts, hair follicles, or CD3+ cells) in order to check the presence/absence of the variant, since unraveling if the disease is originated on the grounds of a genetic predisposition is a crucial piece of information for making therapeutic decisions. Of note, we failed to detect any clinically relevant variants throughout the course of the disease in 2 cases (UPN1, UPN2) although variants of uncertain significance were present in both cases (Figure 3). UPN1 was a 6- year-old girl diagnosed with AML, who showed two VUS in *RAD21* and *IKZF1* genes at relapse. UPN2 was a 23- year-old man, who only presented a VUS in *BCORL1* gene at the time of diagnosis, which was found cleared at follow up.

Therefore, even though the NGS panel failed to detect clinically relevant variants in these two cases, it was useful to show evidence of clonality, especially useful because these cases had no cytogenetic markers (i.e. were found to have a normal karyotype) (Table 1). For cases of this sort, NGS platforms with wider scope (e.g. whole exome sequencing, WES) might provide clinically relevant information, although with a more limited ability to detect minor clones, due to its reduced depth of coverage [16]. Remarkably, our NGS data detected clones harboring pathogenic variants in two patients with negative MRD as measured by flow cytometry. One of them was UPN4, a 58-year-old man diagnosed with AML who received HSCT. At diagnosis, the NGS panel detected a pathogenic variant in *TP53* gene (VAF 76%) and after induction treatment, this clone drastically reduced in size (VAF 1%); at day +28 post HSCT, the pathogenic clone harboring the *TP53* gene mutation could not be detected (VAF 0%; 7641x depth), but at day +180 post HSCT, it was found to have expanded (VAF 2%); at day +306 post HSCT it was detected the *TP53* gene mutation (VAF 65%) and concomitantly a pathogenic variant in *NRAS* gene appeared (VAF 6%).

At post-induction, when the NGS panel detected the pathogenic clone, flow cytometry tested negative for MRD, in clear disagreement with molecular data. The other case was UPN14, a 72-year-old female patient with a VUS in *DNMT3A* gene that, even though it has not been shown to be clinically relevant, still was useful to reveal clonality, proving that the transplant had not been successful, although flow cytometry failed to measure any residual disease. Moreover, at day +186 post HSCT we could detect an additional clone with a different mutation, also in *DNMT3A* gene (Figure 6 & Table 3); remarkably, flow cytometry tested negative for MRD throughout the disease course. These cases are two examples that illustrate how NGS can be a good complement to standard MRD techniques which are already in place in the clinical setting, as has been suggested before [4,9,35].

Overall, our myeloid NGS panel was an excellent tool for the genomic characterization of AML patients during the time course of the disease, since it identified variants that are related to the pathogenicity of the disease and/or the presence of clonality in 100% of the cases included in our study; 90% of them harbored variants described to be valuable for diagnosis, prognosis or choice of treatment. In addition, the high depth of sequencing of the panel achieved detection of clones of minute size, and therefore allowed early detection of clonality, associated with potential relapse. Similarly, the possibility of following the dynamics of those genetic variants led us to identify persistent leukemia-associated mutations which are associated with a significant risk of relapse, and with reduced survival. Of note, NGS data detected clones harboring pathogenic variants in two patients with no MRD as per flow cytometry testing, indicating that NGS could complement the current gold standard follow-up method in some instances. Hence, NGS can help to improve the genetic characterization of AML and be complementary to current routine techniques for follow-up in AML patients.

Conclusion

The present study shows that our NGS panel has been useful for molecular diagnosis, monitoring of treatment efficacy, and early relapse detection in 90% of the AML cases included in our study, and useful for detection of clonality in 100% of them. Our NGS panel was also useful for following mutational clearance and/or clonal evolution in 63% of the total analyzed cases; specifically, it was of clinical utility in 79% of patients undergoing HSCT. Moreover, we could detect variants (and therefore clonality) in two cases who had tested negative on flow cytometry analysis. According to our data, NGS panels could be of clinical utility for routine follow-up in an elevated proportion of AML patients, as a complementary tool to immunophenotypic techniques for MRD monitoring.

Author's Contributions

MJL collected data, analyzed data, contributed to PMP design and wrote the paper, AAD analyzed data and wrote the paper; IV collected data and contributed to PMP design; BA performed data analysis; AM, ZB and PA performed sample preparation and experiments; MCV, MTZ, EB, MCM, JRR, and AAP collected samples, diagnosed patients, and accrued patient clinical history; MFM wrote the paper and contributed to PMP design; FP and MJC conceived the project and contributed to PMP design. All authors reviewed the manuscript and approved its content.

Acknowledgment

This work was funded by the Government of Navarra, Department of Industry, Energy and Innovation (Project DIANA, 0011-1411-2017-000028). We particularly acknowledge the patients for their participation and the Biobank of the University of Navarra for its collaboration. MFM and MJC acknowledge funding from ISCIII (PI16/00159) and from the Spanish Association Againsts Cancer (AECC, AIO2014). FP acknowledges funding from ISCIII (PI17/00701).

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ISSN: 2574-1241

DOI: 10.26717/BJSTR.2023.53.008401

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