Facultad de Ciencias Biológicas

Departamento de Bioquímica y Biología Molecular

Programa de Doctorado en Biomedicina y Biotecnología – 3102

TESIS DOCTORAL

PERFIL GENÓMICO MEDIANTE SECUENCIACIÓN MASIVA PARA ABORDAR LA CARACTERIZACIÓN MOLECULAR DE LA LEUCEMIA MIELOIDE AGUDA

Presentada por:

Claudia Sargas Simarro

Dirigida por:

Dra. Eva Barragán González

Dr. Pau Montesinos Fernández

Enero, 2023

VNIVERSITAT DE VALÈNCIA Escola de Doctorat

Programa de Doctorat en Biomedicina i Biotecnologia

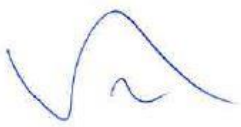
Eva Barragán González, Doctora en Biología por la Universidad de Valencia,

Pau Montesinos Fernández, Doctor en Medicina por la Universidad Autónoma de Barcelona

CERTIFICAN:

Que la tesis doctoral titulada: "**PERFIL GENÓMICO MEDIANTE SECUENCIACIÓN MASIVA PARA ABORDAR LA CARACTERIZACIÓN MOLECULAR DE LA LEUCEMIA MIELOIDE AGUDA**", ha sido realizado bajo nuestra dirección por **Claudia Sargas Simarro**, Graduada en Biotecnología, y reúne los requisitos suficientes para su presentación por compendio de publicaciones y defensa ante el tribunal correspondiente para optar al grado de **Doctora en Biomedicina y Biotecnología**.

Valencia, 27 de enero del 2023.



Fdo.: Dra. Eva Barragán González

Fdo.: Dr. Pau Montesinos Fernández

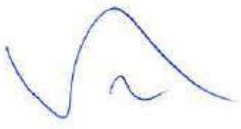
TESIS DOCTORAL PRESENTADA COMO COMPENDIO DE PUBLICACIONES

La tesis doctoral realizada por la doctoranda Claudia Sargas Simarro "**PERFIL GENÓMICO MEDIANTE SECUENCIACIÓN MASIVA PARA ABORDAR LA CARACTERIZACIÓN MOLECULAR DE LA LEUCEMIA MIELOIDE AGUDA**" ha sido realizada por compendio de publicaciones siendo la autora la primera firmante de los tres artículos científicos. De acuerdo a la normativa de tesis por compendio de publicaciones de la Universidad de Valencia, dos de los artículos científicos han sido publicados y aceptados en revistas indexadas y el tercero ha sido enviado y se encuentra en fase de revisión. Los manuscritos no han sido presentados previamente para la defensa de ninguna tesis doctoral.

Primer artículo: "***Networking for advanced molecular diagnosis in acute myeloid leukemia patients is possible: the PETHEMA NGS-AML project***". Publicado en *Haematologica*, una revista abierta que lidera la hematología experimental y clínica con un factor de impacto 11,049; Primer decil.

Segundo artículo: "***Molecular Landscape and Validation of New Genomic Classification in 2668 Adult AML Patients: Real Life Data from the PETHEMA Registry***". Publicado en la revista de acceso abierto *Cancers*, que cuenta con una amplia diseminación en la comunidad científica del campo de la oncología y un factor de impacto 6,575; Primer cuartil.

Tercer artículo: "***Validation of the 2022 European LeukemiaNet risk classification in a real-life cohort of the PETHEMA group"***. Pendiente de aceptación en *Blood Cancer Journal*, revista abierta con alto reconocimiento en el campo de la hematología y la oncología y un factor de impacto 9,812; Primer cuartil.



Fdo.: Dra. Eva Barragán González

Fdo.: Dr. Pau Montesinos Fernández

AGRADECIMIENTOS

En primer lugar, agradecer a mis directores de tesis, la Dra. Eva Barragán González y el Dr. Pau Montesinos Fernández la oportunidad de trabajar a su lado, que me ha permitido aprender de su experiencia y buen hacer.

A todo el personal del laboratorio de Biología Molecular del Hospital La Fe por su labor y esfuerzo diario.

En especial agradecer al Grupo Cooperativo PETHEMA y a todos los centros e instituciones que lo conforman por su incesante trabajo en el campo de la hematología.

Agradecer a la Fundación Española de Hematología y Hemoterapia y al Instituto de Salud Carlos III su apoyo económico durante la realización de este trabajo.

Finalmente, mi mayor agradecimiento a mi familia y amigos. En especial a Javi por acompañarme en el camino.

ÍNDICE TABLAS Y FIGURAS

GLOSARIO DE ABREVIATURAS

bZIP: *Basic-leucine zipper*

CC: Cariotipo complejo

DNA: Ácido desoxirribonucleico

ELN: *European LeukemiaNet*

ERM: Enfermedad residual medible

ICC: *International Consensus Classification*

Indel: Inserción/deleción

ITD: *Internal tandem duplication*

LMA: Leucemia mieloide aguda

MDS: Mielodisplasia

mNOS: *Molecularly not otherwise specified*

MO: Médula ósea

NGS: *Next-Generation Sequencing*

OMS: Organización Mundial de la Salud

PETHEMA: Programa Español de Tratamientos en Hematología

sAML1: Una mutación en un gen relacionado con mielodisplasia

sAML2: Dos o más mutaciones en genes relacionados con mielodisplasia

SG: Supervivencia global

SMD: Síndrome mielodisplásico

SNV: *Single nucleotide variation*

SP: Sangre periférica

TCGA: *The Cancer Genome Atlas*

VAF: *Variant allele frequency*

WT: Wild-type

INTRODUCCIÓN

1. Leucemia mieloide aguda

1.1. Definición

La leucemia mieloide aguda (LMA) es una neoplasia hematológica caracterizada por la expansión clonal de precursores mieloides que han perdido su capacidad de diferenciación, pero mantienen su capacidad proliferativa (Figura 1). La acumulación de células mieloides inmaduras (blastos) en la médula ósea interfiere en la hematopoyesis normal y da lugar a la aparición de manifestaciones clínicas como anemia, trombopenia e infecciones recurrentes (Vakiti & Mewawalla, 2022).

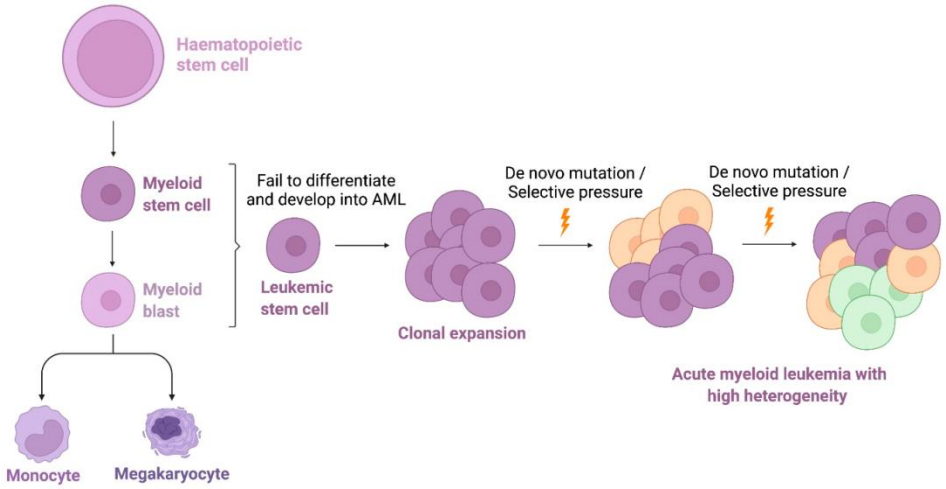


Figura 1. Desarrollo de la LMA caracterizada por la expansión clonal anormal y la diferenciación aberrante de células mieloides inmaduras (Xiang et al., 2022).

1.2 Epidemiología

La LMA es una enfermedad poco frecuente y representa alrededor del 1% de los casos de cáncer. Supone el 90% de las leucemias agudas en adultos, y en torno al 15-20% de las leucemias infantiles (Creutzig et al., 2018; Kuykendall et al., 2018). Su incidencia global es de 3-4 nuevos casos por cada 100.000 habitantes/año con una mediana al diagnóstico de 65 años (Deschler & Lübbert, 2006). A partir de esta edad, su incidencia aumenta hasta los 12 casos por cada 100.000 habitantes/año llegando a alcanzar los 44 nuevos casos por cada 100.000 habitantes/año a partir de los 80 años (Shallis et al., 2019).

1.3 Patogenia

Pese a que hay descritos una serie de factores de riesgo asociados al desarrollo de LMA como la exposición química, radiación ionizante, el alcoholismo o el tabaquismo (Finch, 2007; Poynter et al., 2017; Yi et al., 2020); la etiología de la enfermedad no se conoce con exactitud. La transformación leucémica tiene su origen en la adquisición de alteraciones en genes

implicados en los procesos celulares de proliferación, diferenciación y supervivencia, dando lugar a una trayectoria evolutiva muy heterogénea. La proliferación anormal, la expansión clonal, la diferenciación aberrante y la disminución de la apoptosis determinan el reemplazo de los elementos normales de la sangre por células malignas (Tuval & Shlush, 2019).

1.3.1 Evolución clonal

Un clon leucémico fundador inicia una trayectoria evolutiva durante la que adquiere nuevas alteraciones dando lugar a distintos subclones que son responsables de la evolución y progresión de la enfermedad (Bolandi et al., 2021; Morita et al., 2020).

Mutaciones en reguladores epigenéticos como *DNMT3A*, *TET2* y *ASXL1* aparecen en fases muy tempranas del desarrollo de la leucemia. Estas mutaciones otorgan cierta ventaja proliferativa, sin embargo, son considerados eventos preleucémicos pues son necesarios otros adicionales para el inicio de la leucemogénesis (Murphy et al., 2019). Los clones leucémicos presentes al momento del diagnóstico, pueden persistir tras la terapia siendo una fuente de inestabilidad genómica que eventualmente puede dar lugar a la recaída de la leucemia. Se han descrito distintos patrones de evolutivos que definen la recaída: (1) Un clon fundador presente al diagnóstico adquiere nuevas mutaciones y se convierte en el clon mayoritario en la recaída o (2) un subclon minoritario presente al momento del diagnóstico se expande y acaba causando la recaída (Figura 2). Sin embargo, la evolución clonal supone un proceso dinámico en el que aún se desconocen los eventos exactos que dirigen el patrón evolutivo (Ding et al., 2012; Vosberg & Greif, 2019).

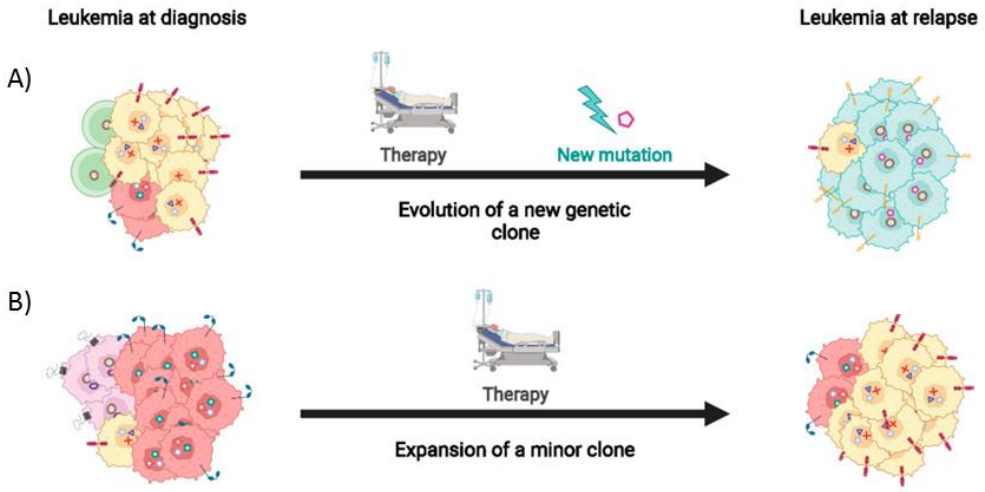


Figura 2. Patrones evolutivos en la recaída. A) Un clon fundador presente al diagnóstico adquiere nuevas mutaciones y se convierte en el clon dominante en la recaída. B) Un clon minoritario al diagnóstico se expande y da lugar a la recaída. Adaptada de (Desai et al., 2022).

1.3.2 Alteraciones genéticas

La presencia de alteraciones genéticas de distinta naturaleza define distintos subtipos biológicos y pronósticos en la LMA (Grimwade et al., 2010, 2016). Aproximadamente un 45% de los pacientes con LMA presenta alteraciones cromosómicas recurrentes que fueron las primeras en establecerse como marcadores de diagnóstico y pronóstico (Betz & Hess, 2010). Sin embargo, más de la mitad de los pacientes con LMA presentan un cariotipo normal (Kumar, 2011). El empleo de técnicas de secuenciación dirigida puso de manifiesto el papel de las alteraciones moleculares en la patogenia de la enfermedad al revelar mutaciones en los genes *FLT3*, *NPM1*, *KIT*, *CEBPA* y *TET2* con posible impacto diagnóstico y pronóstico (Welch et al., 2012). El desarrollo de las tecnologías de alto rendimiento como la secuenciación masiva (NGS) ha aumentado enormemente el conocimiento del perfil molecular de la enfermedad (Döhner et al., 2022) (Figura 3).

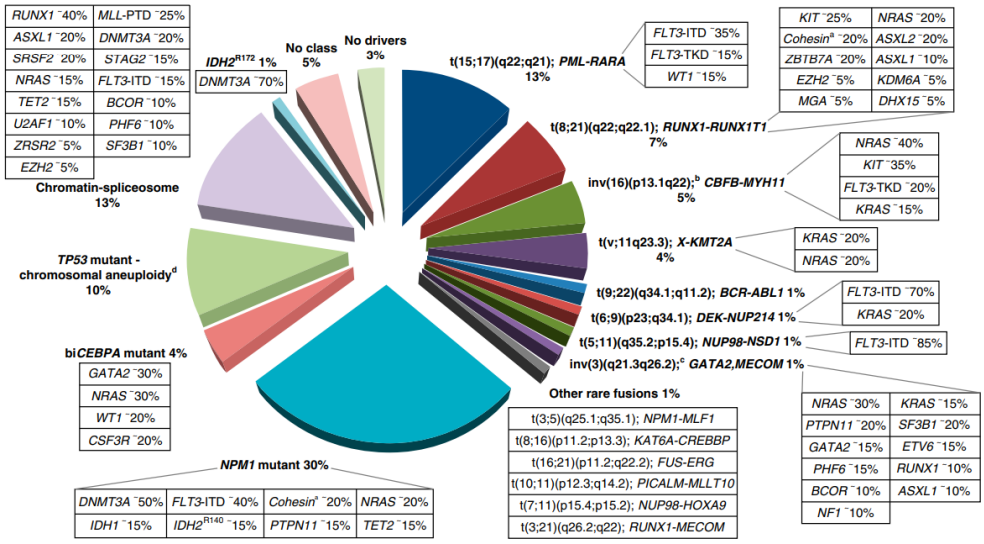


Figura 3. Distribución de alteraciones citogenéticas y moleculares en pacientes jóvenes con LMA (Grimwade et al., 2016).

En el año 2013 el estudio de 200 casos de LMA *de novo* mediante el análisis del genoma o exoma por la red de trabajo *The Cancer Genome Atlas* (TCGA), resaltó por primera vez la importancia de mutaciones somáticas en otros genes a los ya incorporados en el manejo clínico como *NPM1* y *FLT3*, y evidenció una compleja interacción de los eventos genéticos en el desarrollo de la leucemia. Este estudio definió 9 categorías funcionales relevantes en la patogénesis de la LMA (Tabla 1) y mostró que cerca de todos los pacientes analizados poseían al menos una mutación *driver* en genes pertenecientes a estas categorías. Además, se describieron nuevos patrones de cooperación y exclusividad que sugerían fuertes relaciones biológicas entre genes y categorías, identificando nuevas vías de desarrollo (Ley et al., 2013).

Este estudio proporcionó información detallada de nuevos genes recurrentemente mutados y evidenció la necesidad de entender cómo la diversidad genética define la patofisiología de la LMA e informa la práctica clínica.

Tabla 1. Categorías funcionales descritas por el TCGA.

Categorías funcionales
Fusiones de factores de transcripción
PML::RARA
MYH11::CBFB
RUNX1::RUNX1T1
PICALM::MLLT10
NPM1
Genes supresores de tumores
TP53
WT1
PHF6
Metilación del DNA
DNMT3A
DNMT3B
DNMT1
TET1
TET2
IDH1
IDH2
Activación de señales
FLT3
KIT
Otras tirosina quinasa
Serina–Treonina quinasas
KRAS/NRAS
Proteínas tirosina fosfatasas
Factores de transcripción mieloides
RUNX1
CEBPA
Otros factores de transcripción mieloides
Modificadores de la cromatina
Fusiones *KMT2A::X*
Duplicaciones parciales en tándem (PTD) en *KMT2A*
NUP98::NSD1
ASXL1
EZH2
KDM6A
Otros modificadores de la cromatina
Cohesinas
Splicing

2. Secuenciación masiva (NGS)

2.1 Concepto

La NGS es un método de secuenciación de ácidos nucleicos en el que se analizan de forma simultánea varias regiones del genoma (Goodwin et al., 2016). La secuenciación en paralelo de millones de fragmentos de DNA en un único experimento, ha supuesto una revolución en comparación con los métodos tradicionales de secuenciación en los que uno o pocos fragmentos de DNA se secuencian de manera simultánea (Kamps et al., 2017).

2.2 Metodología

Los fundamentos y los métodos de NGS son diversos y están asociados a las plataformas comerciales disponibles, siendo las desarrolladas por Illumina y Ion Torrent las que han experimentado mayor desarrollo y aplicación en clínica (Pervez et al., 2022). Ambas comparten un flujo de trabajo definido por los siguientes pasos:

- **Generación de librerías**: Comienza con la selección de las regiones de interés que puede realizarse mediante amplificación por PCR o por la hibridación con sondas específicas (Figura 4). Además, se realiza la ligación de adaptadores para la posterior amplificación clonal, y de los *barcodes* que permiten multiplexar el análisis de varios pacientes en un mismo experimento. El conjunto de los fragmentos obtenidos se conoce como librería.

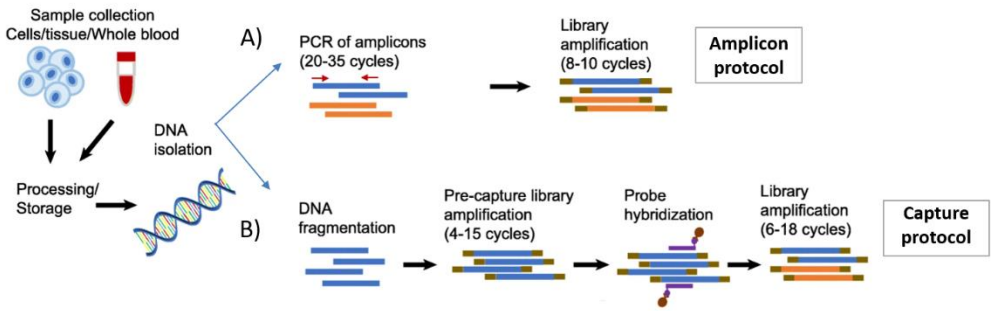


Figura 4. Selección de las regiones diana mediante: A) amplificación por PCR o B) hibridación con sondas específicas. Adaptada de (Ma et al., 2019).

- **Amplificación clonal**: Su finalidad es obtener millones de copias clonales de los fragmentos que conforman la librería. La PCR en emulsión y la PCR en puente son las estrategias más utilizadas (Figura 5).

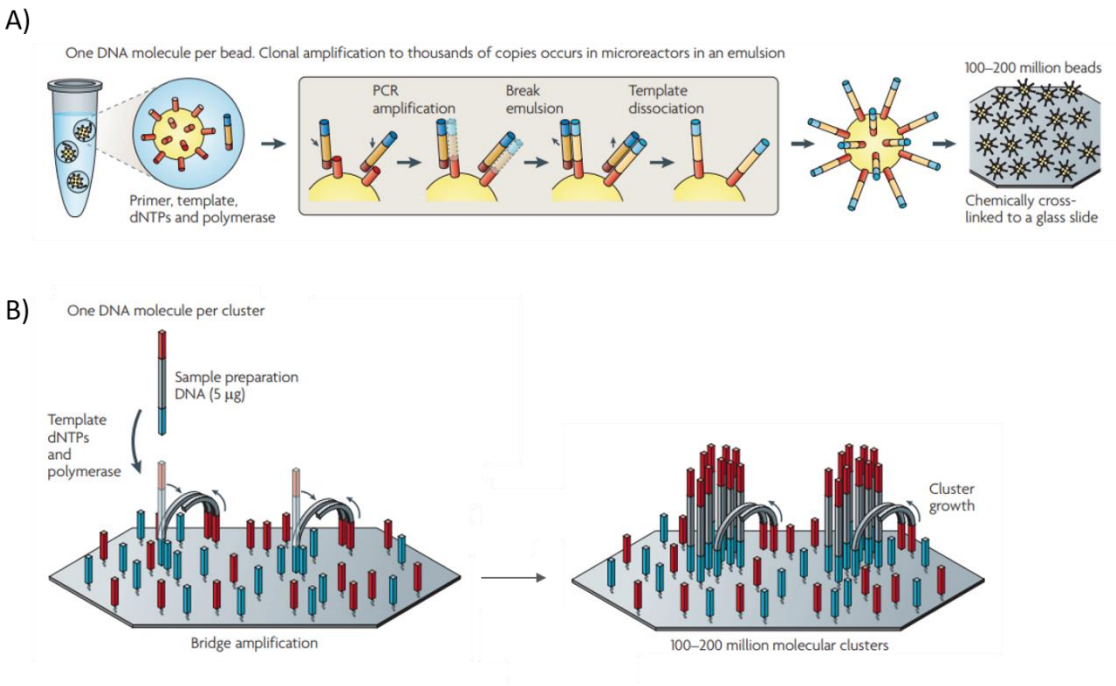


Figura 5. Proceso de amplificación clonal mediante: A) PCR en emulsión y B) PCR en puente. Adaptada de (Metzker, 2009).

- **Secuenciación**: en ambas estrategias se realiza una secuenciación por síntesis. La diferencia radica en la química de secuenciación que puede ser por semiconducción iónica o terminación cíclica reversible.
- **Análisis de datos**: consiste en la conversión de las señales recogidas a datos de secuencia. Posteriormente, se alinean las lecturas frente al genoma de referencia y se anotan las variantes detectadas.
- **Interpretación de las variantes**: Existen bases de datos poblacionales y específicas de tumores que aportan información detallada del impacto de las variantes detectadas, así como herramientas *in silico* que permiten predecir la patogenicidad de variantes no descritas. Definir su carácter (patogénico, polimórfico o de efecto desconocido) y su utilidad clínica es uno de los principales retos en rutina clínica.

La NGS se postula como una técnica con gran potencial en el diagnóstico molecular de distintas enfermedades por su capacidad para evaluar de forma simultánea alteraciones moleculares de distinta naturaleza en un conjunto de genes. La implementación de la NGS en los laboratorios, si bien no está exenta de desafíos, se ha establecido como primera opción frente a otras técnicas de PCR convencional con el fin de aplicar la medicina de precisión (el Achi & Kanagal-Shamanna, 2021; Levine & Valk, 2019).

3. Impacto de la NGS en la clasificación y estratificación pronóstica de la LMA

La exhaustiva caracterización molecular que se ha producido en los últimos años, ha dirigido el desarrollo de nuevos algoritmos y clasificaciones que enfatizan la importancia de estas alteraciones para establecer el diagnóstico, estratificar el riesgo y orientar la aproximación terapéutica, así como definir nuevos marcadores de seguimiento de enfermedad residual medible (ERM)

3.1 Clasificación diagnóstica de la Organización Mundial de la Salud

Históricamente la clasificación de diagnóstico de la LMA se ha definido en base a criterios morfológicos e inmunofenotípicos. En las últimas décadas los avances en la caracterización molecular de la LMA se han traducido en el reconocimiento de entidades definidas por mutaciones. En el año 2008, la Organización Mundial de la Salud (OMS) incluía por primera vez como entidades provisiones la LMA con *NPM1* mutado y LMA con *CEBPA* mutado (Swerdlow et al., 2008). La revisión del año 2016, consolidó estas dos entidades y propuso la LMA con mutaciones en *RUNX1* como una nueva entidad provisional (Arber et al., 2016). En el año 2022, se ha publicado una nueva actualización de la clasificación diagnóstica de la OMS que, si bien descarta la entidad de LMA con *RUNX1* mutado, redefine la entidad de LMA relacionada con mielodisplasia incluyendo como criterio de clasificación la detección de mutaciones en *ASXL1*, *BCOR*, *EZH2*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, o *ZRSR2* (Khoury et al., 2022) (Tabla 2). Esta clasificación, no establece un número mínimo de blastos para el diagnóstico de LMA con alguna de las alteraciones genéticas recurrentes definidas en la tabla 2 a excepción de las entidades LMA con reordenamientos de *BCR::ABL1* y LMA con mutación de *CEBPA*.

Tabla 2. Clasificación diagnóstica de la OMS 2022.

LMA con alteraciones genéticas recurrentes
LPA con reordenamiento *PML::RARA*
LMA con reordenamiento *RUNX1::RUNX1T1*
LMA con reordenamiento *CBFB::MYH11*
LMA con reordenamiento *DEK::NUP214*
LMA con reordenamientos de *KMT2A*
LMA con reordenamientos de *MECOM*
LMA con reordenamientos de *BCR::ABL1* (≥20% blastos)
LMA con mutación de *NPM1*
LMA con mutación de *CEBPA* (≥20% blastos)
LMA con reordenamientos de *NUP98*
LMA con reordenamiento *RBM15::MRTFA*
LMA con otras alteraciones genéticas definidas
LMA relacionada con mielodisplasia (≥20% blastos)

LMA definida por la diferenciación (≥20% blastos)
LMA con mínima diferenciación
LMA sin maduración
LMA con maduración
Leucemia aguda basofílica
Leucemia aguda mielomonocitica
Leucemia aguda monocitica
Leucemia aguda eritroide
Leucemia aguda megacarioblástica
Sarcoma Mieloide

3.2 Clasificación Consenso Internacional de las Neoplasias Mieloides y Leucemia Aguda

Paralelamente a la clasificación de diagnóstico de la OMS surge la nueva Clasificación Consenso Internacional de las Neoplasias Mieloides y Leucemia Aguda (*International Consensus Classification*, ICC). La principal diferencia entre ambas radica en el número mínimo de blastos requerido para el diagnóstico de la LMA. A diferencia de la clasificación de la OMS, la ICC establece un punto de corte de al menos 10% de blastos para el diagnóstico de LMA con alteraciones genéticas recurrentes, excepto para la entidad LMA con reordenamientos de *BCR::ABL1* en la que mantiene el dintel de 20% de blastos. Además, esta clasificación incluye por primera vez la categoría de LMA con *TP53* mutado como entidad diagnóstica (Arber et al., 2022) (Tabla 3).

Tabla 3. Clasificación diagnóstica de la ICC.

LMA con alteraciones genéticas recurrentes (requiere un 10% de blastos en MO o SP)
LPA con t(15;17)(q24.1;q21.2)/*PML::RARA*
LMA con t(8;21)(q22;q22.1)/*RUNX1::RUNX1T1*
LMA con inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB::MYH11*
LMA con t(6;9)(p22.3;q34.1)/*DEK::NUP214*
LMA con t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A*
LMA con otros reordenamientos de *KMT2A*
LMA con inv(3)(q21.3q26.2) o t(3;3)(q21.3;q26.2)/*GATA2*, *MECOM*
LMA con otros reordenamientos de *MECOM*
LMA con t(9;22)(q34.1;q11.2)/*BCR::ABL1* (≥20% de blastos)
LMA con *NPM1* mutado
LMA con mutación *in-frame* en el dominio bZIP de *CEBPA*
LMA con otras translocaciones recurrentes raras
Categorías consideradas LMA (>20% blastos) o SMD/LMA (blastos 10-19%)
LMA con mutaciones génicas asociadas con mielodisplasia
LMA con alteraciones citogenéticas asociadas con mielodisplasia
LMA con *TP53* mutado
LMA no caracterizada de otro modo (NOS) (>20% blastos) o SMD/LMA (10-19% blastos)
Sarcoma Mieloide

3.3 Clasificación pronóstica de la *European LeukemiaNet*

En el año 2022 se han publicado las nuevas recomendaciones de la *European LeukemiaNet* (ELN) para el diagnóstico y el manejo clínico de los pacientes con LMA (ELN 2022) (Döhner et al., 2022). La nueva propuesta de clasificación del riesgo incluye cambios sustanciales respecto a la última revisión publicada en el año 2017 (Döhner et al., 2017) y enfatiza la relevancia de las alteraciones citogenéticas y moleculares en la estratificación pronóstica, así como la importancia del estudio de la ERM para refinar la asignación individual del riesgo.

En la tabla 4 se recogen las alteraciones genéticas que conforman los grupos de riesgo favorable, intermedio y adverso según la clasificación ELN 2022. Las modificaciones introducidas han provocado cambios en todos los grupos de riesgo siendo especialmente relevantes en el grupo de riesgo intermedio y adverso. La eliminación de la ratio alélica de las mutaciones *FLT3*-ITD de la estratificación pronóstica, conlleva la reclasificación de todos los pacientes *FLT3*-ITD mutados al grupo de riesgo intermedio, independientemente de la concurrencia con mutaciones en el gen *NPM1*. La falta de estandarización de los métodos empleados para el cálculo de la ratio alélica, el impacto de la midostaurina en los pacientes con mutaciones *FLT3*-ITD y el papel de la ERM para la toma de decisiones terapéuticas, han sido las principales razones para este cambio. La clasificación de la LMA con mutaciones en genes relacionados con la mielodisplasia (MDS) (*ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1* y *ZRSR2*) en el grupo de riesgo adverso, ha sido otro de los principales cambios incorporados. Respecto al grupo de riesgo favorable, se ha reevaluado el impacto pronóstico de las mutaciones en el gen *CEBPA*. Recientes estudios han demostrado que únicamente las mutaciones en el *basic leucine zipper domain* (bZIP) se correlacionan con buen pronóstico, lo que ha supuesto la clasificación de las mutaciones *in-frame* en el dominio bZIP de *CEBPA* como marcadores de riesgo favorable independientemente de su estado mono- o bialélico (Wakita et al., 2022).

La nueva estratificación del riesgo ELN 2022 también ha incluido numerosos cambios citogenéticos. Las translocaciones cromosómicas que implican la región (3q26.2;v) y dan lugar a reordenamientos del gen *MECOM*, así como la t(8;16)(p11.2;p13.3) asociada al reordenamiento *KAT6A::CREBBP*, se han descrito como nuevos marcadores asociados al grupo de riesgo adverso. En este sentido, las alteraciones cromosómicas pertenecientes al grupo de riesgo adverso definen mal pronóstico independientemente de la concurrencia con *NPM1* mutado (Angenendt et al., 2019). Por último, los cariotipos hiperdiploides con múltiples

trisomías (o polisomías) sin otras alteraciones estructurales no se consideran cariotipos complejos.

Tabla 4. Clasificación pronóstica de la ELN 2022.

Favorable
t(8;21)(q22;q22.1) / *RUNX1::RUNX1T1*
inv(16)(p13.1q22) o t(16;16)(p13.1;q22) / *CBFB::MYH11*
NPM1 mutado sin *FLT3*-ITD
Mutaciones *in-frame* en el dominio bZIP de *CEBPA*
Intermedio
NPM1 mutado con *FLT3*-ITD
NPM1 no mutado con *FLT3*-ITD
t(9;11)(p21.3;q23.3) / *MLLT3::KMT2A*
Alteraciones citogenéticas o moleculares no clasificadas como favorable o adversas
Adverso
t(6;9)(p23;q34.1) / *DEK::NUP214*
t(v;11q23.3) / Reordenamientos de *KMT2A*
t(9;22)(q34.1;q11.2) / *BCR::ABL1*
t(8;16)(p11;p13) / *KAT6A::CREBBP*
inv(3)(q21.3q26.2) o t(3;3)(q21.3;q26.2) / *GATA2*, *MECOM*
t(3q26.2;v) / Reordenamientos de *MECOM*
-5 o del(5q); -7; -17 / abn(17p)
Cariotipo complejo o monosómico
Mutaciones en *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1*, o *ZRSR2*
TP53 mutado

3.4 Clasificación genómica de la LMA

En el año 2016, se publicó la primera clasificación genómica de la LMA que recogía los resultados del análisis por NGS de 111 genes en 1540 pacientes (Papaemmanuil et al., 2016). Este estudio incluyó la mayor cohorte de pacientes con estudios de NGS publicada hasta la fecha y permitió identificar nuevas interacciones entre genes y patrones de co-mutación entre regiones *hotspot* que no habían sido previamente descritas, sugiriendo diferencias funcionales dependientes de los genes y región alterada. Estos resultados permitieron inferir distintos patrones de evolución clonal en el que mutaciones en modificadores epigenéticos como *DNMT3A*, *ASXL1*, *IDH1/2*, y *TET2* constituyen eventos iniciales en el desarrollo de la leucemia, mientras que mutaciones en genes de vías de señalización surgen como eventos tardíos.

La asociación de las características moleculares con la presentación clínica y supervivencia global (SG) compartimentó la LMA en 11 clases moleculares mutuamente excluyentes que validaban las entidades previamente definidas por la clasificación de la OMS [t(15;17), t(8;21), inv(16), t(6;9), inv(3) y fusiones del gen *KMT2A*] y consolidaba las entidades

provisionales de LMA con mutaciones en *NPM1* y LMA con mutaciones en *CEBPA* (Swerdlow et al., 2008). Asimismo, se definieron nuevos subgrupos moleculares como mutaciones en genes implicados en el remodelado de la cromatina o alteraciones en el *splicing*, *TP53*-aneuploidías y mutaciones en el codón R172 del gen *IDH2* (Tabla 5).

Tabla 5. Grupos genómicos propuestos en la primera clasificación genómica de la LMA.

Grupo genómico
LMA con *NPM1* mutado
LMA con mutaciones en genes de la cromatina o *splicing*
LMA con mutaciones en *TP53*, aneuploidías, o ambas
LMA con inv(16)(p13.1q22) o t(16;16)(p13.1;q22); *CBFB::MYH11*
LMA con mutaciones bialélicas en *CEBPA*
LMA con t(15;17)(q22;q12); *PML::RARA*
LMA con t(8;21)(q22;q22); *RUNX1::RUNX1T1*
LMA con reordenamientos de *KMT2A*; t(x;11)(x;q23)
LMA con inv(3)(q21q26.2) o t(3;3)(q21;q26.2); *GATA2*, *MECOM*
LMA con mutación en el codón R172 del gen *IDH2* sin alteraciones que definan grupo
LMA con t(6;9)(p23;q34); *DEK::NUP214*
LMA con mutaciones *driver* que no definen clase
LMA sin eventos *driver* detectados

La reciente publicación de la nueva clasificación genómica de la LMA (Tazi et al., 2022) supone un cambio de paradigma respecto a la primera versión publicada en el año 2016 (Papaemmanuil et al., 2016). El análisis de datos citogenéticos y moleculares de 2113 pacientes ha permitido desarrollar una clasificación jerárquica de 16 clases moleculares no solapantes asociadas a parámetros demográficos y clínicos específicos que permite asignar al 100% de los pacientes con LMA a un grupo molecular y otorgarles valor pronóstico (Tabla 6).

La clasificación jerárquica incluye ocho clases moleculares previamente definidas en la clasificación de la OMS [t(15;17, inv(16), t(8;21), t(11;x), t(6;9), inv(3), *NPM1* y *CEBPA*bi] (Arber et al., 2016) y se proponen ocho nuevas clases con valor pronóstico independiente. Las nuevas clases definidas por alteraciones citogenéticas incluyen a los pacientes con mutaciones en el gen *TP53* y/o cariotipo complejo (CC), que muestran un pronóstico adverso; así como el subgrupo definido por pacientes con ≥1 trisomías asociado a un pronóstico intermedio. Entre las nuevas clases definidas por alteraciones moleculares, los pacientes con mutaciones en genes asociados a la LMA secundaria (*SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR, STAG2, RUNX1, SETBP1* y *KMT2A*-PTD) presentan un valor pronóstico dependiente del número de mutaciones, siendo únicamente los pacientes con ≥2 mutaciones (sAML2) los que se asocian a peor pronóstico. Otras clases moleculares minoritarias incluyen a los pacientes con mutaciones en el gen *WT1* que se asocian a un riesgo intermedio y los pacientes con mutaciones en los

genes *DNMT3A-IDH1/2* que define a un subgrupo muy heterogéneo. Además, se proponen dos nuevos subgrupos definidos por pacientes con alteraciones moleculares distintas a las que definen grupo (mNOS) o pacientes sin ninguna mutación.

Tabla 6. Grupos genómicos y clasificación del riesgo de las 16 clases moleculares definidas en la nueva clasificación genómica de la LMA.

Grupos genómicos	Clase molecular	Favorable	Intermedio	Adverso
1. OMS Set 1	*NPM1*	■		
	t(8;21)	■		
	inv(16)	■		
	t(15;17)	■		
2. *TP53* ± CC	*TP53* ± CC			■
3. OMS Set 2	*CEBPA*bi	■		
	t(11;x)		■	
	t(6;9)		■	
	inv(3)			■
4. sAML1	sAML1		■	
5. sAML2	sAML2			■
6. *WT1*	*WT1*		■	
7. Trisomías	Trisomías		■	
8. *DNMT3A + IDH1/2*	*DNMT3A + IDH1/2*		■	
9. Eventos que no definen clase	mNOS		■	
10. No eventos	No eventos	■		

El análisis de las clases moleculares ha permitido generar un modelo de estratificación del riesgo en tres grupos: Las clases moleculares *NPM1*, inv(16), t(8;21), t(15;17) y *CEBPA*bi se han englobado en el grupo de riesgo favorable. En este grupo de riesgo también se han incluido a los pacientes sin ninguna alteración molecular resaltando así la importancia de hallazgos moleculares negativos. El grupo de riesgo intermedio incluye a pacientes con las alteraciones citogenéticas t(6;9) y t(11;x), así como las nuevas clases moleculares sAML1 (una mutación en genes asociados a mielodisplasia), *WT1*, *DNMT3A-IDH1/2* y mNOS. El grupo de riesgo adverso está formado por clases moleculares de peor pronóstico como los pacientes con *TP53* y/o CC e inv(3), así como la nueva clase molecular sAML2.

Esta clasificación redefine el impacto clínico de las duplicaciones parciales en tándem en el gen *FLT3* (*FLT3*-ITD). Estas mutaciones no se consideran definitorias de grupo debido a su presencia concurrente en todas las clases moleculares y su valor pronóstico se considera

independiente del valor de la ratio. En el modelo de estratificación del riesgo, la presencia de mutaciones *FLT3*-ITD provoca la transición de pacientes con LMA-*NPM1* mutado del grupo de riesgo favorable al intermedio y de pacientes del grupo de riesgo intermedio al grupo de riesgo adverso independientemente de la presencia concomitante de otras mutaciones.

De esta manera, la asociación de los subgrupos biológicos con la presentación clínica y curso de la enfermedad, ha definido un nuevo esquema de estratificación del riesgo basado en la pertenencia a clases moleculares y modulado por la presencia de mutaciones *FLT3*-ITD, unificándose la clasificación y estratificación del riesgo de los pacientes con LMA.

4. Implementación de la secuenciación masiva en la práctica clínica

Los avances metodológicos y las nuevas clasificaciones han convertido a los estudios moleculares en una herramienta fundamental para aumentar el grado de personalización en el manejo clínico de los pacientes con LMA. Las técnicas basadas en el análisis de gen-a-gen han demostrado ser ineficientes para abordar la caracterización completa de la LMA, por lo que ha aumentado la demanda de los estudios de NGS en los laboratorios dedicados al diagnóstico molecular.

Los paneles de genes han sido la estrategia más desarrollada en la práctica clínica al abordar el estudio de un amplio número genes relevantes para establecer el diagnóstico y pronóstico de los pacientes, así como evaluar las opciones terapéuticas con fármacos dirigidos (Alonso et al., 2019). Esta estrategia ofrece una mayor profundidad de lectura, simplifica el análisis de resultados y tiene un coste reducido respecto a otras estrategias de alto rendimiento. Además, ante el cambiante escenario molecular de la LMA, los paneles de genes representan una opción versátil al permitir la adición de nuevos genes o regiones diana (Llop et al., 2022).

Pese a que los estudios de NGS son requeridos en el actual algoritmo diagnóstico de la LMA, constituyen una aproximación no abordable por muchos laboratorios ya que su aplicación en la práctica clínica plantea nuevos retos (Bacher et al., 2018). Además del coste asociado a los equipos de secuenciación, son necesarios grandes volúmenes de muestras para ser coste-efectiva, los flujos de trabajo dificultan obtener resultados en menos de siete días y tanto la parte técnica como el análisis e interpretación de resultados debe realizarse por especialistas cualificados que integren los resultados en el contexto clínico.

Actualmente, los laboratorios dedicados al diagnóstico molecular que integran los estudios de NGS, carecen de recomendaciones consensuadas que guíen su aplicación en la práctica clínica (Mosele et al., 2020). La convivencia de distintas aproximaciones técnicas y

distintos criterios para la translación clínica de los resultados, supone un verdadero desafío para los laboratorios que deben coordinarse con el fin de homogeneizar criterios de calidad, definir las alteraciones que se deben estudiar, cuáles son relevantes en el manejo clínico y cómo deben trasladarse los resultados a la clínica.

HIPÓTESIS Y OBJETIVOS

En los últimos años, la aplicación de la NGS al estudio de los pacientes con LMA ha dado a conocer nuevas alteraciones moleculares que se han incorporado a los sistemas de clasificación y estratificación del riesgo, y han permitido ofrecer tratamientos más adecuados a cada paciente. Los laboratorios clínicos han debido adaptarse tecnológicamente, complementando las técnicas convencionales de diagnóstico con la NGS para abordar el creciente número de alteraciones.

El desarrollo y consolidación de una plataforma nacional de NGS facilitaría el acceso de los pacientes con LMA a la medicina de precisión. Esta nueva estrategia supondría un incremento en la calidad de los estudios moleculares a nivel clínico y también en el ámbito de investigación traslacional.

Los **objetivos** propuestos para esta tesis son:

1. Estandarizar la técnica de NGS entre los laboratorios de referencia en términos de composición de genes, métricas de calidad y elaboración de informes.

2. Establecer un programa de control de calidad interlaboratorios para verificar la calidad de los estudios de NGS.

3. Caracterizar el perfil mutacional de acuerdo al momento de la enfermedad, características clínicas y demográficas de los pacientes.

4. Identificar la existencia patrones de concurrencia y exclusividad entre mutaciones.

5. Inferir patrones de evolución clonal mediante el análisis de la frecuencia alélica (VAF) y la estabilidad de las mutaciones detectadas durante la progresión de la leucemia.

6. Explorar la clasificación genómica de la LMA y validar las nuevas clases moleculares en una cohorte de pacientes de LMA del grupo cooperativo PETHEMA.

7. Validar la estratificación del riesgo propuesta por la *European LeukemiaNet* 2022 en la cohorte de pacientes PETHEMA-LMA. Comparar los distintos grupos de riesgo y su valor pronóstico de acuerdo a los criterios ELN 2017 y ELN 2022.

COMPENDIO PUBLICACIONES

(MÉTODOS Y RESULTADOS)

En la presente tesis doctoral se ha desarrollado el primer control de calidad nacional para la estandarización de los estudios de NGS entre siete laboratorios de referencia pertenecientes al grupo cooperativo PETHEMA con el fin de proporcionar resultados homogéneos a todos los pacientes diagnosticados de LMA en el territorio nacional. Aplicar la NGS como método diagnóstico en una amplia serie de pacientes ha permitido la caracterización molecular exhaustiva de la enfermedad, revelando perfiles moleculares dependientes de características demográficas, así como relaciones de concurrencia y exclusividad. La validación de la nueva clasificación genómica de la LMA ha demostrado la asociación entre los subgrupos moleculares con valor pronóstico, consolidando la utilidad de la NGS en diagnóstico molecular. Asimismo, la validación de la nueva estratificación del riesgo propuesta en la ELN 2022 ha evidenciado nuevos subgrupos genómicos que sugieren la necesidad de refinar estas guías.

Los resultados obtenidos en la presente tesis doctoral se presentan por compendio de los siguientes artículos científicos:

1. *Networking for advanced molecular diagnosis in acute myeloid leukemia patients is possible: the PETHEMA NGS-AML project*. Haematologica, 2021 [Factor de impacto 11,049 (D1)].

2. *Molecular Landscape and Validation of New Genomic Classification in 2668 Adult AML Patients: Real Life Data from the PETHEMA Registry*. Cancers, 2023 [Factor de impacto 6,575 (Q1)].

3. *Validation of the 2022 European LeukemiaNet risk classification in a real-life cohort of the PETHEMA group*. Enviado a Blood Cancer Journal, 2023 [Factor de impacto 9,812 (Q1)].

1. *Networking for advanced molecular diagnosis in acute myeloid leukemia patients is possible: the PETHEMA NGS-AML project.* (Manuscrito original adjunto como anexo)

En este trabajo se recoge el proceso de estandarización de los estudios de NGS entre siete laboratorios dedicados al diagnóstico molecular de la LMA y la descriptiva del perfil de mutaciones en una serie de 823 muestras. El grupo cooperativo PETHEMA designó estos laboratorios como centros de referencia para la centralización de muestras de los hospitales adscritos al grupo.

1.1 Rondas de validación

El proceso de estandarización comenzó con una primera ronda de validación cuya finalidad fue definir la situación inicial de los estudios de NGS entre los centros de referencia. Para ello, se distribuyeron cuatro muestras con 24 variantes que fueron analizadas de forma independiente por cada laboratorio atendiendo a criterios técnicos y de análisis específicos de cada centro. Esta fase de pre-estandarización evidenció falta de consenso en los genes analizados lo que promovió definir un conjunto de genes que incluía el total de alteraciones contempladas en las vigentes clasificaciones de diagnóstico, estratificación del pronóstico y alternativas terapéuticas (*ABL1, ASXL1, BRAF, CALR, CBL, CEBPA, CSF3R, DNMT3A, ETV6, EZH2, FLT3, GATA2, HRAS, IDH1, IDH2, JAK2, KIT, KRAS, MPL, NPM1, NRAS, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, TET2, TP53, U2AF1* y *WT1*).

Seguidamente, se realizó la segunda ronda de validación que tenía como objetivo definir parámetros consenso de métricas de calidad en términos de uniformidad (>85%) y profundidad de lectura (≥1000X), así como evaluar los criterios para el reporte de resultados. Para ello, todos los centros analizaron seis muestras con 30 variantes y se incluyó por primera vez el análisis de variantes con baja VAF (<5%) (rango de 1,8%-4,9%). Los resultados obtenidos mostraron una tasa de error de 12,3% en la detección de variantes con VAF≥5% y de 28,6% en la detección de variantes con VAF<5% (*P*=0,031). Esto promovió establecer un punto de corte de VAF≥5% para el reporte de variantes clínicamente relevantes.

Nuestro análisis no mostró diferencias respecto a la tasa de error en la detección entre inserciones y deleciones (indel) o cambios de secuencia únicos (SNV), o la plataforma de NGS empleada (Illumina o Ion Torrent).

1.2 Perfil de mutaciones

El análisis del perfil mutacional mostró que casi la totalidad de las muestras (90,8%) tenían mutado al menos uno de los genes estudiados. *FLT3* (24,1%)*, DNMT3A* (21,6%) y *NPM1* (21,5%) fueron los genes más frecuentemente mutados y mostraron una fuerte relación de

asociación. También detectamos patrones de co-mutación entre los genes *PTPN11* y *NPM1* (*P*<0.01) o *SRSF2* e *IDH2* (*P*<0.01) (Figura 6); mientras que las mutaciones en el gen *TP53* fueron altamente excluyentes con el resto de genes.

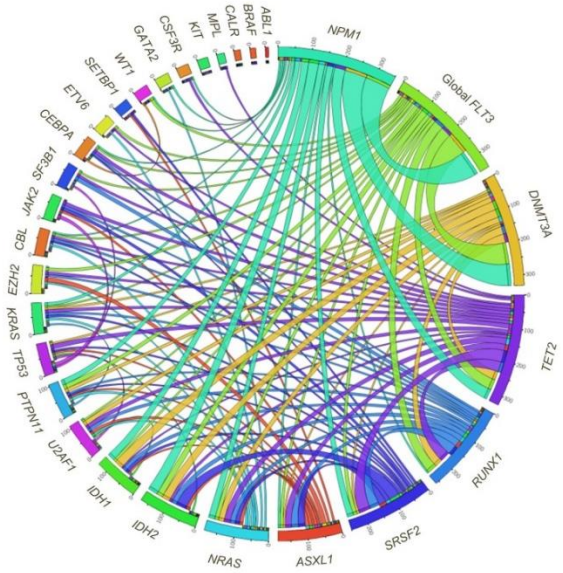


Figura 6. Diagrama de circos que muestra patrones de asociación entre las variantes detectadas.

1.3 Evolución clonal

El análisis de la VAF de las variantes detectadas mostró que la leucemogénesis presenta un patrón de desarrollo definido por alteraciones que aparecen en fases tempranas, como las mutaciones en genes relacionados con las alteraciones epigenéticas (*ASXL1*, *DNMT3A* y *TET2*), que en nuestra serie mostraron la mediana de VAF más elevada (43,3%). Mutaciones en genes implicados en vías de señalización (*FLT3*, *NRAS*, *KRAS*, *KIT* y *PTPN11*) mostraron una mediana de VAF de 17,8% lo que indica su adquisición en fases posteriores (Figura 7).

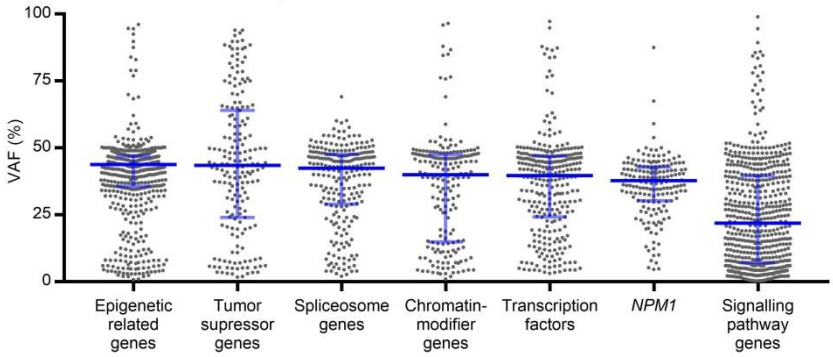


Figura 7. Mediana de la VAF de acuerdo a la clasificación funcional de los genes. Cada punto gris representa una variante.

El análisis de la estabilidad mutacional en muestras pareadas diagnóstico-recaída (N=14) y diagnóstico-resistencia (N=20) mostró distintos patrones de evolución clonal. La tasa de estabilidad de las mutaciones en *NPM1* fue del 100% ya que en nuestro estudio no se detectó ningún caso de cambio del estado mutacional en la resistencia o en recaída de la leucemia. Las mutaciones en *DNMT3A* también mostraron una tasa de estabilidad del 100% probablemente debido a su adquisición temprana como eventos preleucémicos y su persistencia durante la remisión y progresión de la enfermedad. Mutaciones en genes de vías de señalización (*FLT3*, *NRAS*, *KRAS*, *BRAF*, *KIT* y *PTPN11*) fueron altamente inestables con tasas de estabilidad promedio del 33%. Esto tiene gran impacto a nivel de terapias dirigidas ya que en nuestra serie el 26,5% de los pacientes cambió el estado mutacional de *FLT3* en la resistencia o recaída.

1.4 Detección de variantes con impacto clínico

La NGS ha permitido la detección de mutaciones en genes con impacto en el diagnóstico, estratificación pronóstica y terapia dirigida en los pacientes con LMA. Dado que este estudio es anterior a las nuevas clasificaciones de diagnóstico y pronóstico que han redefinido el perfil de alteraciones moleculares con impacto clínico, los genes analizados como clínicamente relevantes fueron: *ASXL1, CEBPA, FLT3, IDH1, IDH2, NPM1, RUNX1* y *TP53*. Un 72,3% de los pacientes mostraron mutaciones en alguno de estos genes siendo especialmente relevantes aquellos susceptibles de tratamiento con terapias dirigidas como las mutaciones en *FLT3* detectadas en el 21,1% de los pacientes o las mutaciones en los genes IDH1/2 detectadas en el 22.6%.

Entre los genes clínicamente relevantes, las mutaciones en el gen *FLT3* fueron las más frecuentes (24,1%) con predominio de las mutaciones *FLT3*-ITD (16,5%). Un 39% de los pacientes presentó al menos una mutación en los genes *ASXL1*, *RUNX1* o *TP53* siendo las mutaciones en estos genes la única variante detectada en el 26,1% de los casos. Esto fue especialmente relevante teniendo en cuenta que estos genes pueden presentar mutaciones a lo largo de toda la secuencia y su estudio únicamente es abordable mediante técnicas de NGS. Las mutaciones en *NPM1* se detectaron en un 21,5% de los casos y, aunque la mayoría correspondieron a los tipos más frecuentes A (78,5%), B (6,21%) y D (6,21%), un 9,04% presentó otras variantes minoritarias. Mutaciones en los genes IDH1/2 se detectaron en el 22,6% de las muestras con predominio de mutaciones en el gen *IDH2* (13,9%). En nuestra serie las mutaciones en *CEBPA* fueron minoritarias detectándose únicamente en el 5,4% de los casos.

El primer artículo científico que conforma la presente tesis doctoral muestra el desarrollo de la primera estrategia nacional de estandarización de los estudios de NGS en el contexto del grupo cooperativo PETHEMA. Esta estrategia ha permitido unificar criterios de análisis y disminuir la variabilidad en el informe de resultados, superando los retos que plantea la implementación de la NGS en los laboratorios dedicados al diagnóstico molecular. El análisis del perfil mutacional en una serie prospectiva de pacientes de nuevo diagnóstico en recaída o resistencia, muestra el complejo panorama molecular de la LMA.

2. *Molecular Landscape and Validation of New Genomic Classification in 2668 Adult AML Patients: Real Life Data from the PETHEMA Registry*. (Manuscrito original adjunto como anexo)

En este trabajo se recogen los resultados de la tercera ronda de validación cuya finalidad fue revisar los criterios establecidos en las dos primeras rondas y explorar la detección de variantes de baja VAF (<5%). Asimismo, se llevó a cabo la caracterización molecular de 2856 muestras que permitió identificar patrones de co-mutación y exclusividad entre genes y entre categorías funcionales propuestas por el TCGA. También se aplicó la nueva clasificación genómica de la LMA a una serie de 956 pacientes de acuerdo a la disponibilidad de datos de citogenética, moleculares y clínicos.

2.1 Resultados tercera ronda de validación

En la tercera ronda de validación se analizaron cuatro muestras con 32 variantes (11 con VAF≤5%). La tasa de error en la detección de variantes con VAF>5% fue de 4,8% y en las variantes con VAF≤5% fue de 59,6%. La comparativa de estos resultados con los obtenidos en la primera y segunda ronda de validación (primer artículo de la tesis doctoral), mostró que la tasa de error en la detección de variantes con VAF>5% disminuyó en las sucesivas rondas [1ª RV: 39%, 2ª RV: 14,4% y 3ª RV: 4,8%]. En cambio, la inclusión de variantes de muy baja VAF en la tercera ronda de validación (VAF media 1,2%) provocó un aumento del 31% en la tasa de error respecto a la segunda ronda de validación (VAF media 3,3%). Estos resultados revalidaron el criterio de VAF≥5% para el reporte de variantes clínicamente relevantes.

2.2 Descripción molecular

El análisis del perfil de mutaciones en una amplia serie de muestras (N=2856) permitió identificar patrones de mutación asociados al momento de la enfermedad. Mutaciones en el gen *NPM1* y en genes implicados en vías de señalización como *KRAS* y *NRAS,* fueron más frecuentes en el diagnóstico, mientras que la LMA refractaria o en recaída mostró mayor frecuencia de mutaciones en *WT1 (P*<0,001*), RUNX1 (P*=0,037*), DNMT3A (P*=0,018*)* e *IDH1 (P*=0,017*)*. Los cambios en el perfil de mutaciones según el momento de la enfermedad, permitieron sugerir distintos mecanismos subyacentes a la progresión de la leucemia ya que un 49,2% de los pacientes con LMA refractaria pero solo un 24,1% de los pacientes en recaída, mantuvo el perfil de mutaciones observado al diagnóstico.

El análisis del perfil mutacional según la edad, mostró que los pacientes jóvenes (<65 años) tienen menor número de mutaciones al diagnóstico que los pacientes mayores (≥65 años) [2,5 vs. 2,9; *P*<0,001]. Mutaciones en los genes *FLT3* (*P*<0,001)*, NPM1* (*P*<0,001) y

DNMT3A (*P*=0,032) fueron más frecuentes en los pacientes <65 años, mientras que los pacientes mayores mostraron mayor frecuencia de mutaciones en *TET2* (*P*<0.001), *RUNX1* (*P*<0.001), *TP53* (*P*<0.001), *IDH2* (*P*<0.01), *ASXL1* (*P*<0.001), *SRSF2* (*P*<0.001), *U2AF1* (*P*<0.01), *JAK2* (*P*<0.001) y *EZH2* (*P*<0.001) (Figura 8A).

También observamos distinto perfil de mutaciones según el sexo de los pacientes. Las mujeres mostraron menor número de mutaciones que los hombres en el momento del diagnóstico (2,6 vs. 2,8; *P*<0,01), y mayor frecuencia de mutaciones en *DNMT3A* (*P*<0,001), *FLT3* (*P*<0,01) y *NPM1* (*P*<0,001). En cambio, los hombres mostraron mayor frecuencia de mutaciones en *TET2* (*P*=0,014), *RUNX1* (*P*<0,001), *ASXL1* (*P*<0,001), *SRSF2* (*P*<0,001), *EZH2* (*P*<0,001), *U2AF1* (*P*<0,001) y *JAK2* (*P*=0,01) (Figura 8B). Estas diferencias suponen un fuerte impacto en términos de estratificación pronóstica, ya que las mutaciones en genes MDS han sido recientemente incluidas en la estratificación pronóstica de la ELN 2022 como marcadores de riesgo advero. Asimismo, mutaciones en el gen *FLT3* confieren beneficio en términos de tratamiento con terapias dirigidas mientras que las mutaciones en *NPM1* permiten monitorizar la evolución de la enfermedad y evaluar la respuesta al tratamiento.

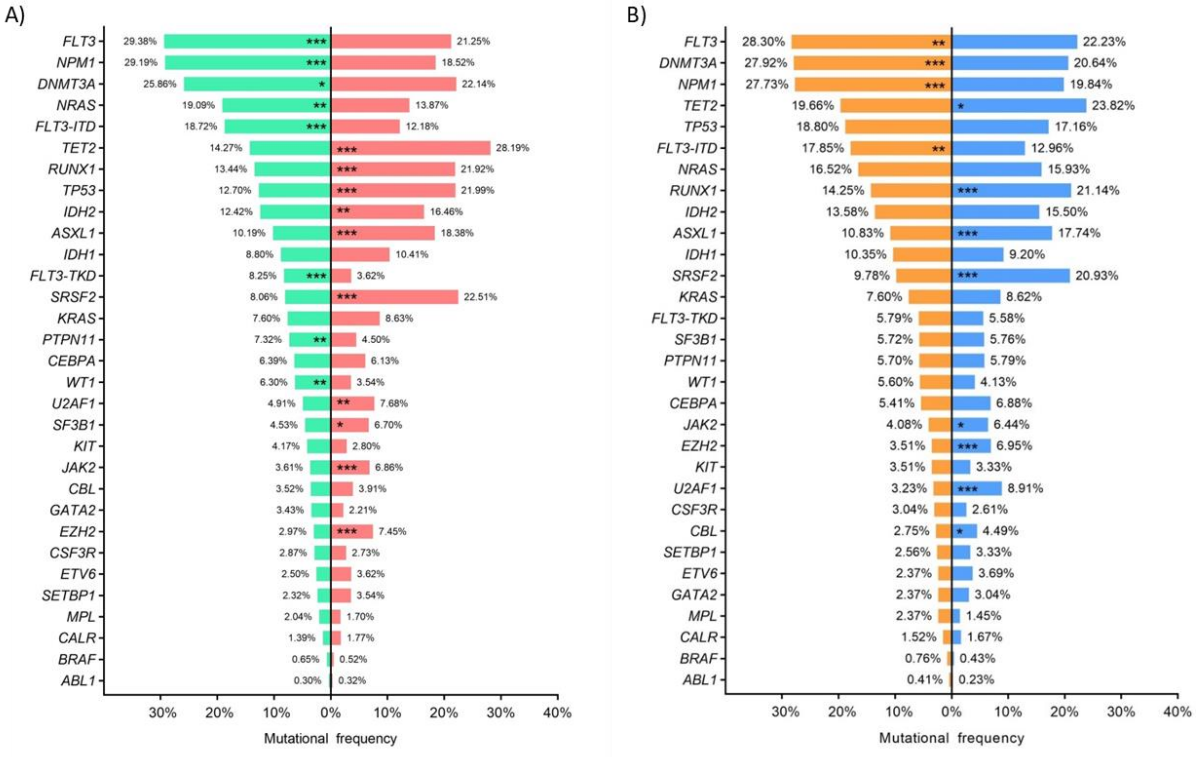


Figura 8. Perfil mutacional asociado a A) la edad y B) sexo de los pacientes. Barras verdes: <65 años; barras rojas ≥65 años. Barras naranjas: mujeres; barras azules: hombres. **P*<0.05, ***P*<0.01, ****P*<0.001.

2.3 Nueva clasificación genómica de la LMA

La aplicación de la nueva clasificación genómica a la serie de 954 pacientes, mostró una elevada representatividad del grupo molecular definido por mutaciones en el gen *NPM1* (23,9%) así como otros no descritos hasta el momento como el subgrupo sAML2 (25,6%) (Definido por la presencia de mutaciones en dos o más genes relacionados con la mielodisplasia) y el subgrupo de mutaciones en *TP53* y/o CC (19,2%). Otros grupos menos frecuentes fueron la clase sAML1 (8,7%) y mNOS (8,3%) (Figura 9).

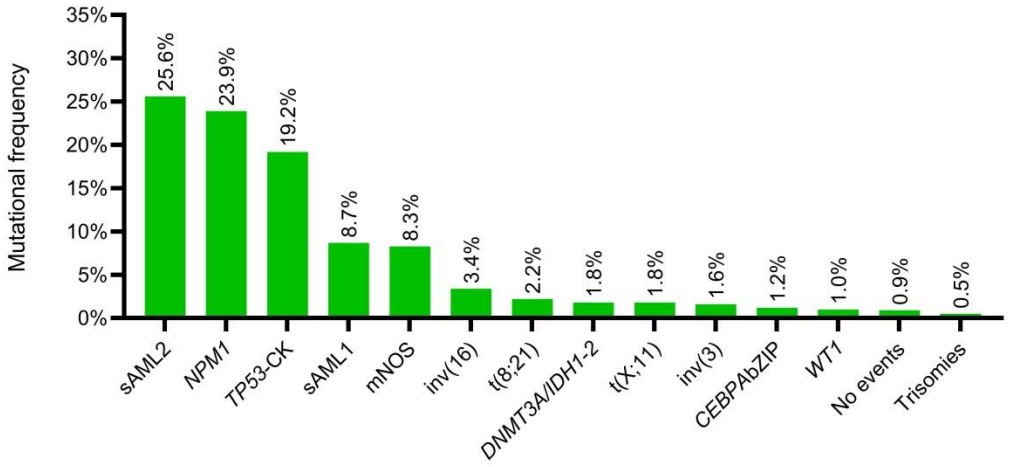


Figura 9. Distribución en clases moleculares según la clasificación genómica de la LMA.

Estas clases moleculares se asociaron con distinto valor pronóstico. Entre las clases previamente definidas en la clasificación de la OMS, las alteraciones inv(16) (mediana de SG no alcanzada a los 42 meses), *CEBPA*-bZIP (mediana de SG no alcanzada a los 32 meses) y *NPM1* (mediana de SG 29 meses; 95%CI 19,9-38,0) mostraron los mejores valores de SG, mientras que la alteración inv(3) se asoció con peor pronóstico (4,9 meses; 95%CI 0,8-9,1). Esta asociación con el valor pronóstico también se detectó en las clases moleculares nuevamente descritas. Los pacientes en los que no se detectó ningún evento (mediana de SG no alcanzada a los 33 meses) o los pacientes del grupo mNOS (23,3 meses; 95%CI 11,0-35,6) se asociaron a buen pronóstico mientras que las clases moleculares de *TP53* y/o CC (5,3 meses; 95%CI 2,9-7,6), sAML2 (12,1 meses; 95%CI 9,9-14,2) y mutaciones en *WT1* (4 meses; 95%CI 0-18,4) se asociaron con peores valores de SG y mayor riesgo de muerte que el resto de categorías.

De acuerdo al modelo de estratificación del riesgo generado a partir de las clases moleculares, un 23,8% de los pacientes se clasificó en el grupo de riesgo favorable, 27,1% en el grupo de riesgo intermedio y 49,1% en el grupo de riesgo adverso. Sin embargo, esta distribución cambió en función de la edad: los pacientes <65 años mostraron una distribución equilibrada entre los grupos de riesgo favorable (31,2%), intermedio (32,4%) y adverso

(36,4%); mientras que los pacientes ≥65 años se clasificaron mayoritariamente en el grupo de riesgo adverso (59,3%). Además, los pacientes con edad avanzada mostraron peores valores de SG y mayor riesgo de muerte para todos los grupos de riesgo [Favorable: 4,7 (95%CI 2,8–8,0; *P*<0,001), intermedio: 1,8 (95%CI 1,2–2,6; *P*<0,01) y adverso: 2,6 (95%CI 2,0–3,5; *P* <0,001)].

El segundo artículo científico que conforma la presente tesis doctoral muestra que sucesivas rondas de validación realizadas en el contexto de un grupo cooperativo han permitido obtener resultados de NGS fiables y consistentes mediante la unificación de los criterios de análisis y reporte de resultados. Los datos moleculares generados, suscriben la complejidad molecular de la LMA y han permitido describir perfiles moleculares asociados a la edad y el sexo de los pacientes. Asimismo, la validación de la clasificación genómica de la LMA en una amplia serie de pacientes de práctica clínica habitual, ha demostrado la asociación de las clases moleculares con el pronóstico clínico, lo que refleja la importancia de abordar el diagnóstico molecular mediante técnicas de alto rendimiento como la NGS.

3. *Validation of the 2022 European LeukemiaNet risk classification in a real-life cohort of the PETHEMA group*. (Manuscrito original adjunto como anexo)

El último trabajo que comprende la presente tesis doctoral, incluye la comparativa de la nueva estratificación pronóstica propuesta por la *European LeukemiaNet* en el año 2022 con la última revisión del año 2017. Esta clasificación se ha validado en una serie de 925 pacientes (546 tratados de forma intensiva y 379 de forma no intensiva) de práctica clínica habitual cuyo perfil molecular se ha analizado mediante estudios de NGS estandarizados en el grupo cooperativo PETHEMA.

3.1 Distribución y valor pronóstico de los grupos de riesgo

Ambas ediciones proponen una clasificación pronóstica en tres grupos de riesgo: favorable, intermedio y adverso, definidos por distintas alteraciones genéticas. De acuerdo a la estratificación del riesgo de la ELN 2017, 31% de los pacientes fueron asignados al grupo de riesgo favorable, 24,7% al grupo de riesgo intermedio y 44,3% al grupo de riesgo adverso. En cambio, la clasificación de la ELN 2022 ha supuesto una reducción del número de pacientes clasificados en el grupo de riesgo favorable (27,8%) e intermedio (20,3%) en favor del grupo de riesgo adverso (51,8%). Esto ha supuesto un incremento de 7,5% de pacientes clasificados en el grupo de riesgo adverso debido principalmente a la asociación de las mutaciones MDS con mal pronóstico. En la ELN 2022, la edad avanzada (≥ 65 años) y el sexo masculino se asociaron al grupo de riesgo adverso debido a la mayor frecuencia de alteraciones genéticas que definen esta categoría (Figura 10).

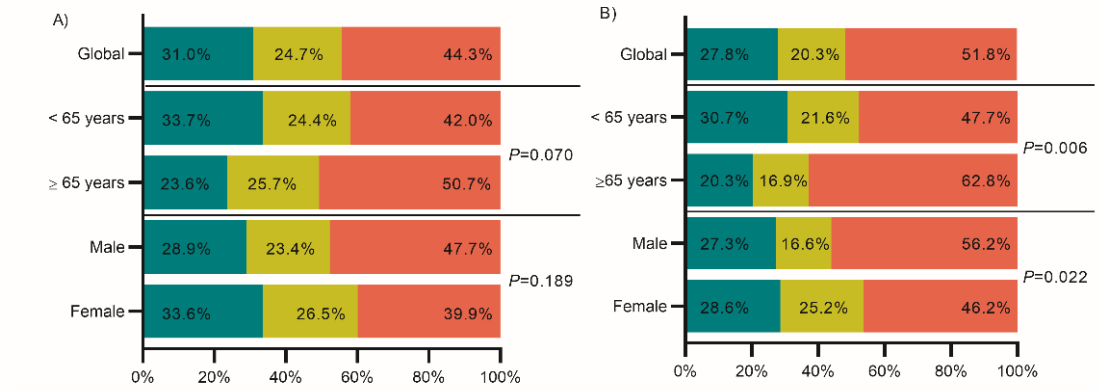


Figura 10. Distribución en grupos de riesgo según las clasificaciones A) ELN 2017 y B) ELN 2022. Barra verde: riesgo favorable; Barra amarilla: riesgo intermedio; Barra roja: riesgo adverso.

Respecto al pronóstico, en ambas ediciones, los pacientes del grupo de riesgo favorable e intermedio no alcanzaron la mediana de SG mientras que los pacientes clasificados en el grupo de riesgo adverso mostraron una SG de 15,7 meses (95%CI 11,3-20,1) según la ELN 2017 y de 15,2 meses (95%CI 11,8-18,6) según la ELN 2022. En ambas ediciones, los pacientes ≥65 años mostraron peor SG que los pacientes <65 años independientemente del grupo de riesgo.

La estratificación pronóstica de la ELN está dirigida a pacientes tratados de forma intensiva. Sin embargo, se analizó la distribución en grupos de riesgo y la SG de 379 pacientes tratados con regímenes no intensivos. Según la ELN 2017, un 18,2% de los pacientes se clasificó en el grupo de riesgo favorable, 19,8% en el intermedio y 62% en el grupo de riesgo adverso. Según la propuesta de la ELN 2022, 16,1% de los pacientes se clasificó en el grupo de riesgo favorable, 11,9% en el intermedio y 72% en el grupo de riesgo adverso. Todos los pacientes tratados de forma no intensiva, independientemente del grupo de riesgo y de la clasificación pronóstica utilizada, mostraron un pronóstico muy desfavorable con medianas de SG entre 6 y 11 meses.

3.2 Reasignación de grupo de riesgo entre la ELN 2017 y la ELN 2022

Los cambios introducidos en las alteraciones genéticas que definen los grupos de riesgo de la ELN 2017 y la ELN 2022, provocó la reasignación de grupo de riesgo de 79 (14,5%) pacientes tratados de forma intensiva. La mayoría de las transiciones (12,5%) implicaron el cambio a un grupo de peor pronóstico. En este sentido, 20 pacientes (3,7%) fueron reclasificados del grupo de riesgo favorable-2017 al grupo de riesgo intermedio-2022 debido a que la ELN 2022 no otorga valor pronóstico a la ratio de *FLT3*-ITD. Asimismo, 47 pacientes (8,6%) pertenecientes al grupo de riesgo intermedio-2017 fueron reasignados al grupo de riesgo adverso-2022 debido a la presencia de mutaciones en genes MDS. Las transiciones a grupos de mejor pronóstico fueron minoritarias. Únicamente cinco pacientes (0,9%) con mutaciones de *FLT3*-ITD de alto ratio en ausencia de co-mutación en *NPM1*, fueron reasignados del grupo de riesgo adverso-2017 al grupo de riesgo intermedio-2022. Finalmente, cuatro pacientes con mutación en *CEBPA*-bZIP fueron reclasificados del grupo de riesgo intermedio-2017 al grupo de riesgo favorable-2022 (Figura 11). El análisis de supervivencia de los pacientes reclasificados permitió validar el impacto pronóstico de los cambios introducidos en la ELN 2022 al no observarse diferencias en la SG entre los pacientes reasignados y sus respectivos grupos de riesgo favorable (*P*=0,369), intermedio (*P*=0,679) y adverso (*P*=0,794).

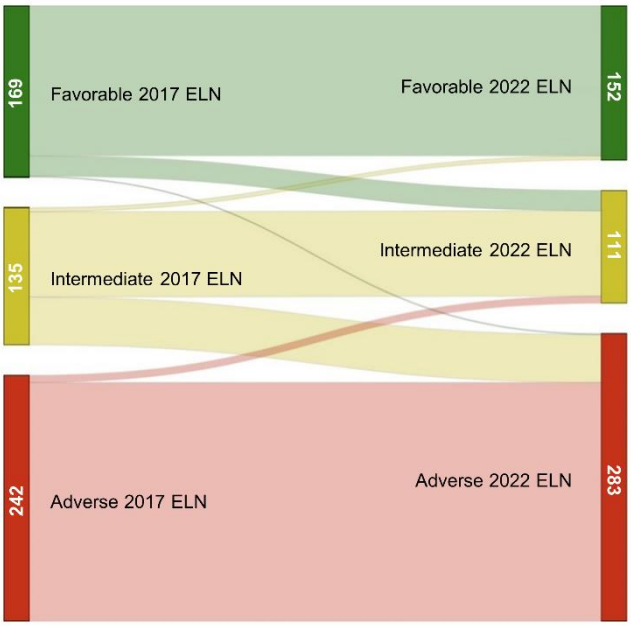


Figura 11. Diagrama de Sankey que muestra la reasignación de grupos entre la ELN 2017 y la ELN 2022.

3.3 Subgrupos genéticos de la clasificación ELN 2022

Se evaluó el valor pronóstico de las alteraciones genéticas que definen las categorías de riesgo de la ELN 2022. No detectamos diferencias en la SG entre los subgrupos genéticos que definen el grupo de riesgo favorable [*NPM1*mut sin *FLT3*-ITD, *CEBPA*-bZIP, inv(16) y t(8;21); *P*=0,741] lo que validó su clasificación como un mismo grupo de riesgo. Tampoco se detectaron diferencias entre las alteraciones genéticas que definen el grupo de riesgo intermedio [*NPM1*mut con *FLT3*-ITD, *NPM1*-WT con *FLT3*-ITD, t(9;11) y otras alteraciones citogenéticas y moleculares; *P*=0,201]. Sin embargo, se observaron diferentes valores de SG entre los subgrupos genéticos que conforman el grupo de riesgo adverso [inv(3), (-5, -7, -17), *TP53* y/o CC, mutaciones MDS, t(X;11), t(6;9) y t(9;22)]. Los pacientes con inv(3) [SG 8,3 meses; 95%CI 0-16,8] y mutaciones en *TP53* y/o CC [SG 9,5 meses; 95%CI 5,0-14,0] mostraron los peores valores de SG respecto al resto de subgrupos genéticos. La agrupación de estas alteraciones definió un nuevo grupo de riesgo con muy mal pronóstico [9,5 meses; 95%CI 5,4-13,6] y con 1,9 veces mayor riesgo de muerte que los pacientes clasificados en el grupo de riesgo adverso (*P*=0,001) (Figura 12).

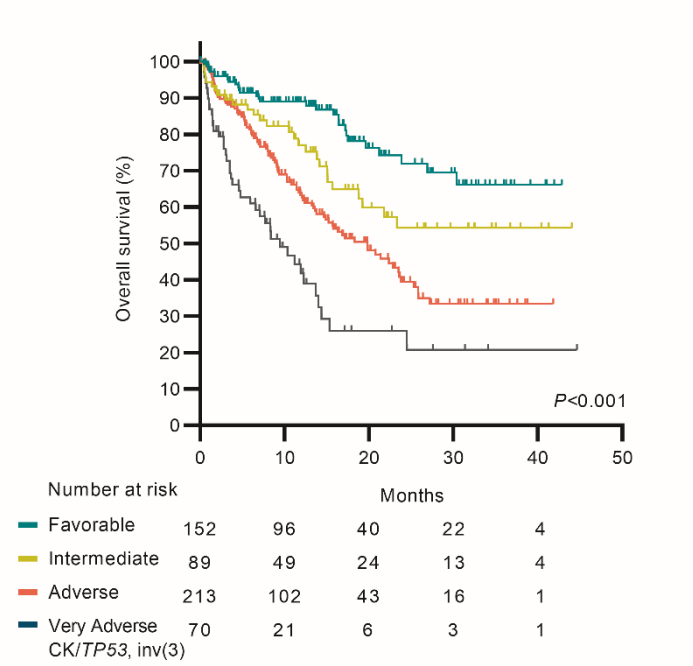


Figura 12. SG de acuerdo a la nueva propuesta de estratificación del riesgo descrita en la cohorte PETHEMA.

Las mutaciones en los genes MDS han adquirido mayor relevancia en los últimos estudios de las bases moleculares de la LMA, aunque su valor pronóstico carece de consenso. En la última clasificación genómica de la LMA se establece una asociación específica entre el valor pronóstico y el número de mutaciones en estos genes, sin embargo, la estratificación pronóstica de la ELN 2022 no hace referencia al respecto. En nuestra serie, observamos que los pacientes con solo un gen MDS mutado no alcanzaron la mediana de SG mientras que los pacientes con ≥2 mutaciones mostraron una SG de 13,6 meses (95%CI 9,0-18,1) (*P*=0,002). Cuando comparamos estos subgrupos con las categorías de riesgo, observamos que el pronóstico de los pacientes con una mutación MDS fue similar a la categoría de riesgo intermedio con tasas de SG a los 3 años de 57,6% y 59,6% (*P*=0,978), respectivamente. Los pacientes con ≥2 mutaciones mostraron una SG similar al grupo de riesgo adverso: tasas de SG a los 3 años: 25,7% y 30,6%; *P*=0,391) (Figura 13).

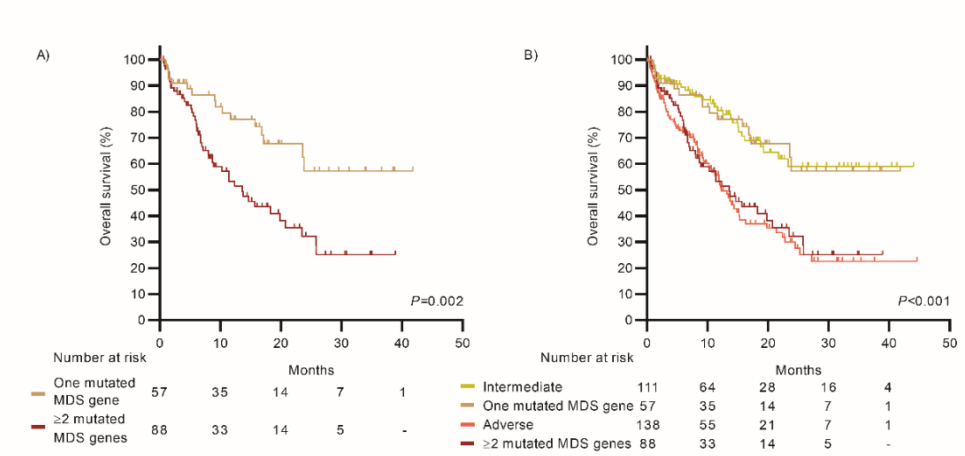


Figura 13. SG de los pacientes con mutaciones en genes MDS como única alteración clínicamente relevante. A) Comparativa según el número de mutaciones; y B) Comparativa con los grupos de riesgo intermedio y adverso.

El tercer artículo científico que conforma la presente tesis doctoral muestra la comparativa entre las clasificaciones de la ELN 2017 y la ELN 2022. Para ello, ambas clasificaciones se han aplicado a una serie de 546 pacientes tratados de forma intensiva de acuerdo a la práctica clínica habitual cuyo perfil molecular se ha estudiado mediante protocolos de NGS estandarizados en la plataforma diagnóstica definida por el grupo PETHEMA. Esto ha permitido validar las modificaciones introducidas en la ELN 2022 así como definir nuevos subgrupos con valor pronóstico independiente.

DISCUSIÓN

La LMA representa un ejemplo de la necesidad de disección molecular de la enfermedad con el fin de implementar una medicina de precisión capaz de mejorar el pronóstico en muchos pacientes. En la última década, el desarrollo de tecnologías de alto rendimiento ha provocado un notable progreso en la elucidación de los mecanismos moleculares de la LMA y ha promovido el desarrollo de nuevos algoritmos de clasificación y de estrategias de tratamiento adaptadas al riesgo. Sin embargo, las pruebas diagnósticas utilizadas habitualmente en la práctica clínica, se basan en técnicas dirigidas al estudio de un escaso número de genes con impacto diagnóstico o candidatos a terapia dirigida.

En este contexto, la NGS se ha establecido como una tecnología con gran potencial en la práctica clínica debido a su capacidad de analizar diversas alteraciones moleculares en un amplio número de genes. Su implementación en los laboratorios dedicados al diagnóstico molecular ha supuesto un importante reto debido al elevado coste, los requerimientos de número de muestras, la necesidad de equipamientos específicos y de personal cualificado. Además, las iniciativas individuales de trasladar estos estudios a la práctica clínica, carecen de procedimientos estandarizados, consenso de genes, criterios mínimos de calidad y requisitos del informe de resultados (Bacher et al., 2018).

El desarrollo de la plataforma nacional de estudios de NGS descrita en el presente proyecto de tesis, ha logrado poner en marcha una red cooperativa de diagnóstico molecular avanzado mediante el envío de muestras, de forma centralizada, a siete centros nacionales de referencia. Para tal fin, se ha implantado el primer control de calidad de NGS de ámbito nacional. Este control metodológico y las sucesivas rondas de validación han permitido armonizar los procedimientos de diagnóstico molecular y aumentar la tasa en la detección de variantes hasta alcanzar una concordancia global del 96,6% entre centros. Esta nueva estrategia ha supuesto un incremento notable en la accesibilidad a los estudios de NGS, calidad de las pruebas y homogenización de resultados en los pacientes con LMA (Sargas et al., 2021).

La consolidación de esta red cooperativa ha permitido obtener una exhaustiva caracterización molecular en una serie de 2668 pacientes tratados homogéneamente con los protocolos del grupo cooperativo PETHEMA. Los resultados del perfil mutacional mostraron que más del 96% de los pacientes presentaba al menos una mutación en alguno de los genes consenso (*ABL1, ASXL1, BCOR, BRAF, CALR, CBL, CEBPA, CSF3R, DNMT3A, ETV6, EZH2, FLT3, GATA2, HRAS, IDH1, IDH2, JAK2, KIT, KRAS, MPL, NPM1, NRAS, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, STAG2, TET2, TP53, U2AF1, WT1* y *ZRSR2*). Además, se detectaron numerosos

patrones de asociación y exclusividad entre genes y entre categorías funcionales lo que sugiere distintos mecanismos de cooperación en el desarrollo de la leucemia que además pueden modular la respuesta al tratamiento (Lachowiez et al., 2022; Qin et al., 2020).

El actual manejo clínico de la LMA requiere realizar un estudio molecular completo al momento del diagnóstico. Sin embargo, durante la recaída o refractariedad al tratamiento no existen recomendaciones que guíen el estudio molecular. Esto supone que, en muchos casos, se asume que el perfil molecular del diagnóstico se mantiene invariable en la recaída. Sin embargo, este trabajo en concordancia con otros recientes estudios, ha identificado diferentes perfiles mutacionales según el momento de la enfermedad (Ding et al., 2012; Niu et al., 2022; van Gils et al., 2021; Zhang et al., 2019). Se ha observado que el 49% de los pacientes con LMA refractaria mantiene el perfil mutacional identificado al diagnóstico, frente al 24% de los pacientes en recaída. En este sentido, la LMA se concibe como una enfermedad caracterizada por cambios dinámicos en su estructura clonal lo que es especialmente relevante a nivel de las alteraciones accionables. Por ejemplo, las mutaciones en el gen *FLT3* mostraron una baja estabilidad (<50%) entre el diagnóstico y la recaída remarcando la necesidad de repetir los estudios moleculares en los distintos momentos de la enfermedad (Daver et al., 2019). Asimismo, el análisis de la VAF de las variantes detectadas, mostró que las mutaciones en genes de las vías de señalización (*NRAS, KRAS, FLT3* y *PTPN11*) tenían los valores de VAF más bajos debido a su adquisición en fases tardías de la leucemogénesis y únicamente en subclonas leucémicas (di Nardo & Cortes, 2016). Es por ello, que disponer de estudios moleculares mediante NGS cobra especial importancia en el momento de la recaída con el fin de abordar de forma rigurosa el estudio de mutaciones subclonales.

A pesar del potencial de la NGS en la detección de alteraciones moleculares de distinta naturaleza, su aplicabilidad puede verse limitada para evaluar el impacto clínico de mutaciones en determinados genes. Las mutaciones bialélicas en *CEBPA* se han asociado numerosas veces con un pronóstico favorable (Li et al., 2015). De hecho, la nueva clasificación genómica también incluye la clase molecular la LMA con mutaciones *CEBPA*bi y las asocia con buen pronóstico. Sin embargo, recientes estudios han reportado que únicamente las mutaciones en el dominio bZIP se asocian con un pronóstico favorable independientemente de su estado mono- o bialélico (Taube et al., 2022; Wakita et al., 2022). Esto supone un punto muy controvertido, ya que la NGS no permite discernir de manera inequívoca si las alteraciones detectadas afectan a uno o ambos alelos del gen (Mannelli et al., 2017). Sin embargo, ambas categorías pueden considerarse solapantes en algunos casos, ya que diversos estudios sugieren que los pacientes con *CEBPA* doble mutado frecuentemente incluyen mutaciones en

el dominio C-terminal en el que se localiza bZIP (Lin et al., 2005; van Waalwijk van Doorn-Khosrovani et al., 2003). De hecho, el porcentaje de pacientes con *CEBPA*-bZIP (1,2%) obtenido en la serie de estudio fue similar al porcentaje de pacientes con mutaciones bialélicas descrito por Tazi et al., (1,8%).

Diversos estudios sugieren que la incidencia, perfil molecular y el pronóstico de los pacientes con LMA está influenciado por el sexo (Hellesøy et al., 2021). Los hombres generalmente presentan mayor incidencia de LMA y peor pronóstico. En este sentido, los resultados obtenidos en la presente tesis, mostraron que el perfil molecular en los hombres es similar al de los pacientes de edad avanzada y está caracterizado por alteraciones en genes del *splicing* (*SRSF2, U2AF1* y*, SF3B1*), reguladores epigenéticos (*IDH2*, *TET2*, *ASXL1* y *EZH2*), factores de transcripción (*RUNX1*) y supresores tumorales (*TP53*). Además, este perfil molecular se incluye mayoritariamente en el grupo de riesgo adverso de acuerdo a la última estratificación pronóstica de la ELN (Ayala et al., 2021; Buege et al., 2018; Döhner et al., 2022). Sin embargo, las mujeres y los pacientes jóvenes tienen un perfil molecular caracterizado por mayor frecuencia de mutaciones en los genes *FLT3* y *NPM1.*

Como consecuencia de la progresiva incorporación de los estudios de NGS en la práctica asistencial, existe una creciente necesidad de evaluar el impacto clínico de las alteraciones detectadas. Este fue el principal objetivo de la reciente clasificación genómica de la LMA, que ha revelado nuevos subgrupos moleculares asociados con distinta presentación clínica y valor pronóstico. Aplicar esta clasificación genómica a 954 pacientes de la cohorte PETHEMA mostró que la mayoría de los pacientes presentaban mutaciones en genes MDS (34,3%) siendo el subgrupo sAML2 (dos o más mutaciones) la clase molecular mayoritaria (25%). Estas alteraciones también han sido recientemente incluidas en la última edición de estratificación del riesgo de la ELN; sin embargo, no existe un consenso entre ambas propuestas. La clasificación genómica otorga valor pronóstico adverso únicamente a la presencia de dos o más mutaciones mientras que la clasificación de la ELN no indica ninguna diferencia al respecto. En este sentido, los resultados presentados en esta tesis sugieren un valor pronóstico dependiente del número de mutaciones. Los pacientes con una mutación presentaron mayor SG que los pacientes con dos o más mutaciones. Asimismo, esta clasificación genómica ha definido otras clases moleculares nuevas como mutaciones en el gen *TP53* y/o CC. Este subgrupo también mostró una alta representación en la cohorte de estudio (19,2%) y mostró valores de mediana de SG muy adversos (5,3 meses; 95%CI 2,9-7,6) en comparación con otras clases moleculares asociadas a mal pronóstico, debido a su alta

refractariedad a la terapia de inducción y su previamente establecido pronóstico adverso (Molica et al., 2021).

De manera muy similar a lo ocurrido en los sistemas de clasificación diagnóstica, la incorporación de un mayor número de marcadores moleculares en la estratificación del riesgo ha permitido refinar los grupos pronóstico. La estratificación del pronóstico propuesta por la ELN ha sido revisada recientemente en el año 2022. Esta nueva edición actualiza la propuesta del año 2017 y enfatiza la relevancia de las alteraciones moleculares, de tal manera que su aplicación requiere disponer de un estudio molecular completo realizado mediante NGS. Esto puede comprometer su aplicabilidad en la práctica clínica habitual de muchos centros y refuerza la necesidad de estudios cooperativos como el descrito en la presente tesis.

La validación realizada en la serie PETHEMA muestra que, en los pacientes tratados con quimioterapia intensiva, los criterios de la estratificación pronóstica de las ediciones ELN 2017 y ELN 2022 permiten discriminar entre los grupos de riesgo favorable, intermedio y adverso. El grupo de riesgo adverso fue el más representado en ambas clasificaciones, aunque se ha observado un incremento de 7,5% en la ELN 2022 debido principalmente a la incorporación de las mutaciones en genes MDS como marcadores de riesgo adverso. Estas mutaciones son altamente frecuentes en la LMA secundaria y en los pacientes con edad avanzada lo que explica que en nuestra serie más del 60% de los pacientes ≥65 años se clasifiquen en la categoría de riesgo adverso (Prassek et al., 2018; Wang et al., 2019). Por el contrario, la mayor frecuencia de mutaciones en los genes *NPM1* y *FLT3* en los pacientes jóvenes y en las mujeres explica su mayor pertenencia a los grupos de riesgo favorable e intermedio.

En nuestra serie, la edad avanzada se asoció con peor pronóstico independientemente del grupo de riesgo lo que sugiere menor aplicabilidad de la estratificación del riesgo en los pacientes ancianos (Appelbaum et al., 2006; Sekeres et al., 2020). Esto ha sido ampliamente descrito por distintos grupos cooperativos y estudios poblacionales que han demostrado que la edad avanzada al momento del diagnóstico está claramente asociada con un pronóstico adverso (de Moor et al., 2013; Juliusson et al., 2009; Pulte et al., 2008). De forma similar, los resultados obtenidos muestran que la aplicabilidad de la clasificación del riesgo propuesta por la ELN queda restringida a pacientes tratados con esquemas de tratamiento intensivo ya que los pacientes tratados de forma no intensiva presentan un pronóstico muy desfavorable independientemente del grupo de riesgo.

La nueva estratificación del riesgo ELN 2022 propone la clasificación de todos los pacientes con mutaciones *FLT3*-ITD en el grupo de riesgo intermedio independientemente del valor de la ratio alélica y de la concurrencia de mutaciones en el gen *NPM1*. Esto ha supuesto la reasignación de 4.6% de los pacientes clasificados en los grupos de riesgo favorable y adverso según la ELN 2017 al grupo de riesgo intermedio de la ELN 2022. Sin embargo, los recientes estudios que validan el valor pronóstico de la ratio de las mutaciones *FLT3*-ITD implican que este cambio sea uno de los puntos más controvertidos de la nueva propuesta ELN 2022 (Ayala et al., 2022).

Por otro lado, los resultados obtenidos muestran que los pacientes con mutaciones en *TP53* y/o CC y los pacientes con inv(3) pueden agruparse en una categoría de riesgo independiente con muy mal pronóstico. Este hallazgo ya había sido previamente descrito por (Herold et al., 2020) que sugirió un refinamiento de la clasificación ELN 2017, estratificando a los pacientes con mutaciones en *TP53* y/o CC como un subgrupo de riesgo muy adverso. Además, estos resultados son consistentes con los obtenidos en la validación de la clasificación genómica de la LMA en la que la mediana de SG de los pacientes con estas alteraciones fue muy inferior a la detectada en otras clases.

La inclusión de mutaciones en genes MDS como marcadores de riesgo adverso ha sido otro de los principales cambios introducidos en la clasificación ELN 2022. La falta de consenso respecto al valor pronóstico de estos marcadores entre la clasificación genómica de la LMA y la estratificación de la ELN, resalta la importancia de estudios adicionales que evalúen el impacto pronóstico de estas mutaciones. Nuestros resultados en la clasificación genómica de la LMA mostraron diferente pronóstico en función del número de mutaciones en los genes sAML. Este hallazgo ha sido revalidado al analizar los grupos de riesgo definidos en la ELN 2022, ya que los pacientes con una única mutación en genes MDS presentaron una SG similar a la de los pacientes del grupo de riesgo intermedio mientras que los pacientes con dos o más mutaciones MDS mostraron unos valores de SG correspondientes al grupo de riesgo adverso. Estos resultados apoyan las observaciones descritas por Tazi et al., de menor tasa de respuesta a la inducción y mayor beneficio del trasplante de progenitores hematopoyéticos en los pacientes con dos o más mutaciones MDS.

Los estudios de NGS desarrollados en el contexto de la red cooperativa PETHEMA objeto de la presente tesis doctoral, se han establecido como una herramienta imprescindible en la práctica clínica diaria para abordar la caracterización molecular de los pacientes con LMA. Su aplicación ha permitido generar gran cantidad de información genómica potenciando así la investigación traslacional.

CONCLUSIONES

1. Las rondas de validación han permitido definir un grupo de genes consenso relevantes para la clasificación diagnóstica y pronóstica de la LMA, unificar criterios de análisis y disminuir la variabilidad en los informes de resultados.
2. El 96,5% de los pacientes mostraron al menos una mutación con una media de 2,7 variantes/muestra. Los genes más frecuentemente mutados fueron *FLT3*, *DNMT3A*, *NPM1* y *TET2.*
3. Las mutaciones detectadas mostraron distintos patrones de asociación y exclusividad. Mutaciones en *FLT3*, *DNMT3A* y *NPM1* asociaron en todas sus combinaciones mientras que las mutaciones en *TP53* fueron altamente excluyentes.
4. Las mutaciones en genes reguladores epigenéticos mostraron los valores de VAF más elevados con alta estabilidad en los distintos momentos de la enfermedad. Mutaciones en los genes de vías de señalización presentaron los valores más bajos de VAF y fueron altamente inestables debido a su adquisición en etapas tardías de la leucemogénesis.
5. Se detectaron perfiles mutacionales asociados al momento de la enfermedad, sexo y edad de los pacientes.
6. De acuerdo a la clasificación genómica de la LMA, las clases moleculares sAML2, *NPM1*, y mutaciones en *TP53* y/o CC fueron las más frecuentes en la cohorte PETHEMA.
7. Nuestro estudio valida la utilidad pronóstica de las nuevas clases moleculares definidas en la clasificación genómica de la LMA y su unificación en tres grupos de riesgo con pronóstico favorable, intermedio y adverso.
8. Las clases moleculares “No eventos” y “Alteraciones que no definen clase” mostraron el mejor valor pronóstico mientras que “mutaciones en *TP53* y/o cariotipo complejo”, “sAML2” y “*WT1”* se asociaron a mal pronóstico.
9. La comparativa de las dos últimas propuestas de estratificación del riesgo (ELN 2017 y ELN 2022) ha supuesto la reclasificación del 14,5% de los pacientes y un incremento del grupo de riesgo adverso en un 7,5% debido principalmente al valor pronóstico otorgado a las mutaciones en genes MDS.
10. En ambas ediciones, la edad avanzada (≥65 años) se ha asociado a peor pronóstico independientemente de la categoría de riesgo.

11. Nuestra validación muestra que la estratificación pronóstica de la ELN 2022 no es aplicable a pacientes tratados de forma no intensiva.
12. El análisis de subgrupos genéticos sugiere la existencia de un grupo de riesgo muy adverso con valor pronóstico independiente que engloba a los pacientes con mutaciones en *TP53* y/o CC o inv(3).
13. El número de mutaciones en genes MDS confiere valor pronóstico. Los pacientes con una mutación pueden considerarse como grupo de riesgo intermedio, mientras que los pacientes con dos o más mutaciones se clasifican con el grupo de riesgo adverso.
14. La estandarización de los estudios de NGS en el contexto del grupo cooperativo PETHEMA ha proporcionado datos moleculares robustos que han definido el complejo paisaje molecular de la LMA estableciendo así a la NGS como una técnica esencial para abordar las actuales clasificaciones de diagnóstico y pronóstico con el fin de aplicar la medicina de precisión.

BIBLIOGRAFÍA

Alonso, C. M., Llop, M., Sargas, C., Pedrola, L., Panadero, J., Hervás, D., Cervera, J., Such, E., Ibáñez, M., Ayala, R., Martínez-López, J., Onecha, E., de Juan, I., Palanca, S., Martínez-Cuadrón, D., Rodríguez-Veiga, R., Boluda, B., Montesinos, P., Sanz, G., … Barragán, E. (2019). Clinical Utility of a Next-Generation Sequencing Panel for Acute Myeloid Leukemia Diagnostics. *The Journal of Molecular Diagnostics : JMD*, *21*(2), 228–240. https://doi.org/10.1016/J.JMOLDX.2018.09.009

Angenendt, L., Röllig, C., Montesinos, P., Martínez-Cuadrón, D., Barragan, E., García, R., Botella, C., Martínez, P., Ravandi, F., Kadia, T., Kantarjian, H. M., Cortes, J., Juliusson, G., Lazarevic, V., Höglund, M., Lehmann, S., Recher, C., Pigneux, A., Bertoli, S., … Schliemann, C. (2019). Chromosomal Abnormalities and Prognosis in NPM1-Mutated Acute Myeloid Leukemia: A Pooled Analysis of Individual Patient Data From Nine International Cohorts. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *37*(29), 2632–2642. https://doi.org/10.1200/JCO.19.00416

Appelbaum, F. R., Gundacker, H., Head, D. R., Slovak, M. L., Willman, C. L., Godwin, J. E., Anderson, J. E., & Petersdorf, S. H. (2006). Age and acute myeloid leukemia. *Blood*, *107*(9), 3481–3485. https://doi.org/10.1182/BLOOD-2005-09-3724

Arber, D. A., Orazi, A., Hasserjian, R. P., Borowitz, M. J., Calvo, K. R., Kvasnicka, H. M., Wang, S. A., Bagg, A., Barbui, T., Branford, S., Bueso-Ramos, C. E., Cortes, J. E., Dal Cin, P., DiNardo, C. D., Dombret, H., Duncavage, E. J., Ebert, B. L., Estey, E. H., Facchetti, F., … Tefferi, A. (2022). International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. *Blood*, *140*(11), 1200–1228. https://doi.org/10.1182/BLOOD.2022015850

Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., le Beau, M. M., Bloomfield, C. D., Cazzola, M., & Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*, *127*(20), 2391–2405. https://doi.org/10.1182/BLOOD-2016-03-643544

Ayala, R., Carreño-Tarragona, G., Barragán, E., Boluda, B., Larráyoz, M. J., Chillón, M. C., Carrillo-Cruz, E., Bilbao, C., Sánchez-García, J., Bernal, T., Martinez-Cuadron, D., Gil, C., Serrano, J., Rodriguez-Medina, C., Bergua, J., Pérez-Simón, J. A., Calbacho, M., Alonso-Domínguez, J. M., Labrador, J., … Montesinos, P. (2022). Impact of FLT3-ITD Mutation Status and Its Ratio in a Cohort of 2901 Patients Undergoing Upfront Intensive Chemotherapy: A PETHEMA Registry Study. *Cancers*, *14*(23). https://doi.org/10.3390/CANCERS14235799

Ayala, R., Rapado, I., Onecha, E., Martínez-Cuadrón, D., Carreño-Tarragona, G., Bergua, J. M., Vives, S., Algarra, J. L., Tormo, M., Martinez, P., Serrano, J., Herrera, P., Ramos, F., Salamero, O., Lavilla, E., Gil, C., Lorenzo, J. L. L., Vidriales, M. B., Labrador, J., … Montesinos, P. (2021). The Mutational Landscape of Acute Myeloid Leukaemia Predicts Responses and Outcomes in Elderly Patients from the PETHEMA-FLUGAZA Phase 3 Clinical Trial. *Cancers*, *13*(10). https://doi.org/10.3390/CANCERS13102458

Bacher, U., Shumilov, E., Flach, J., Porret, N., Joncourt, R., Wiedemann, G., Fiedler, M., Novak, U., Amstutz, U., & Pabst, T. (2018). Challenges in the introduction of next-generation sequencing (NGS) for diagnostics of myeloid malignancies into clinical routine use. *Blood Cancer Journal*, *8*(11). https://doi.org/10.1038/S41408-018-0148-6

Betz, B. L., & Hess, J. L. (2010). Acute myeloid leukemia diagnosis in the 21st century. *Archives of Pathology & Laboratory Medicine*, *134*(10), 1427–1433. https://doi.org/10.5858/2010-0245-RA.1

Bolandi, S. M., Pakjoo, M., Beigi, P., Kiani, M., Allahgholipour, A., Goudarzi, N., Khorashad, J. S., & Eiring, A. M. (2021). A Role for the Bone Marrow Microenvironment in Drug Resistance of Acute Myeloid Leukemia. *Cells*, *10*(11). https://doi.org/10.3390/CELLS10112833

Buege, M. J., DiPippo, A. J., & DiNardo, C. D. (2018). Evolving Treatment Strategies for Elderly Leukemia Patients with IDH Mutations. *Cancers 2018, Vol. 10, Page 187*, *10*(6), 187. https://doi.org/10.3390/CANCERS10060187

Creutzig, U., Kutny, M. A., Barr, R., Schlenk, R. F., & Ribeiro, R. C. (2018). Acute myelogenous leukemia in adolescents and young adults. *Pediatric Blood & Cancer*, *65*(9), e27089. https://doi.org/10.1002/PBC.27089

Daver, N., Schlenk, R. F., Russell, N. H., & Levis, M. J. (2019). Targeting FLT3 mutations in AML: review of current knowledge and evidence. In *Leukemia* (Vol. 33, Issue 2, pp. 299–312). Nature Publishing Group. https://doi.org/10.1038/s41375-018-0357-9

de Moor, J. S., Mariotto, A. B., Parry, C., Alfano, C. M., Padgett, L., Kent, E. E., Forsythe, L., Scoppa, S., Hachey, M., & Rowland, J. H. (2013). Cancer survivors in the United States: prevalence across the survivorship trajectory and implications for care. *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *22*(4), 561–570. https://doi.org/10.1158/1055-9965.EPI-12-1356

Desai, R. H., Zandvakili, N., & Bohlander, S. K. (2022). Dissecting the Genetic and Non-Genetic Heterogeneity of Acute Myeloid Leukemia Using Next-Generation Sequencing and In Vivo Models. *Cancers*, *14*(9). https://doi.org/10.3390/CANCERS14092182

Deschler, B., & Lübbert, M. (2006). Acute myeloid leukemia: epidemiology and etiology. *Cancer*, *107*(9), 2099–2107. https://doi.org/10.1002/CNCR.22233

di Nardo, C. D., & Cortes, J. E. (2016). Mutations in AML: prognostic and therapeutic implications. *Hematology*, *2016*(1), 348–355. https://doi.org/10.1182/ASHEDUCATION-2016.1.348

Ding, L., Ley, T. J., Larson, D. E., Miller, C. A., Koboldt, D. C., Welch, J. S., Ritchey, J. K., Young, M. A., Lamprecht, T., McLellan, M. D., McMichael, J. F., Wallis, J. W., Lu, C., Shen, D., Harris, C. C., Dooling, D. J., Fulton, R. S., Fulton, L. L., Chen, K., … Dipersio, J. F. (2012). Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*, *481*(7382), 506–510. https://doi.org/10.1038/NATURE10738

Döhner, H., Estey, E., Grimwade, D., Amadori, S., Appelbaum, F. R., Büchner, 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., … Bloomfield, C. D. (2017). Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*, *129*(4), 424–447. https://doi.org/10.1182/BLOOD-2016-08-733196

Döhner, H., Wei, A. H., Appelbaum, F. R., Craddock, C., DiNardo, C. D., Dombret, H., Ebert, B. L., Fenaux, P., Godley, L. A., Hasserjian, R. P., Larson, R. A., Levine, R. L., Miyazaki, Y., Niederwieser, D., Ossenkoppele, G., Röllig, C., Sierra, J., Stein, E. M., Tallman, M. S., … Löwenberg, B. (2022). Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. *Blood*, *140*(12), 1345–1377. https://doi.org/10.1182/BLOOD.2022016867

el Achi, H., & Kanagal-Shamanna, R. (2021). Biomarkers in Acute Myeloid Leukemia: Leveraging Next Generation Sequencing Data for Optimal Therapeutic Strategies. *Frontiers in Oncology*, *11*. https://doi.org/10.3389/FONC.2021.748250

Finch, S. C. (2007). Radiation-induced leukemia: lessons from history. *Best Practice & Research. Clinical Haematology*, *20*(1), 109–118. https://doi.org/10.1016/J.BEHA.2006.10.009

Goodwin, S., McPherson, J. D., & McCombie, W. R. (2016). Coming of age: ten years of next-generation sequencing technologies. *Nature Reviews. Genetics*, *17*(6), 333–351. https://doi.org/10.1038/NRG.2016.49

Grimwade, D., Hills, R. K., Moorman, A. v., Walker, H., Chatters, S., Goldstone, A. H., Wheatley, K., Harrison, C. J., & Burnett, A. K. (2010). Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood*, *116*(3), 354–365. https://doi.org/10.1182/BLOOD-2009-11-254441

Grimwade, D., Ivey, A., & Huntly, B. J. P. (2016). Molecular landscape of acute myeloid leukemia in younger adults and its clinical relevance. *Blood*, *127*(1), 29–41. https://doi.org/10.1182/BLOOD-2015-07-604496

Hellesøy, M., Engen, C., Grob, T., Löwenberg, B., Valk, P. J. M., & Gjertsen, B. T. (2021). Sex disparity in acute myeloid leukaemia with FLT3 internal tandem duplication mutations: implications for prognosis. *Molecular Oncology*, *15*(9), 2285. https://doi.org/10.1002/1878-0261.13035

Herold, T., Rothenberg-Thurley, M., Grunwald, V. v., Janke, H., Goerlich, D., Sauerland, M. C., Konstandin, N. P., Dufour, A., Schneider, S., Neusser, M., Ksienzyk, B., Greif, P. A., Subklewe, M., Faldum, A., Bohlander, S. K., Braess, J., Wörmann, B., Krug, U., Berdel, W. E., … Metzeler, K. H. (2020). Validation and refinement of the revised 2017 European LeukemiaNet genetic risk stratification of acute myeloid leukemia. *Leukemia*, *34*(12), 3161–3172. https://doi.org/10.1038/S41375-020-0806-0

Juliusson, G., Antunovic, P., Derolf, Å., Lehmann, S., Möllgård, L., Stockelberg, D., Tidefelt, U., Wahlin, A., & Höglund, M. (2009). Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. *Blood*, *113*(18), 4179–4187. https://doi.org/10.1182/BLOOD-2008-07-172007

Kamps, R., Brandão, R. D., van den Bosch, B. J., Paulussen, A. D. C., Xanthoulea, S., Blok, M. J., & Romano, A. (2017). Next-Generation Sequencing in Oncology: Genetic Diagnosis, Risk Prediction and Cancer Classification. *International Journal of Molecular Sciences*, *18*(2). https://doi.org/10.3390/IJMS18020308

Khoury, J. D., Solary, E., Abla, O., Akkari, Y., Alaggio, R., Apperley, J. F., Bejar, R., Berti, E., Busque, L., Chan, J. K. C., Chen, W., Chen, X., Chng, W. J., Choi, J. K., Colmenero, I., Coupland, S. E., Cross, N. C. P., de Jong, D., Elghetany, M. T., … Hochhaus, A. (2022). The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. *Leukemia*, *36*(7), 1703–1719. https://doi.org/10.1038/S41375-022-01613-1

Kumar, C. C. (2011). Genetic Abnormalities and Challenges in the Treatment of Acute Myeloid Leukemia. *Genes & Cancer*, *2*(2), 95. https://doi.org/10.1177/1947601911408076

Kuykendall, A., Duployez, N., Boissel, N., Lancet, J. E., & Welch, J. S. (2018). Acute Myeloid Leukemia: The Good, the Bad, and the Ugly. *American Society of Clinical Oncology Educational Book. American Society of Clinical Oncology. Annual Meeting*, *38*(38), 555–573. https://doi.org/10.1200/EDBK_199519

Lachowiez, C. A., Reville, P. K., Kantarjian, H., Jabbour, E., Borthakur, G., Daver, N., Issa, G., Furudate, K., Tanaka, T., Pierce, S., Tang, G., Patel, K. P., Medeiros, J., Abbas, H., Haddad, F., Hammond, D., Short, N. J., Maiti, A., Yilmaz, M., … DiNardo, C. D. (2022). Contemporary outcomes in IDH-mutated acute myeloid leukemia: The impact of co-occurring NPM1 mutations and venetoclax-based treatment. *American Journal of Hematology*. https://doi.org/10.1002/AJH.26694

Levine, R. L., & Valk, P. J. M. (2019). Next-generation sequencing in the diagnosis and minimal residual disease assessment of acute myeloid leukemia. *Haematologica*, *104*(5), 868. https://doi.org/10.3324/HAEMATOL.2018.205955

Ley, T. J., Miller, C., Ding, L., Raphael, B. J., Mungall, A. J., Robertson, G., Hoadley, K., Triche, T. J., Laird, P. W., Baty, J. D., Fulton, L. L., Fulton, R., Heath, S. E., Kalicki-Veizer, J., Kandoth, C., Klco, J. M., Koboldt, D. C., Kanchi, K. L., Kulkarni, S., … Wilson, R. K. (2013). Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *New England Journal of Medicine*, *368*(22), 2059–2074. https://doi.org/10.1056/NEJMoa1301689

Li, H. Y., Deng, D. H., Huang, Y., Ye, F. H., Huang, L. L., Xiao, Q., Zhang, B., Ye, B. B., Lai, Y. R., Mo, Z. N., & Liu, Z. F. (2015). Favorable prognosis of biallelic CEBPA gene mutations in acute myeloid leukemia patients: a meta-analysis. *European Journal of Haematology*, *94*(5), 439–448. https://doi.org/10.1111/EJH.12450

Lin, L. I., Chen, C. Y., Lin, D. T., Tsay, W., Tang, J. L., Yeh, Y. C., Shen, H. L., Su, F. H., Yao, M., Huang, S. Y., & Tien, H. F. (2005). Characterization of CEBPA mutations in acute myeloid leukemia: most patients with CEBPA mutations have biallelic mutations and show a distinct immunophenotype of the leukemic cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *11*(4), 1372–1379. https://doi.org/10.1158/1078-0432.CCR-04-1816

Llop, M., Sargas, C., & Barragán, E. (2022). The role of next-generation sequencing in acute myeloid leukemia. *Current Opinion in Oncology*, *34*(6), 723–728. https://doi.org/10.1097/CCO.0000000000000899

Ma, X., Shao, Y., Tian, L., Flasch, D. A., Mulder, H. L., Edmonson, M. N., Liu, Y., Chen, X., Newman, S., Nakitandwe, J., Li, Y., Li, B., Shen, S., Wang, Z., Shurtleff, S., Robison, L. L., Levy, S., Easton, J., & Zhang, J. (2019). Analysis of error profiles in deep next-generation sequencing data. *Genome Biology*, *20*(1), 1–15. https://doi.org/10.1186/S13059-019-1659-6/FIGURES/7

Mannelli, F., Ponziani, V., Bencini, S., Bonetti, M. I., Benelli, M., Cutini, I., Gianfaldoni, G., Scappini, B., Pancani, F., Piccini, M., Rondelli, T., Caporale, R., Gelli, A. M. G., Peruzzi, B., Chiarini, M., Borlenghi, E., Spinelli, O., Giupponi, D., Zanghì, P., … Bosi, A. (2017). CEBPA-double-mutated acute myeloid leukemia displays a unique phenotypic profile: a reliable screening method and insight into biological features. *Haematologica*, *102*(3), 529–540. https://doi.org/10.3324/HAEMATOL.2016.151910

Metzker, M. L. (2009). Sequencing technologies — the next generation. *Nature Reviews Genetics 2010 11:1*, *11*(1), 31–46. https://doi.org/10.1038/nrg2626

Molica, M., Mazzone, C., Niscola, P., & de Fabritiis, P. (2021). TP53 Mutations in Acute Myeloid Leukemia: Still a Daunting Challenge? *Frontiers in Oncology*, *10*. https://doi.org/10.3389/FONC.2020.610820

Morita, K., Wang, F., Jahn, K., Hu, T., Tanaka, T., Sasaki, Y., Kuipers, J., Loghavi, S., Wang, S. A., Yan, Y., Furudate, K., Matthews, J., Little, L., Gumbs, C., Zhang, J., Song, X., Thompson, E., Patel, K. P., Bueso-Ramos, C. E., … Takahashi, K. (2020). Clonal evolution of acute myeloid leukemia revealed by high-throughput single-cell genomics. *Nature Communications 2020 11:1*, *11*(1), 1–17. https://doi.org/10.1038/s41467-020-19119-8

Mosele, F., Remon, J., Mateo, J., Westphalen, C. B., Barlesi, F., Lolkema, M. P., Normanno, N., Scarpa, A., Robson, M., Meric-Bernstam, F., Wagle, N., Stenzinger, A., Bonastre, J., Bayle, A., Michiels, S., Bièche, I., Rouleau, E., Jezdic, S., Douillard, J. Y., … André, F. (2020). Recommendations for the use of next-generation sequencing (NGS) for patients with metastatic cancers: a report from the ESMO Precision Medicine Working Group. *Annals of Oncology*, *31*(11), 1491–1505. https://doi.org/10.1016/J.ANNONC.2020.07.014

Murphy, T., Zou, J., Daher-Reyes, G. S., Arruda, A., Gupta, V., McNamara, C. J., Minden, M. D., Schimmer, A. D., Sibai, H., Yee, K. W. L., Korulla, M., Stockley, T., Kamel-Reid, S., Maze, D., Tierens, A., Bratman, S. v., Schuh, A. C., & Chan, S. M. (2019). Impact of preleukemic mutations and their persistence on hematologic recovery after induction chemotherapy

for AML. *Blood Advances*, *3*(15), 2307–2311. https://doi.org/10.1182/BLOODADVANCES.2019000306

Niu, J., Peng, D., & Liu, L. (2022). Drug Resistance Mechanisms of Acute Myeloid Leukemia Stem Cells. *Frontiers in Oncology*, *12*. https://doi.org/10.3389/FONC.2022.896426

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., … Campbell, P. J. (2016). Genomic Classification and Prognosis in Acute Myeloid Leukemia. *New England Journal of Medicine*, *374*(23), 2209–2221. https://doi.org/10.1056/nejmoa1516192

Pervez, M. T., Hasnain, M. J. U., Abbas, S. H., Moustafa, M. F., Aslam, N., & Shah, S. S. M. (2022). A Comprehensive Review of Performance of Next-Generation Sequencing Platforms. *BioMed Research International*, *2022*. https://doi.org/10.1155/2022/3457806

Poynter, J. N., Richardson, M., Roesler, M., Blair, C. K., Hirsch, B., Nguyen, P., Cioc, A., Cerhan, J. R., & Warlick, E. (2017). Chemical exposures and risk of acute myeloid leukemia and myelodysplastic syndromes in a population-based study. *International Journal of Cancer*, *140*(1), 23–33. https://doi.org/10.1002/IJC.30420

Prassek, V. v., Rothenberg-Thurley, M., Sauerland, M. C., Herold, T., Janke, H., Ksienzyk, B., Konstandin, N. P., Goerlich, D., Krug, U., Faldum, A., Berdel, W. E., Wörmann, B., Braess, J., Schneider, S., Subklewe, M., Bohlander, S. K., Hiddemann, W., Spiekermann, K., & Metzeler, K. H. (2018). Genetics of acute myeloid leukemia in the elderly: mutation spectrum and clinical impact in intensively treated patients aged 75 years or older. *Haematologica*, *103*(11), 1853–1861. https://doi.org/10.3324/HAEMATOL.2018.191536

Pulte, D., Gondos, A., & Brenner, H. (2008). Improvements in survival of adults diagnosed with acute myeloblastic leukemia in the early 21st century. *Haematologica*, *93*(4), 594–600. https://doi.org/10.3324/HAEMATOL.12304

Qin, G., Ilya, S., Kim, T.-K., Tercan, B., Martins, T. J., Dai, J., Chien, S., Carson, A., Patay, B., Estey, E. H., Loeb, L. A., Monnat, R. J., & Becker, P. S. (2020). Co-Occurring Mutation Clusters Predict Drug Sensitivity in Acute Myeloid Leukemia. *Blood*, *136*(Supplement 1), 12–13. https://doi.org/10.1182/BLOOD-2020-142727

Sargas, C., Ayala, R., Chillón, M. C., Larráyoz, M. J., Carrillo-Cruz, E., Bilbao, C., Yébenes-Ramírez, M., Llop, M., Rapado, I., García-Sanz, R., Vázquez, I., Soria, E., Florido-Ortega, Y., Janusz, K., Botella, C., Serrano, J., Martínez-Cuadrón, D., Bergua, J., Amigo, M. L., … Montesinos, P. (2021). Networking for advanced molecular diagnosis in acute myeloid leukemia patients is possible: the PETHEMA NGS-AML project. *Haematologica*, *106*(12), 3079–3089. https://doi.org/10.3324/HAEMATOL.2020.263806

Sekeres, M. A., Guyatt, G., Abel, G., Alibhai, S., Altman, J. K., Buckstein, R., Choe, H., Desai, P., Erba, H., Hourigan, C. S., LeBlanc, T. W., Litzow, M., MacEachern, J., Michaelis, L. C., Mukherjee, S., O'Dwyer, K., Rosko, A., Stone, R., Agarwal, A., … Brignardello-Petersen, R. (2020). American Society of Hematology 2020 guidelines for treating newly diagnosed

acute myeloid leukemia in older adults. *Blood Advances*, *4*(15), 3528–3549. https://doi.org/10.1182/BLOODADVANCES.2020001920

Shallis, R. M., Wang, R., Davidoff, A., Ma, X., & Zeidan, A. M. (2019). Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges. *Blood Reviews*, *36*, 70–87. https://doi.org/10.1016/J.BLRE.2019.04.005

Swerdlow, S., Campo, E., Harris, N., Jaffe, E., & Pileri, S. (2008). *WHO classification of tumours of haematopoietic and lymphoid tissues*. https://hero.epa.gov/hero/index.cfm/reference/details/reference_id/786623

Taube, F., Georgi, J. A., Kramer, M., Stasik, S., Middeke, J. M., Röllig, C., Krug, U., Krämer, A., Scholl, S., Hochhaus, A., Brümmendorf, T. H., Naumann, R., Petzold, A., Mulet-Lazaro, R., Valk, P. J. M., Steffen, B., Einsele, H., Schaich, M., Burchert, A., … Thiede, C. (2022). CEBPA mutations in 4708 patients with acute myeloid leukemia: differential impact of bZIP and TAD mutations on outcome. *Blood*, *139*(1), 87–103. https://doi.org/10.1182/BLOOD.2020009680

Tazi, Y., Arango-Ossa, J. E., Zhou, Y., Bernard, E., Thomas, I., Gilkes, A., Freeman, S., Pradat, Y., Johnson, S. J., Hills, R., Dillon, R., Levine, M. F., Leongamornlert, D., Butler, A., Ganser, A., Bullinger, L., Döhner, K., Ottmann, O., Adams, R., … Papaemmanuil, E. (2022). Unified classification and risk-stratification in Acute Myeloid Leukemia. *Nature Communications*, *13*(1). https://doi.org/10.1038/S41467-022-32103-8

Tuval, A., & Shlush, L. I. (2019). Evolutionary trajectory of leukemic clones and its clinical implications. *Haematologica*, *104*(5), 872–880. https://doi.org/10.3324/HAEMATOL.2018.195289

Vakiti, A., & Mewawalla, P. (2022). Acute Myeloid Leukemia. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK507875/

van Gils, N., Denkers, F., & Smit, L. (2021). Escape From Treatment; the Different Faces of Leukemic Stem Cells and Therapy Resistance in Acute Myeloid Leukemia. *Frontiers in Oncology*, *11*. https://doi.org/10.3389/FONC.2021.659253

van Waalwijk van Doorn-Khosrovani, S. B., Erpelinck, C., Meijer, J., van Oosterhoud, S., van Putten, W. L. J., Valk, P. J. M., Beverloo, H. B., Tenen, D. G., Löwenberg, B., & Delwel, R. (2003). Biallelic mutations in the CEBPA gene and low CEBPA expression levels as prognostic markers in intermediate-risk AML. *The Hematology Journal : The Official Journal of the European Haematology Association*, *4*(1), 31–40. https://doi.org/10.1038/SJ.THJ.6200216

Vosberg, S., & Greif, P. A. (2019). Clonal evolution of acute myeloid leukemia from diagnosis to relapse. *Genes, Chromosomes & Cancer*, *58*(12), 839–849. https://doi.org/10.1002/GCC.22806

Wakita, S., Sakaguchi, M., Oh, I., Kako, S., Toya, T., Najima, Y., Doki, N., Kanda, J., Kuroda, J., Mori, S., Satake, A., Usuki, K., Ueki, T., Uoshima, N., Kobayashi, Y., Kawata, E., Tajika, K.,

Nagao, Y., Shono, K., … Yamaguchi, H. (2022). Prognostic impact of CEBPA bZIP domain mutation in acute myeloid leukemia. *Blood Advances*, *6*(1), 238–247. https://doi.org/10.1182/BLOODADVANCES.2021004292

Wang, S. Y., Cheng, W. Y., Mao, Y. F., Zhu, Y. M., Liu, F. J., Ma, T. T., & Shen, Y. (2019). Genetic alteration patterns and clinical outcomes of elderly and secondary acute myeloid leukemia. *Hematological Oncology*, *37*(4), 456–463. https://doi.org/10.1002/HON.2656

Welch, J. S., Ley, T. J., Link, D. C., Miller, C. A., Larson, D. E., Koboldt, D. C., Wartman, L. D., Lamprecht, T. L., Liu, F., Xia, J., Kandoth, C., Fulton, R. S., McLellan, M. D., Dooling, D. J., Wallis, J. W., Chen, K., Harris, C. C., Schmidt, H. K., Kalicki-Veizer, J. M., … Wilson, R. K. (2012). The origin and evolution of mutations in acute myeloid leukemia. *Cell*, *150*(2), 264–278. https://doi.org/10.1016/J.CELL.2012.06.023

Xiang, W., Lam, Y. H., Periyasamy, G., & Chuah, C. (2022). Application of High Throughput Technologies in the Development of Acute Myeloid Leukemia Therapy: Challenges and Progress. *International Journal of Molecular Sciences*, *23*(5). https://doi.org/10.3390/IJMS23052863

Yi, M., Li, A., Zhou, L., Chu, Q., Song, Y., Wu, K., & Wu, K. (2020). The global burden and attributable risk factor analysis of acute myeloid leukemia in 195 countries and territories from 1990 to 2017: estimates based on the global burden of disease study 2017. *Journal of Hematology & Oncology*, *13*(1). https://doi.org/10.1186/S13045-020-00908-Z

Zhang, J., Gu, Y., & Chen, B. (2019). Mechanisms of drug resistance in acute myeloid leukemia. *OncoTargets and Therapy*, *12*, 1937–1945. https://doi.org/10.2147/OTT.S191621

DOCUMENTOS ANEXOS

COMPENDIO ARTÍCULOS ORIGINALES

ARTICLE

Networking for advanced molecular diagnosis in acute myeloid leukemia patients is possible: the PETHEMA NGS-AML project

Claudia Sargas,[1] Rosa Ayala,[2] María Carmen Chillón,[3] María J. Larráyoz,[4] Estrella Carrillo-Cruz,[5] Cristina Bilbao,[6] Manuel Yébenes-Ramírez,[7] Marta Llop,[1] Inmaculada Rapado,[2] Ramón García-Sanz,[3] Iria Vázquez,[4] Elena Soria,[5] Yanira Florido-Ortega,[6] Kamila Janusz,[7] Carmen Botella,[8] Josefina Serrano,[7] David Martínez-Cuadrón,[9,10] Juan Bergua,[11] Mari Luz Amigo,[12] Pilar Martínez-Sánchez,[2] Mar Tormo,[13] Teresa Bernal,[14] Pilar Herrera-Puente,[15] Raimundo García,[16] Lorenzo Algarra,[17] María J. Sayas,[18] Lisette Costilla-Barriga,[19] Esther Pérez-Santolalla,[20] Inmaculada Marchante,[21] Esperanza Lavilla-Rubira,[22] Víctor Noriega,[23] Juan M. Alonso-Domínguez,[24] Miguel Á. Sanz,[9,10] Joaquín Sánchez-Garcia,[7] María T. Gómez-Casares,[6] José A. Pérez-Simón,[5] María J. Calasanz,[4] Marcos González-Díaz,[3] Joaquín Martínez-López,[2] Eva Barragán,[1,10#] and Pau Montesinos,[9,10#] on behalf of the PETHEMA group

[1]Molecular Biology Unit, Hospital Universitari i Politècnic-IIS La Fe, Valencia; [2]Hematology Department, Hospital Universitario 12 de Octubre, CNIO, Complutense University, Madrid; [3]Hospital Universitario de Salamanca (HUS/IBSAL), CIBERONC and Center for Cancer Research-IBMCC (USAL/CSIC), Salamanca; [4]CIMA LAB Diagnostics-Universidad de Navarra, Pamplona; [5]Hospital Universitario Virgen del Rocío, Instituto de Biomedicina (IBIS/CSIC/CIBERONC), Universidad de Sevilla, Sevilla; [6]Hospital Universitario de Gran Canaria Dr. Negrín, Las Palmas de Gran Canaria; [7]IMIBIC, Hematology, Hospital Universitario Reina Sofía, UCO, Córdoba; [8]Hospital General Universitario de Alicante, Alicante; [9]Hematology Department, Hospital Universitari i Politécnic-IIS La Fe, Valencia; [10]CIBERONC Instituto de Salud Carlos III, Madrid; [11]Hospital Universitario San Pedro de Alcántara, Cáceres; [12]Hospital Universitario Morales Messeguer, Murcia; [13]Hematology Department, Hospital Clínico Universitario-INCLIVA, Valencia; [14]Hospital Universitario Central de Asturias, Oviedo; [15]Hospital Universitario Ramón y Cajal, Madrid; [16]Hospital Universitari General de Castelló, Castellón; [17]Hospital Universitario General de Albacete, Albacete; [18]Hospital Univerisitario Dr. Peset, Valencia; [19]Hospital Universitario Miguel Servet, Zaragoza; [20]Hospital de Donosti, San Sebastián; [21]Hospital Universitario Puerta del Mar, Cádiz; [22]Complexo Hospitalario Lucus Augusti, Lugo; [23]Complexo Hospitalario Universitario A Coruña, A Coruña and [24]Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain

#EB and PM contributed equally as co-senior authors.

Ferrata Storti Foundation

Haematologica 2021
Volume 106(12):3079-3089

ABSTRACT

Next-generation sequencing (NGS) has recently been introduced to efficiently and simultaneously detect genetic variations in acute myeloid leukemia (AML). However, its implementation in the clinical routine raises new challenges focused on the diversity of assays and variant reporting criteria. In order to overcome this challenge, the PETHEMA group established a nationwide network of reference laboratories aimed to deliver molecular results in the clinics. We report the technical cross-validation results for NGS panel genes during the standardization process and the clinical validation in 823 samples of 751 patients with newly diagnosed or refractory/relapse AML. Two cross-validation rounds were performed in seven nationwide reference laboratories in order to reach a consensus regarding quality metrics criteria and variant reporting. In the pre-standardization cross-validation round, an overall concordance of 60.98% was obtained with a great variability in selected genes and conditions across laboratories. After consensus of relevant genes and optimization of quality parameters the overall concordance rose to 85.57% in the second cross-validation round. We show that a diagnostic network with harmonized NGS analysis and reporting in seven experienced laboratories is feasible in the context of a scientific group. This cooperative nationwide strategy provides advanced molecular diagnostic for AML patients of the PETHEMA group (clinicaltrials gov. Identifier: NCT03311815).

Correspondence:

EVA BARRAGÁN GONZÁLEZ
barragan_eva@gva.es

Received: June 24, 2020.

Accepted: November 3, 2020.

Pre-published: November 12, 2020.

https://doi.org/10.3324/haematol.2020.263806

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by a wide spectrum of molecular alterations that lead malignant transformation of normal hematopoietic cells.[1] The relevance of chromosomal alterations and gene variants for diagnosis, risk stratification and choice of targeted therapies (i.e, *FLT3* and *IDH1/2* inhibitors) has remarkably increased the complexity of routine molecular diagnostic strategies.[2–5] Next-generation sequencing (NGS) has been established as a new molecular diagnostic tool rapidly adopted by clinical laboratories, being able to simultaneously assess different genetic alterations such as rearrangements, single nucleotide variants (SNV), insertions-deletions (*indels*) and copy number variations (CNV) in a wide variety of genes.[6] NGS gene panels have been preferentially adopted rather than whole genome or exome sequencing due to an easier results interpretation, lower cost and less time consumption, as well as higher read deep needed for low frequency variant detection. Compared to NGS, conventional single-gene approaches by polymerase chain reaction (PCR)[7] are laborious, time-consuming and less efficient to detect minor clones, but they are still needed as rapid-screening tests for druggable variants.[8]

The new scenario for AML molecular diagnosis, requiring rapid screening by conventional PCR and comprehensive characterization by NGS, is a great challenge for molecular biology laboratories. For this purpose, the PETHEMA (*Programa Español de Tratamientos en Hematología*) group established a nationwide network involving seven central laboratories aimed to deliver molecular results to clinics for newly diagnosed and relapsed/refractory AML patients. The first step was to ensure appropriate logistic support, including geographical localization of highly skilled central laboratories strategically distributed according to population density and distance. The second step was to harmonize NGS and PCR techniques methodology and result reporting across the seven central laboratories, establishing consensus panel genes, quality metrics cutoffs and variant reporting criteria.

In this work, we performed the first analysis of a NGS-AML study (clinicaltrials gov. Identifier: NCT03311815), reporting the technical cross-validation results for NGS panel genes during the standardization process and the clinical validation in 823 samples of 751 patients with newly diagnosed or refractory/relapse AML.

Methods

Study design and reference laboratories

This was a prospective, multi-center, non-interventional study, performed in seven Spanish PETHEMA central laboratories: Hospital Universitario La Fe (HULF, Valencia), Hospital Universitario de Salamanca (HUS, Salamanca), Hospital Universitario 12 de Octubre (H12O, Madrid), Hospital Universitario Virgen del Rocío (HUVR, Sevilla), Hospital Universitario Reina Sofía (HURS, Córdoba), Hospital Universitario de Gran Canaria Dr. Negrín (HUDN, Las Palmas de Gran Canaria) and CIMA LAB Diagnostics (UNAV, Pamplona) (see the *Online Supplementary Appendix* for further details).

Inclusion criteria

All adult patients (≥18 years) with newly diagnosed or relapsed/refractory AML (excluding acute promyelocytic leukemia) according to the World Health Organization criteria (2008), regardless of the treatment received, were eligible for the NGS-AML study. The Institutional Ethics Committee for Clinical Research of each institution approved this study. Written informed consent in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki, and institutional regulations were obtained from all patients.

Cross-validation

The first cross-validation round was developed to evaluate the starting situation of reference laboratories (see the *Online Supplementary Appendix*). For this purpose, four samples harboring 24 variants were distributed from HULF (coordinator center) and each laboratory carried out NGS analysis according to their already implemented protocols. Reports were sent to the coordinator center to analyze the results.

Taking into account the obtained results, the collaborative group established a set of relevant AML genes and minimum quality metrics criteria. Then, a second cross-validation round was designed to strengthen the established quality parameters, the consensus recommendations, and variant reporting for NGS analysis among the seven reference laboratories. Variant detection, variant allele frequency (VAF), dispersion among centers and variant reporting (clinically and non-clinically relevant variants) were assessed in six samples with 30 variants (five with a lower VAF than 5%). Reports were sent to the coordinator center to analyze the results.

Consensus genes establishment

Thrirty genes were established as key genes for AML pathogenesis: *ABL1*, *ASXL1*, *BRAF*, *CALR*, *CBL*, *CEBPA*, *CSF3R*, *DNMT3A*, *ETV6*, *EZH2*, *FLT3*, *GATA2*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *KIT*, KRAS, *MPL*, *NPM1*, *NRAS*, *PTPN11*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, *U2AF1* and *WT1*. *ASXL1*, *CEBPA*, *FLT3*, *IDH1*, *IDH2*, *NPM1*, *RUNX1*, and *TP53* were mandatory for their implication in clinical guidelines, targeted therapy and risk stratification. The remaining genes were recommended for NGS panels, according to laboratory features and sequencing panel options.

Sequencing platforms and panels

The sequencing platform and panel were selected by each laboratory using the following criteria: i) to include all eight mandatory genes and, ii) to include the maximum number of other 22 relevant genes (sequencing platforms and panels data are shown in the *Online Supplementary Appendix* and *Online Supplementary Table S1*).

Clinical validation

NGS was performed according to already implemented protocols and the consensus parameters established in both cross-validation rounds. Samples meeting the quality metrics criteria established in previous standardization rounds were considered in the clinical validation.

Statistical analyses

All statistics were performed using SPSS version 22 (IBM, Armonk, NY, USA) and GraphPad Prism 4 (GraphPad, La Jolla, CA, USA) software programs. A *P*-value (*P*) <0.05 was considered statistically significant (see the *Online Supplementary Appendix*).

Results

Cross-validation

In the first cross-validation round (pre-standardization),

we observed a great diversity in the included genes in each NGS panel. Some AML relevant genes such as *NPM1* and *CEBPA* were not studied, while other non-relevant genes for AML pathogenesis were included. The global error rate was 39.02% with a higher error rate in variants showing a VAF lower than 10% (77.04 ± 6.98% *vs.* 18.56 ± 29.24%, *P*<0.001) (Table 1).

In the second round (post-standardization), the mean read depth was 4,032 (range, 1,463-4,532) with a median uniformity of 98.34%. The error rate for all variants was reduced to 14.43% (Table 2). In this round, the error rate was significantly higher in variants with a VAF lower than 5% (28.57 ± 14.28% *vs.* 12.27 ± 14.39%, *P*=0.031) (Figure 1). All centers provided a correct clinical classification of the detected variants. No differences in the error rate were observed between *indels* and SNV. Regarding the accuracy of VAF determination among the different centers, VAF standard deviation (SD) was higher in *indel* variants than SNV (5.44 ± 2.80 *vs.* 2.15 ± 2.03, *P*=0.001).

After cross-validation results, uniformity (>85%) and mean read depth of 1,000X were established as quality control parameters for a valid NGS assay. Synonymous, intronic and polymorphic variants (minor allele frequency [MAF] ≥1% and/or included in the dbSNP database) were filtered out. VAF ≥5% was established as a cutoff value for variant reporting with the exception of pathogenic variants with strong clinical evidence which were reported with a VAF ≥1% (e.g, *TP53* or *FLT3*). Variants accomplishing all these requirements were considered.

Platform performance

The performance of the NGS platforms (Ion Torrent vs. Illumina) regarding the error rate and the VAF SD was assessed after the results of the standardization rounds were obtained. No significant differences were observed when analyzing all the included variants or in any of the sub groups (*Indel*, SNV, variants with VAF ≤5% and variants with VAF >5%) (*Online Supplementary Table S2*).

Clinical validation

From October 2017 to October 2019 a total of 823 samples from 751 AML patients were sent to the laboratory network. Disease status at sample collection was: newly diagnosis (DX) (n=639), refractoriness (RS) (n=82), and relapse (RP) (n=102). Patient characteristics are summarized in Table 3. NGS was performed according to already implemented protocols and the consensus parameters established in both cross-validation rounds.

Mutation distribution

A total of 2,052 variants were reported in the 823 samples, with 90.81% of patients showing at least one mutated gene (*Online Supplementary Figure S1A*). The mean number of variants per sample was 2.49 (range, 0-8). Most patients had three variants (24.37%), followed by patients with two (21.04%) and one (20.77%) variants, respectively (*Online Supplementary Figure S1B*).

A high frequency of variants in genes involved in signal transduction and epigenetic regulation was observed. *FLT3* (24.06%: *FLT3* internal tandem duplications [ITD] 16.52%, *FLT3*-point mutations [PM] 8.87%) was the most prevalent mutated gene followed by *IDH* (22.60%: *IDH1* 9.11%, *IDH2* 13.85%), *DNMT3A* (21.63%) and *NPM1* (21.51%) (*Online Supplementary Figure S2*).

Co-mutations

FLT3, *NPM1* and *DNMT3A* were significantly co-mutated for all combinations (*P*<0.001). *PTPN11* variants also

Table 1. First cross-validation round results.

ID	Gene	Coding	Protein	Detected	Included	Error Rate	Mean VAF	SD
1	*NPM1* (NM_002520)	c.860_863dup	p.Trp288Cysfs*12	5	5	0.00%	41.68%	18.90%
	IDH2 (NM_002168.3)	c.419G>A	p.Arg140Gln	6	6	0.00%	44.73%	3.31%
	DNMT3A (NM_022552)	c.2645G>A	p.Arg882His	6	6	0.00%	43.77%	1.92%
	STAG2 (NM_001042749.2)	c.2124del	p.Leu708Phefs*9	1	3	66.67%	NA	NA
	RUNX1 (NM_001754.4)	c.736A>C	p.Thr246Pro	1	6	83.33%	NA	NA
	ASXL1 (NM_015338.5)	c.1934dup	p.Gly646Trpfs*12	1	6	83.33%	NA	NA
2	*CEBPA* (NM_004364.4)	c.68_78del	p.Pro23Glnfs*81	4	5	20.00%	51.32%	7.17%
	CEBPA (NM_004364.4)	c.895A>G	p.Ser299Gly	5	5	0.00%	45.32%	3.86%
	IDH2 (NM_002168.3)	c.419G>A	p.Arg140Gln	6	6	0.00%	49.82%	5.22%
	NRAS (NM_002524.4)	c.37G>C	p.Gly13Arg	6	6	0.00%	46.32%	2.01%
	EZH2 (NM_004456.4)	c.952del	p.Thr318Glnfs*3	3	4	25.00%	47.54%	2.16%
	EZH2 (NM_004456.4)	c.1321G>A	p.Glu441Lys	4	4	0.00%	50.13%	2.81%
	DNMT3A (NM_022552)	c.1961G>A	p.Gly654Asp	1	6	83.33%	NA	NA
	KMT2A (NM_001197104.1)	c.3253G>A	p.Val1085Met	1	4	75.00%	NA	NA
	GATA2 (NM_032638.4)	c.1084C>T	p.Arg362*	1	5	80.00%	NA	NA
	ASXL1 (NM_015338.5)	c.1934dup	p.Gly646Trpfs*12	2	6	66.67%	39.82%	4.41%
3	*DNMT3A* (NM_022552)	c.2678G>C	p.Trp893Ser	6	6	0.00%	44.54%	2.32%
	TP53 (NM_000546.5)	c.652_670del	p.Val218fs	5	6	16.67%	67.58%	19.42%
	STAG2 (NM_001042749.2)	c.2858G>A	p.Arg953Gln	1	3	66.67%	NA	NA
	CUX1 (NM_181552.4)	c.1588A>C	p.Lys530Gln	1	3	66.67%	NA	NA
	ASXL1 (NM_015338.5)	c.1934dup	p.Gly646Trpfs*12	1	6	83.33%	NA	NA
4	*TP53* (NM_000546.5)	c.392A>T	p.Asn131Ile	6	6	0.00%	47.33%	1.83%
	EZH2 (NM_004456.4)	c.553G>C	p.Asp185His	1	4	75.00%	NA	NA
	ASXL1 (NM_015338.5)	c.1934dup	p.Gly646Trpfs*12	1	6	83.33%	NA	NA

Detected: number of centers which have detected the mutation; Included: number of centers which include each variant in its next-generation sequencing assay; Error Rate: number of centers which failed to detect the variant regarding the total of centers; VAF: variant allele frequency; SD: standard deviation of VAF establishment among centers; NA: not applicable; variants only were detected by one center.

Table 2. Second cross-validation round results.

ID		Gene	Coding	Protein	Detected	Included	Error Rate	Mean VAF	SD
1	CRV	*NPM1* (NM_002520)	c.863_864insCCTG	p.Trp288Cysfs*12	7	7	0.00%	34.88%	8.47%
		FLT3 (NM_004119.2)	c.1801_1802ins30	p.Asp600_Leu601ins10	7	7	0.00%	29.62%	9.32%
		FLT3 (NM_004119.2)	c.2505T>A	p.Asp835Glu	5	7	28.57%	2.46%	0.53%
	NCRV	*PHF6* (NM_032458.2)	c.548C>T	p.Ser183Phe	4	6	33.33%	50.32%	2.23%
		DNMT3A (NM_022552)	c.2264T>C	p.Phe755Ser	7	7	0.00%	42.10%	5.85%
		NRAS (NM_002524.4)	c.34G>A	p.Gly12Ser	4	7	42.86%	1.75%	0.30%
2	CRV	*RUNX1* (NM_001754.4)	c.1306dupT	p.Ser436Phefs*164	6	7	14.29%	43.24%	4.33%
		IDH1 (NM_005896.3)	c.394C>T	p.Arg132Cys	7	7	0.00%	16.25%	2.31%
	NCRV	*TET2* (NM_001127208.2)	c.3866G>T	p.Cys1289Phe	7	7	0.00%	43.02%	6.82%
		PHF6 (NM_032458.2)	c.346C>T	p.Arg116*	4	5	20.00%	42.25%	1.90%
		EZH2 (NM_004456.4)	c.2255G>C	p.*752Ser	7	7	0.00%	10.39%	1.59%
		SRSF2 (NM_003016.4)	c.161C>T	p.Ser54Phe	6	7	14.29%	5.73%	0.66%
		JAK2 (NM_004972.3)	c.1849G>T	p.Val617Phe	6	7	14.29%	2.73%	0.66%
3	CRV	*FLT3* (NM_004119.2)	c.2028C>G	p.Asn676Lys	5	7	28.57%	22.17%	3.63%
		FLT3 (NM_004119.2)	c.2504A>C	p.Asp835Ala	6	7	14.29%	5.40%	0.62%
	NCRV	*SH2B3* (NM_005475.2)	c.557G>T	p.Ser186Ile	2	3	33.33%	56.40%	0.57%
4	CRV	*PHF6* (NM_032458.2)	c.129_130insGG	p.Lys44Glyfs*38	4	5	20.00%	49.44%	3.48%
		EZH2 (NM_004456.4)	c.2212_2231del	p.Ala738Argfs*18	6	7	14.29%	29.49%	6.96%
	NCRV	*NRAS* (NM_002524.4)	c.35G>A	p.Gly12Asp	7	7	0.00%	17.08%	2.33%
		EZH2 (NM_004456.4)	c.796G>A	p.Gly266Arg	4	7	42.86%	4.86%	0.93%
5	CRV	*ASXL1* (NM_015338.5)	c.1772dup	p.Tyr591*	7	7	0.00%	21.69%	2.32%
		ASXL1 (NM_015338.5)	c.1745_1758del	p.Pro582Argfs*32	6	7	14.29%	13.96%	2.87%
		TP53 (NM_000546.5)	c.916C>T	p.Arg306*	6	7	14.29%	4.49%	0.28%
	NCRV	*SF3B1* (NM_012433.3)	c.1873C>T	p.Arg625Cys	6	7	14.29%	8.02%	1.06%
6	CRV	*RUNX1* (NM_001754.4)	c.593A>G	p.Asp198Gly	7	7	0.00%	77.98%	2.75%
		ASXL1 (NM_015338.5)	c.2463_2478del	p.Asp821Glufs*12	7	7	0.00%	40.47%	8.29%
	NCRV	*ASXL1* (NM_015338.5)	c.2537G>A	p.Ser846Asn	7	7	0.00%	54.70%	6.82%
		SF3B1 (NM_012433.3)	c.2098A>G	p.Lys700Glu	6	7	14.29%	47.47%	1.85%
		CSF3R (NM_156039.3)	c.1853C>T	p.Thr618Ile	6	7	14.29%	47.06%	1.50%
		CSF3R (NM_156039.3)	c.2346dup	p.Ser783Glnfs*6	3	7	57.14%	37.77%	2.92%

Detected: number of centers which have detected the mutation; Included: number of centers which include each variant in its next-generation sequencing assay; Error Rate: number of centers which failed to detect the variant regarding the total of centers; VAF: variant allele frequency; SD: standard deviation of VAF establishment among centers; CRV: clinically-relevant variants; NCRV: non-clinically relevant variants.

associated with these genes (P<0.01 for *NPM1* and P<0.05 for *DNMT3A* and *FLT3*). *IDH* variants associated with *NPM1* (P=0.01 for *IDH1* and P<0.01 for *IDH2*) as well as *DNMT3A* (P<0.01 for *IDH1* and P<0.05 for *IDH2*). *SRSF2* was strongly co-mutated with *IDH2* and *TET2* (P<0.01) (Figure 2).

Exclusion analysis provided 17 MEGS which are defined as lists of exclusive mutated genes. *NPM1*, *TP53*, *RUNX1* and *KIT* set was defined as the most significant mutually exclusive gen set (p-nominal: 2,25E-24). *TP53* was present in all MEGS being the most exclusive gene. *RUNX1* was highly exclusive with *NPM1*. *CEBPA* and *NRAS* were included in several MEGS of three blocks suggesting their exclusive nature (*Online Supplementary Figure S3*; *Online Supplementary Table S3*).

Variant allele frequency analysis

Variants in genes involved in signaling pathways (*NRAS*, *FLT3*, *KIT*, *KRAS*, and *PTPN11*) showed lower median VAF, whereas genes involved in clonal hematopoiesis of indeterminate potential (CHIP) (*ASXL1*, *DNMT3A* and *TET2*) showed a VAF around 50%. *TP53* variants showed the highest median VAF value (Figure 3A). According to the functional categories, epigenetically related genes and tumor suppressor genes were characterized by a high VAF while genes related to signaling pathways genes showed the lowest VAF (Figure 3B).

Mutational landscape according to disease stage

NPM1 and *PTPN11* variants were more frequent at diagnosis (*NPM1* 23.16% *vs*. 9.76%, P=0.004, and *PTPN11* 6.62% *vs*. 1.10%, P=0.048). *RUNX1* and *SF3B1* variants were more frequent in refractory as compared to diagnosis (*RUNX1* 30.49% *vs*. 16.43%, P=0.003 and *SF3B1* 9.76% *vs*. 4.23%, P=0.049). *IDH1*, *IDH2* and *WT1* variants were more frequent at relapse as compared to

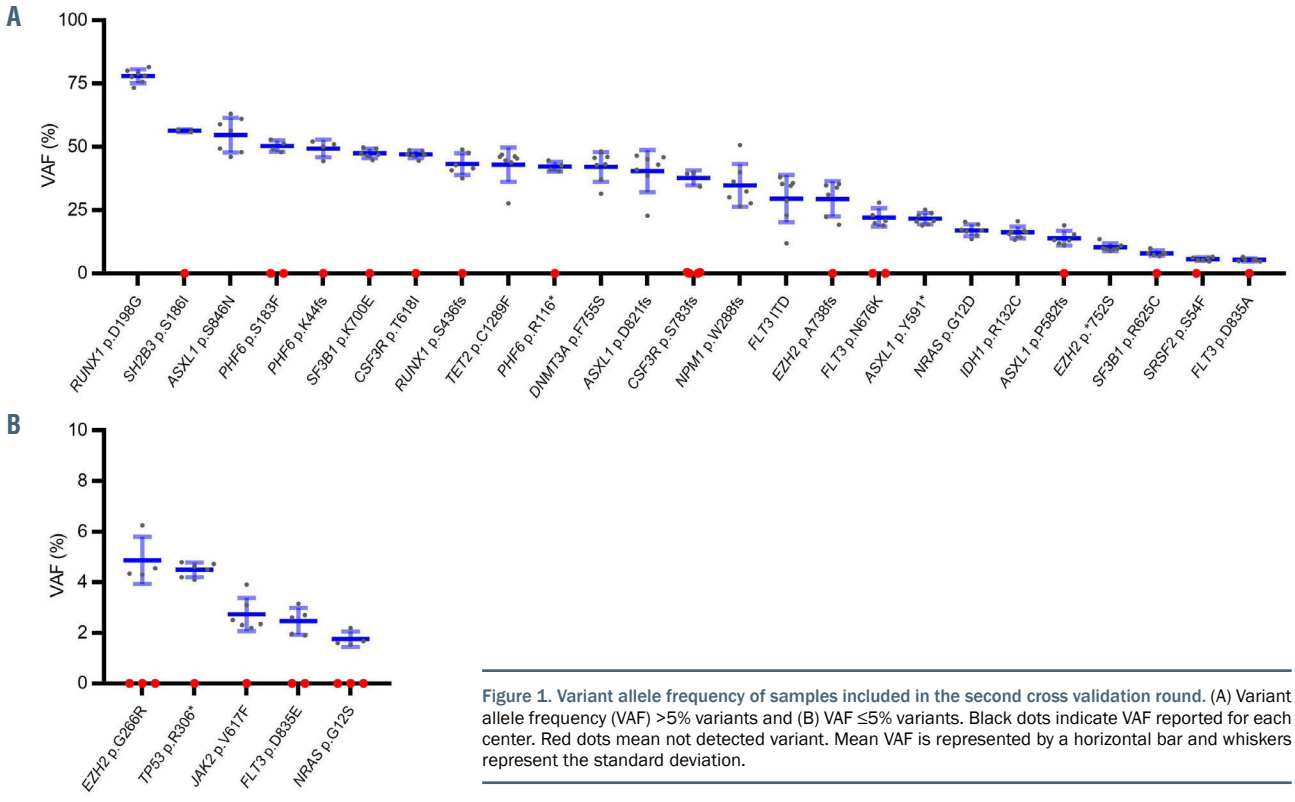


Figure 1. Variant allele frequency of samples included in the second cross validation round. (A) Variant allele frequency (VAF) >5% variants and (B) VAF ≤5% variants. Black dots indicate VAF reported for each center. Red dots mean not detected variant. Mean VAF is represented by a horizontal bar and whiskers represent the standard deviation.

diagnosis (*IDH1* 14.71% *vs.* 8.14%, *P*=0.040; *IDH2* 21.57% *vs.* 12.52%, *P*=0.020, and *WT1*: 7.84% *vs.* 3.13%, *P*=0.043). *KRAS* and *PTPN11* variants were more frequent in refractory as compared to relapse stage (*KRAS* 9.76% *vs.* 1.96%, *P*=0.025, and *PTPN11* 8.54% *vs.* 1.10%, *P*=0.028) (Figure 4A and B).

Age-related mutations

At diagnosis, patients aged ≥65 years had more variants than younger (<65 years) patients (2.74 ± 0.81 *vs.* 2.18 ± 0.74 variants per patient, *P*<0.001). The following genes were more frequently mutated in patients aged ≥65 years *vs.* <65 years: *ASXL1*, *EZH2*, *IDH2*, *JAK2*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, and *U2AF1*. *FLT3*-ITD and *NPM1* mutations were more frequent in younger AML patients (Figure 5).

In relapsed AML, *ASXL1* (20.00% *vs.* 3.57%, *P*=0.011) and *IDH* variants (46.70% *vs.* 25%, *P*=0.035) were associated with patients aged ≥65 years (*Online Supplementary Table S4*).

Mutational stability in paired samples

Paired samples at DX-RP (n=14) and DX-RS (n=20) were obtained to assess clonal evolution. The following were stable variants: *NPM1* (100%, as no patients acquired or lost variants), *TP53*, *IDH2* (one acquisition at RS and one at RP in each gene), *DNMT3A* (100% stable at RS and one acquisition at RP), and *RUNX1* (stable at RP and one loss at RS). The following variants were unstable: activating signaling pathways genes such as *FLT3*, *NRAS*, *KRAS*, *BRAF*, *KIT* and *PTPN11* (*Online Supplementary Figure S4*). Interestingly for targeted therapy, 26.47% of patients changed the mutational status of *FLT3* at RS or RP (*FLT3*-ITD: two gains and three losses; *FLT3*-PM: one gain and three losses). In all cases, the loss of function mutation of *FLT3*-PM was located on the Asp835 codon.

Clinically relevant mutations

Overall, 72.30% of patients harbored at least one clinically relevant variant included in the AML clinical guidelines, clinical trials inclusion criteria or as a risk stratification biomarker (*ASXL1*, *CEBPA*, *FLT3*, *IDH1/2*, *NPM1*, *RUNX1* and *TP53*) (*Online Supplementary Figure S5*). Moreover, druggable mutations were present in a significant proportion of patients (*FLT3* in 21.14% and *IDH1/2* in 22.60%).

NPM1 mutations

NPM1 variants were found in 21.51% of samples being 78.53% type A (c.860_863dupTCTG), 6.21% type B (c.863_864insCATG), 6.21% type D (c.863_864insCCTG), and 9.04% had uncommon variants (*Online Supplementary Figure S6*; *Online Supplementary Table S5*).

FLT3 mutations

FLT3 was the most frequently mutated gene (24.06%). *FLT3* ITD was the most frequent *FLT3* aberration (16.52%) followed by D835 and I836 variants (5.71%) and other variants (3.16%) (*Online Supplementary Figure S7A*). Other variants were mostly SNV (95.18%) located in the tyrosine kinase 1 domain (TKD1; 41.18%), juxtamembrane domain (JMD; 23.53%), tyrosine kinase 2 domain (TKD2; 20.59%), extracellular domain (ED; 11.76%) and kinase insert domain (KID 2.94%). No variants were detected in transmembrane (TMD) and C-terminal domains (CTD). (*Online Supplementary Figure S7B*). 84.75% of all *FLT3* variants were targetable with *FLT3* inhibitors and had a direct clinical impact in 21.14% of patients through targeted therapy or clinical trials.

CEBPA mutations

CEBPA variants were found in 5.35% of samples, 3.52% were monoallelic variants and 1.82% were biallelic variants

(both *CEBPA* alleles mutated). Two variants with similar VAF were reported as probably biallelic variants *(Online Supplementary Table S6)*. Although *CEBPA* variants were distributed along the entire coding sequence, biallelic variants were frequently detected in BZIP (43.33%) and the N-terminal domain (30%). Monoallelic variants, were mostly detected in BZIP (44.83%) and almost equally distributed among TAD1 (13.79%), TAD2 (17.94%) and the N-terminal domain (6.90%).

IDH1 and *IDH2* mutations

IDH variants were detected in 22.60% of samples. In mutated *IDH1* samples (9.11%), all variants were detected in the Arg132 codon. *IDH2* (13.85%) was exclusively mutated in the Arg140 or Arg172 codons (84.20% and 15.79%, respectively). Three patients (1.61%) showed simultaneous variants in *IDH1* and *IDH2* (*Online Supplementary Table S7*). All *IDH1* and *IDH2* variants were targetable mutations by IDH inhibitors as no atypical variants were detected.

ASXL1, *RUNX1* and *TP53* mutations

Overall, 39.00% of samples showed variants in at least one of them: 18.23% *RUNX1*, 14.70% *TP53* and 12.39% *ASXL1*. In 26.12% of samples a variant detected in one of these genes was the only clinically relevant variant. Moreover, 28.19% of patients were classified to an unfavorable risk group according to *ASXL1*, *RUNX1* and *TP53* mutations, following European LeukemiaNet 2017 recommendations.

Discussion

This study shows that a network platform involving many highly skilled laboratories can successfully deliver robust molecular data for AML patients. This strategy allows for testing NGS in the majority of newly diagnosed and relapsed/refractory AML patients involved in the PETHEMA studies, overcoming the current challenging needs for a high-standard diagnosis in cooperative groups. Our descriptive analysis performed in a large series of real-life patients depicts the complex molecular landscape of AML.

In the last 5 years, NGS has irrupted as a potential routine tool for molecular diagnosis, allowing for precise and simultaneous detection of relevant variants in AML. However, this technique is still non-affordable for many institutions due to: i) remarkable cost as compared to conventional PCR tests, ii) batch of samples, ranging from eight to more than 30, to run the test, and consequently high time consumption, both making it difficult to report results in less than 7-10 days; and iii) the need of expensive machinery and highly-qualified teams for biostatistical and molecular biologists. Moreover, the majority of prognostic or druggable mutations can be rapidly and relatively easily detected by conventional PCR. In fact, from the mandatory NGS panel genes selected by the PETHEMA central laboratories (i.e, *ASXL1*, *CEBPA*, *FLT3*, *IDH1*, *IDH2*, *NPM1*, *RUNX1*, and *TP53*), a mutation screening by conventional PCR is still required for *FLT3*, *IDH1*, *IDH2* and *NPM1*, as a positive result could allow for rapid implementation of targeted or risk-adapted therapeutic approaches.[8] In addition, rapid PCR is also needed for core-binding factor (CBF), *PML-RARA* and *BCR-ABL* rearrangements. Under this scenario, our cooperative group designed a nationwide network involving seven central laboratories aimed to deliver homogeneous and comparable molecular results for newly diagnosed and relapsed/refractory AML patients. As far as we know, this is a different strategy as compared to other cooperative groups that usually rely on only one or two central laboratories for molecular diagnostics (e.g, British NCRI).[9] Several reasons guided us to make this decision: i) the economic and work burden required to collect samples from the whole group, which covers a wide territory and population, was not affordable for a single laboratory; ii) a minimum referral population is required to permit an efficient diagnosis by studying the appropriate number of samples in every run; iii) the involvement many on-site teams in order

Table 3. Demographic and baseline characteristics of the study population (n=751).

Characteristic	NGS population Mean	Median	Range	n (%)
Age, years	62.5	65	8-93	751 (100)
<60				284 (38)
≥60				467 (62)
Sex				751 (100)
Male				423 (56)
Female				328 (44)
ECOG	0.9	1	0-4	751 (100)
0				184 (25)
1				203 (27)
2				48 (6)
3				26 (3)
4				6 (1)
Not available				284 (38)
Type of AML				751 (100)
De novo				378 (50)
Secondary				155 (21)
Not available				218 (29)
WBC, $\times 10^9$/L	31.2	8.4	0.3-305	751 (100)
≤ 5				205 (27)
5-10				58 (8)
10-50				146 (19)
> 50				95 (13)
Not available				247 (33)
BM blast cells, %	55	53	2-100	751 (100)
≤ 30				109 (15)
30-70				203 (27)
> 70				157 (21)
Not available				282 (38)
Creatinine, mg/dL	1.0	0.87	0.23-3.78	751 (100)
≤ 1,2				400 (53)
> 1,2				68 (9)
Not available				283 (38)
Cytogenetic risk				751 (100)
Favorable				25 (3)
Normal				224 (30)
Intermediate				66 (9)
Adverse				125 (17)
Not available				311 (41)
Therapeutic approach				751 (100)
Intensive				297 (40)
Non-intensive				125 (17)
Clinical Trial				33 (4)
Supportive care				20 (3)
Not available				276 (37)

AML: acute myeloid leukemia, BM: bone marrow, WBC: white blood cell; ECOG: Eastern Cooperative Oncology Group.

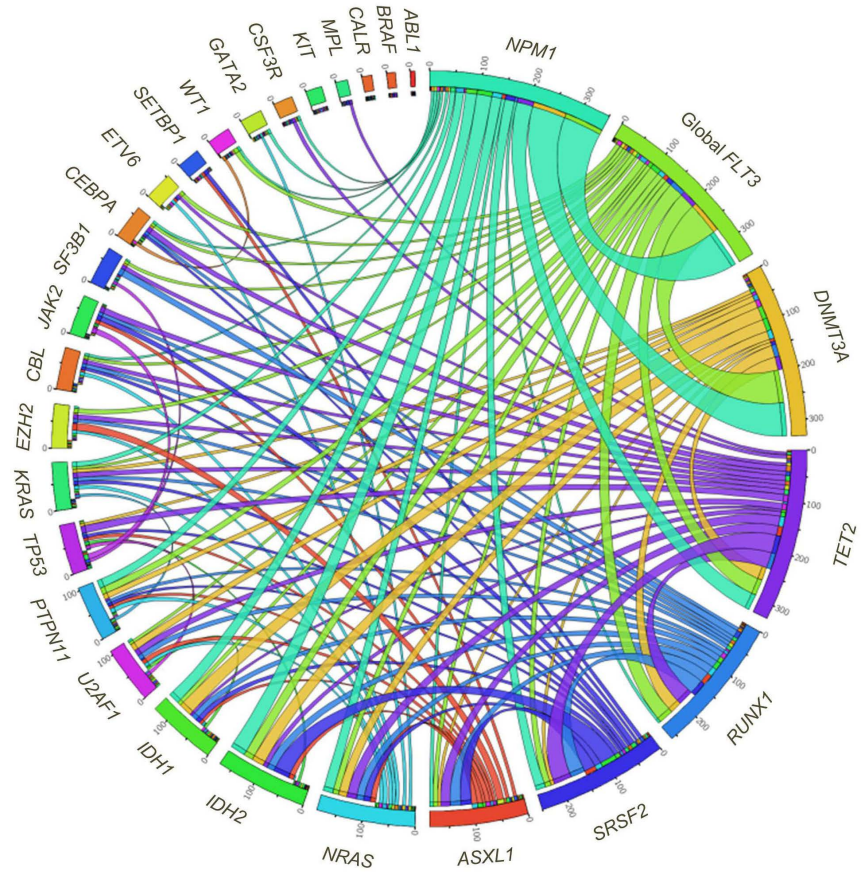


Figure 2. Circos diagram showing mutation concurrences in our cohort.

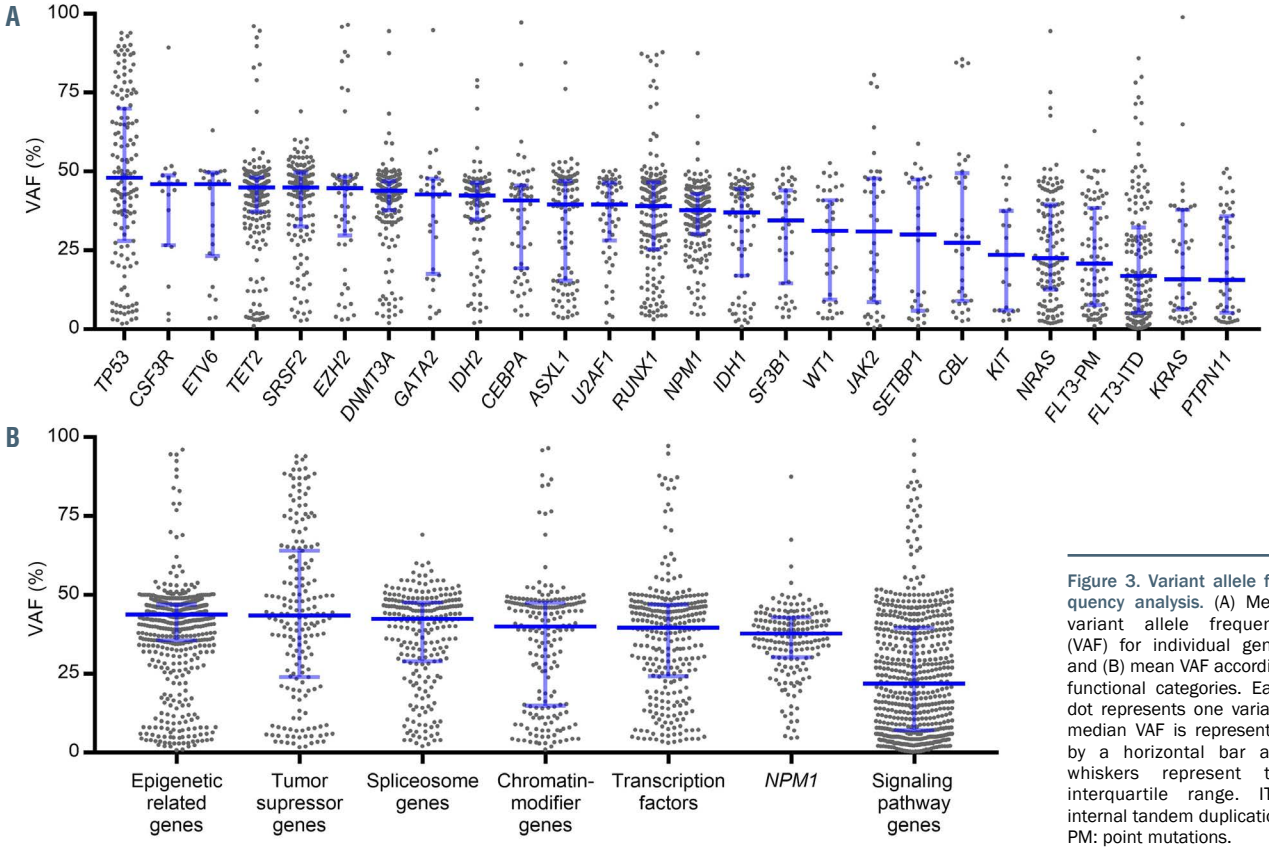


Figure 3. Variant allele frequency analysis. (A) Mean variant allele frequency (VAF) for individual genes and (B) mean VAF according functional categories. Each dot represents one variant, median VAF is represented by a horizontal bar and whiskers represent the interquartile range. ITD: internal tandem duplication; PM: point mutations.

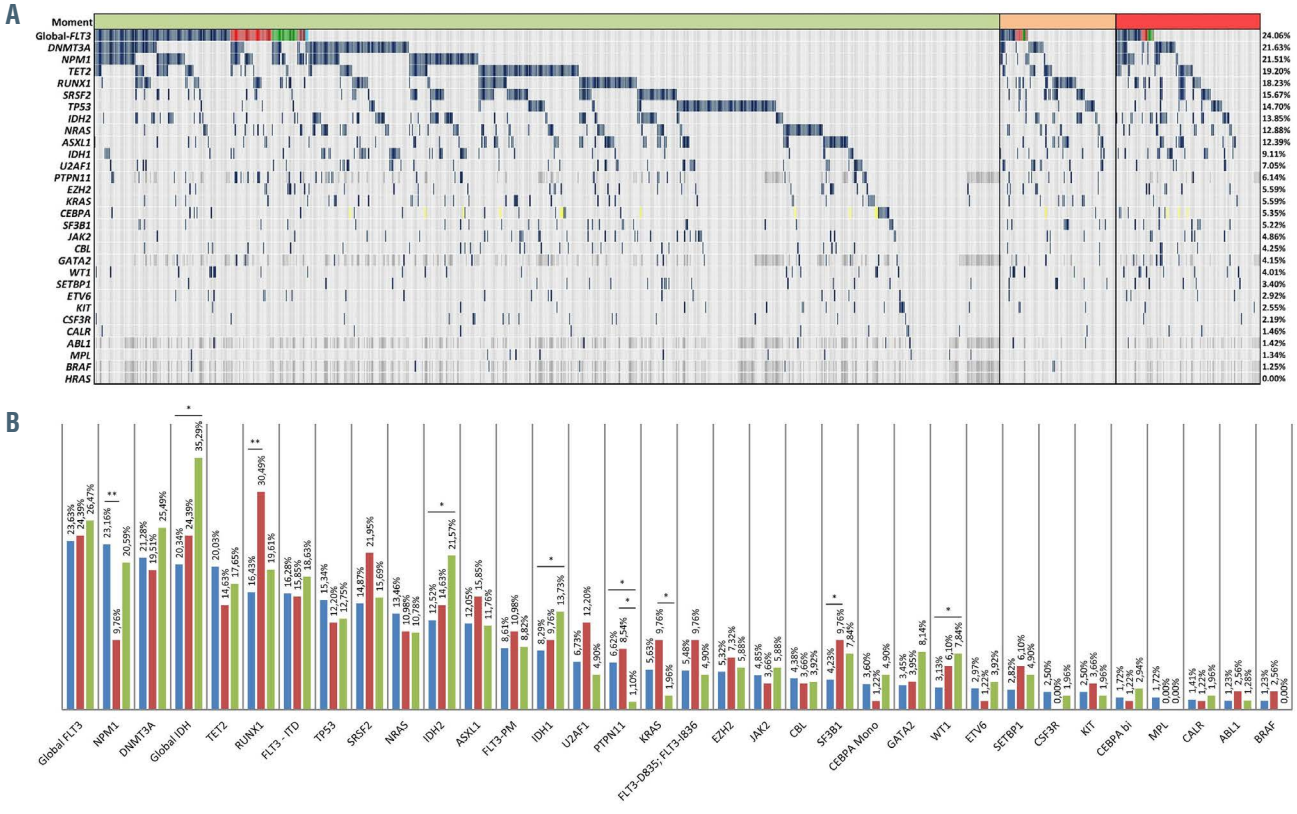


Figure 4. Distribution of gene alterations in acute myeloid leukemia samples. (A) Mutational landscape in the global cohort. Horizontal green bars: diagnosis, orange: refractory, and red: relapse.Vertical dark blue bars: positive (in global *FLT3* row represents internal tandem duplications [ITD]), red: *FLT3*-D835/I836, green: other *FLT3* point mutations (PM), orange: *FLT3*-ITD and D835/I836, light blue: *FLT3*-ITD and other *FLT3*-PM, light grey: negative, dark grey: not tested, yellow: bi-allelic variants in *CEBPA*. (B) Mutational prevalence according to disease stage. Diagnosis are represented as blue bars, refractory as red bars and relapse as green bars. **P*<0.05, ***P*<0.01.

to create a true research network; and iv) the need to facilitate rapid delivery of samples while preserving closer and well established relationships between the sample referral institution and the assigned central laboratory. We can affirm that the PETHEMA model for centralized diagnosis has been successful collecting samples from 751 patients in roughly 1 year, enabling the use of this network in routine clinical practice and research.

Our study demonstrates that harmonized and reliable NGS results can be achieved across several laboratories, even if they are using their own diagnostic platforms. As shown in the pre-standardization cross-validation round, an overall concordance of 60.98% was obtained with a great variability in selected genes and conditions across laboratories. After consensus of AML relevant genes and optimization of quality parameters (uniformity >85%; mean read depth of 1,000X) the overall concordance rose to 85.57% in the second cross-validation round. This was a remarkable achievement for all laboratories taking into account that low VAF (≤5%) variants were included in this second round. To the best of our knowledge, there are no similar studies reported in the literature for AML.

Clinical validation of our AML cohort was consistent with previous reports. Roughly 91% of AML patients had at least one variant, and many harbored three, four and up to eight variants reflecting the heterogeneous AML mutational profile.[10] *FLT3*, *IDH1/2*, *DNMT3A* and *NPM1* were the most frequently mutated genes,[11,12] and we showed that up to 73% of patients had variants with clinical implications for risk stratification or targeted therapy-based approaches (i.e, *ASXL1*, *CEBPA*, *FLT3*, *IDH1/2*, *NPM1*, *RUNX1* and *TP53*).[13] Moreover, *ASXL1*, *RUNX1* and *TP53* variants which are not easily analyzed with conventional molecular techniques,[14–16] were the unique clinically relevant alteration detected in up to 28.19% of patients, highlighting that NGS-based mutational profiling seems crucial to categorize AML risk according to the European LeukemiaNet 2017 guidelines.[3]

As reported by other groups,[17–20] elderly patients had a higher number of variants, which were enriched in spliceosome machinery, epigenetic regulators and in DNA repair (i.e, *SRSF2*, *U2AF1*, *SF3B1*, *ASXL1*, *TET2*, *IDH2* and *TP53*). In line with previous studies, *NPM1* variants were more frequent in younger AML patients, and we noticed a striking decrease of *FLT3* variants in older patients.[21] We can affirm that a lower number of older patients may benefit from tyrosine kinase inhibitors-based approaches,[22] but more from novel IDH-inhibitors.[23] In our experience, NGS has efficiently screened *FLT3* gene variants, including less frequent variants, which could also be informative for therapeutic decisions.[24,25] Furthermore, NGS is a promising tool to assess *FLT3*-ITD duplicated region, which could have prognostic impact regarding its location and extension.[26]

We also provide insights on clonal evolution and leukemogenesis: i) variants in signalling pathway genes (*FLT3*, *KIT*, *RAS*) had lower VAF, reflecting their role as late events,[27]; ii) genes related to CHIP showed higher VAF values,[28] and iii) median VAF in AML patients with *TP53* variants was above 50%, indicating the frequent loss of the wild-type *TP53* allele. Recent studies suggest that a higher

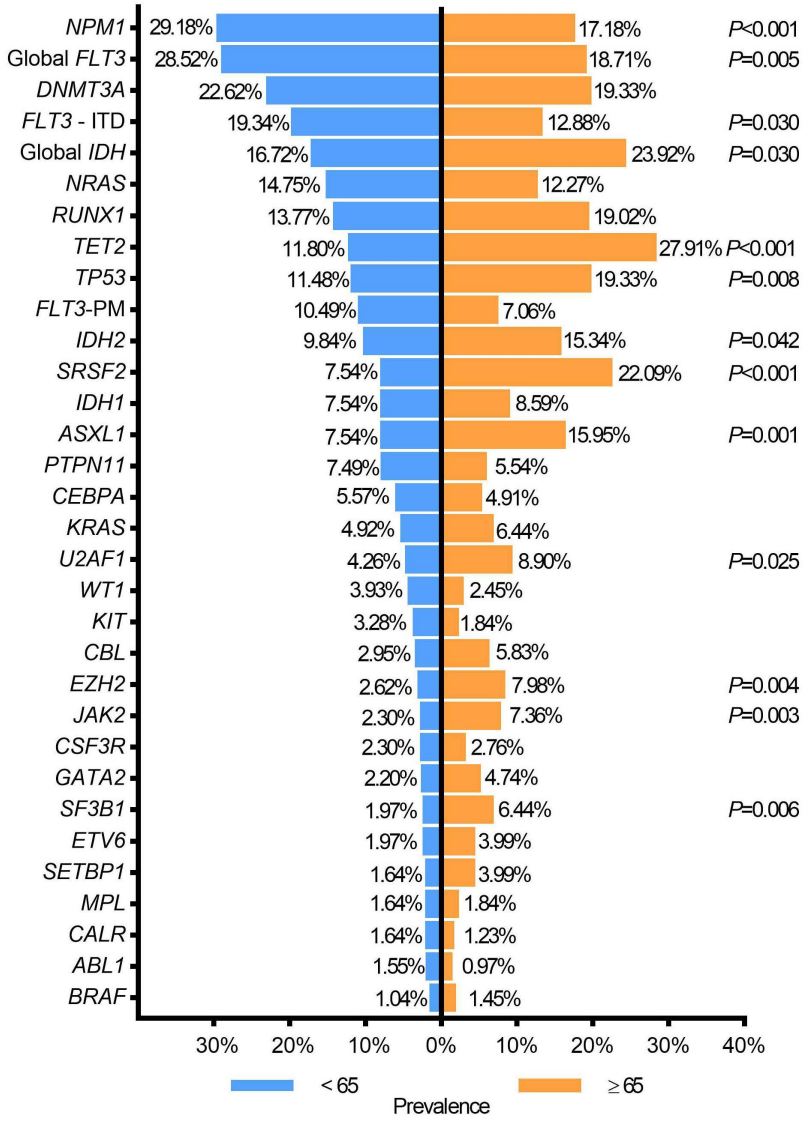


Figure 5. Age-related mutational profile. *P*-values (*P*) for statistically significant results are shown. ITD: internal tandem duplication; PM: point mutations.

clonal size of *TP53* variants, determined by VAF and chromosomal aberrations (del 17p), could discriminate patients with worse prognosis among *TP53* mutated AML.[29,30]

Our study allowed to observe differences in the mutational profile of relapsed/refractory patients as compared to newly diagnosed/untreated subjects. Interestingly, *RUNX1* variants were more frequent among refractory AML patients, which is consistent with previously reported poorer outcomes after intensive chemotherapy and its association with older age.[31] The same was observed with *SFRS2*, depicting poorer outcomes when commutated with *IDH2*[32] which was highly associated in our cohort. *NPM1* variants were significantly less frequent in refractory patients, as compared to newly diagnosed or relapsed, reflecting the known high complete remission rates in this setting.[33,34] Interestingly, *IDH* variants were the most frequent alteration at relapse, suggesting that they are associated with higher relapse risk,[35] but also have more possibilities to obtain an initial response with front-line therapies.[18,36]

We analyzed 35 patients with paired samples at diagnosis *versus* relapse/refractory setting, confirming some findings from scarce studies on clonal evolution: i) as a founder variant, *NPM1* was very stable,[37,38] ii) *DNMT3A* variants were very stable, probably due to its early acquisition and preleukemic occurrence,[39,40] as well as their persistence during remission and disease progression,[41] and iii) activating signaling pathways genes were unstable (*FLT3*, *NRAS*, *KRAS*, *BRAF*, *KIT* and *PTPN11*). This is particularly relevant for the management of patients who acquire *FLT3* mutations during relapse and refractoriness which may benefit from second generation inhibitors such as gilteritinib, available for the treatment of relapsed/refractory AML with *FLT3* mutation.[42,43] In line with the study by Kronke *et al.*, we show that *IDH2* variants were very stable, contrarily to *IDH1*, but numbers are low and should be cautiously interpreted.[38]

The main limitation of our study is that, apart from age and disease phase, baseline clinical characteristics, treatment patterns, and outcomes, were not available for a minor proportion of patients at the time of this interim analysis of the NGS-AML trial. This is why the results herein presented focused on the overall network building strategy, as well as in the cross-validation of samples and reporting harmonization, showing a mutational landscape of AML consistent with the current knowledge. We should mention that our diagnostic platform has many areas of improvement: i) time to NGS reporting, which is longer than conventional molecular analysis (approximately 2-3

weeks), ii) need to promote larger participation of PETHEMA clinical sites, as a sizable proportion of patients are not yet benefiting from advanced laboratory centralization (especially in the relapse/refractory setting), and ii) budgetary vulnerability.

In conclusion, the PETHEMA cooperative scientific group has adopted the reported nationwide strategy network with centralized NGS analyses. Sample and information exchange allowed us to unify analysis criteria and decrease reporting variability in order to offer reliable and consistent NGS results. This cooperative strategy has also been applied to rapid screening by conventional PCR and quantitive real-time PCR to measure residual disease, and is being expanded to other AML diagnostic areas (e.g., biobanking and multiparametric flow cytometry). Ongoing therapeutic guidelines (NCT01296178) and clinical trials (clinicaltrials gov. Identifier: NCT04230239, NCT04107727, NCT04112589, NCT04090736) by the PETHEMA group are benefiting from this diagnostic network.

Disclosures

No conflicts of interest to disclose.

Contributions

EB and PM conceived the study; CS, EB and PM analyzed, interpreted the data and wrote the paper; CS performed the statistical analyses; CS, RA, CC, MJL, EC, CB, MYR, MLL, IR, RGS, IVU, ES, YFO, KJ, CB, JS, DMC, JB, MA, PMS, MT, TB, PHP, RG, LA, MJS, LCB, EPS, IM, ELR, VN, JMA, MAS, JSG, MTGC, JAPS, MJC, MG, JML, EB and PM included data of patients treated in their institutions, reviewed the manuscript and contributed to the final draft.

Acknowledgments

The authors would like to thank María D. García, Carlos Pastorini, Rafael Vianney, and Mar Benlloch for data collection and management; and Data Science, Biostatistics and Bioinformatics Unit from IIS La Fe for its collaboration in statistical analysis.

Funding

This work was partially supported by a Celgene grant, the Subdirección General de Investigación Sanitaria (Instituto de Salud Carlos III, Spain) Spanish Ministry of Economy and Competitiveness: PI15/01706, PI16/00517 PI16/0665, PI16/01530, PI18/01340, PI18/01946 PI19/00730, PI19/01518, FI19/00059, Fundación Española de Hematología y Hemoterapia (FEHH) grant, CRIS against Cancer foundation 2018/001. CIBERONC-CB16/12/00233 and "Una manera de hacer Europa" (Innocampus; CEI-2010-1-0010), Instituto de Investigación Sanitaria La Fe (Contrato de Investigación postresidentes 2019-052-1)

References

1. Saultz J, Garzon R. Acute myeloid leukemia: a concise review. J Clin Med. 2016;5(3):33.
2. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-2405.
3. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129(4):424-447.
4. Hackl H, Astanina K, Wieser R. Molecular and genetic alterations associated with therapy resistance and relapse of acute myeloid leukemia. J Hematol Oncol. 2017;10(1):51.
5. Luppi M, Fabbiano F, Visani G, Martinelli G, Venditti A. Novel agents for acute myeloid leukemia. Cancers (Basel). 2018;10(11):429.
6. Riva L, Luzi L, Pelicci PG. Genomics of acute myeloid leukemia: the next generation. Front Oncol. 2012;2:40.
7. Kuo FC, Mar BG, Lindsley RC, Lindeman NI. The relative utilities of genome-wide, gene panel, and individual gene sequencing in clinical practice. Blood. 2017;130(4):433-439.
8. Bacher U, Shumilov E, Flach J, et al. Challenges in the introduction of next-generation sequencing (NGS) for diagnostics of myeloid malignancies into clinical routine use. Blood Cancer J. 2018;8(11):113.
9. Macklin PS, Pillay N, Lee JL, et al. CM-Path Molecular Diagnostics Forum—consensus statement on the development and implementation of molecular diagnostic tests in the United Kingdom. Br J Cancer. 2019;121(9):738-743.
10. Ley TJ, Miller C, Ding L, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013;368(22):2059-2074.
11. Tyner JW, Tognon CE, Bottomly D, et al. Functional genomic landscape of acute myeloid leukaemia. Nature. 2018;562(7728):526-531.
12. Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic classification and prognosis in acute myeloid leukemia. N Engl J Med. 2016;374(23):2209-2221.
13. Castelli G, Pelosi E, Testa U. Targeted therapies in the treatment of adult acute myeloid leukemias: current status and future perspectives. Int J Hematol Oncol. 2016;5(4):143-164.
14. Gelsi-Boyer V, Brecqueville M, Devillier R, Murati A, Mozziconacci MJ, Birnbaum D. Mutations in ASXL1 are associated with poor prognosis across the spectrum of malignant myeloid diseases. J Hematol Oncol. 2012;5:12.
15. Kohlmann A, Nadarajah N, Alpermann T, et al. Monitoring of residual disease by next-generation deep-sequencing of RUNX1 mutations can identify acute myeloid leukemia patients with resistant disease. Leukemia. 2014;28(1):129-137.
16. Barbosa K, Li S, Adams PD, Deshpande AJ. The role of TP53 in acute myeloid leukemia: challenges and opportunities. Genes Chromosomes Cancer. 2019;58(12):875-888.
17. Dinardo CD, Ravandi F, Agresta S, et al. Characteristics, clinical outcome, and prognostic significance of IDH mutations in AML. Am J Hematol. 2015;90(8):732-736.
18. Medeiros BC, Fathi AT, DiNardo CD, Pollyea DA, Chan SM, Swords R. Isocitrate dehydrogenase mutations in myeloid malignancies. Leukemia. 2017;31(2):272-281.
19. Prassek VV, Rothenberg-Thurley M, Sauerland MC, et al. Genetics of acute myeloid leukemia in the elderly: Mutation spectrum and clinical impact in intensively treated patients aged 75 years or older. Haematologica. 2018;103(11):1853-1861.
20. Metzeler KH, Herold T, Rothenberg-Thurley M, et al. Spectrum and prognostic relevance of driver gene mutations in acute myeloid leukemia. Blood. 2016;128(5):686-698.
21. Schneider F, Hoster E, Schneider S, et al. Age-dependent frequencies of NPM1 mutations and FLT3-ITD in patients with normal karyotype AML (NK-AML). Ann Hematol. 2012;91(1):9-18.
22. Daver N, Schlenk RF, Russell NH, Levis MJ. Targeting FLT3 mutations in AML: review of current knowledge and evidence. Leukemia. 2019;33(2):299-312.
23. Liu X, Gong Y. Isocitrate dehydrogenase inhibitors in acute myeloid leukemia. Biomark Res. 2019;7(1):22.
24. Williams AB, Nguyen B, Li L, et al. Mutations of FLT3/ITD confer resistance to multiple tyrosine kinase inhibitors. Leukemia. 2013;27(1):48-55.
25. Heidel F, Solem FK, Breitenbuecher F, et al. Clinical resistance to the kinase inhibitor PKC412 in acute myeloid leukemia by mutation of Asn-676 in the FLT3 tyrosine kinase domain. Blood. 2006;107(1):293-300.
26. Liu SB, Dong HJ, Bao XB, et al. Impact of FLT3-ITD length on prognosis of acute myeloid leukemia. Haematologica. 2019;104(1):e9-e12.
27. Hirsch P, Zhang Y, Tang R, et al. Genetic hierarchy and temporal variegation in the clonal history of acute myeloid leukaemia. Nat Commun. 2016;7(1):1-13.
28. Martignoles JA, Delhommeau F, Hirsch P. Genetic hierarchy of acute myeloid leukemia: from clonal hematopoiesis to molecular residual disease. Int J Mol Sci. 2018;19(12):3850.
29. Goel S, Hall J, Pradhan K, et al. High prevalence and allele burden-independent prognostic importance of p53 mutations in an inner-city MDS/AML cohort. Leukemia. 2016;30(8):1793-1795.
30. Rücker FG, Schlenk RF, Bullinger L, et al.

TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. Blood. 2012;119(9):2114-2121.
31. Gaidzik VI, Bullinger L, Schlenk RF, et al. RUNX1 mutations in acute myeloid leukemia: results from a comprehensive genetic and clinical analysis from the AML study group. J Clin Oncol. 2011;29(10): 1364-1372.
32. Yoshimi A, Lin KT, Wiseman DH, et al. Coordinated alterations in RNA splicing and epigenetic regulation drive leukaemogenesis. Nature. 2019;574(7777):273-277.
33. Jain P, Kantarjian H, Patel K, et al. Mutated NPM1 in patients with acute myeloid leukemia in remission and relapse. Leuk Lymphoma. 2014;55(6):1337-1344.
34. Schneider F, Hoster E, Unterhalt M, et al. NPM1 but not FLT3-ITD mutations predict early blast cell clearance and CR rate in patients with normal karyotype AML (NK-AML) or high-risk myelodysplastic syndrome (MDS). Blood. 2009;113(21):5250-5253.
35. Ok CY, Loghavi S, Sui D, et al. Persistent IDH1/2 mutations in remission can predict relapse in patients with acute myeloid leukemia. Haematologica. 2019;104(2):305-311.
36. Megías-Vericat JE, Ballesta-López O, Barragán E, Montesinos P. IDH1-mutated relapsed or refractory AML: current challenges and future prospects. Blood Lymphat Cancer Targets Ther. 2019;9:19-32.
37. Cocciardi S, Dolnik A, Kapp-Schwoerer S, et al. Clonal evolution patterns in acute myeloid leukemia with NPM1 mutation. Nat Commun. 2019;10(1):2031.
38. Krönke J, Bullinger L, Teleanu V, et al. Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. Blood. 2013;122(1):100-108.
39. Bhatnagar B, Eisfeld AK, Nicolet D, et al. Persistence of DNMT3A R882 mutations during remission does not adversely affect outcomes of patients with acute myeloid leukaemia. Br J Haematol. 2016;175(2):226-236.
40. Hou HA, Kuo YY, Liu CY, et al. DNMT3A mutations in acute myeloid leukemia: stability during disease evolution and clinical implications. Blood. 2012;119(2):559-568.
41. Gaidzik VI, Weber D, Paschka P, et al. DNMT3A mutant transcript levels persist in remission and do not predict outcome in patients with acute myeloid leukemia. Leukemia. 2018;32(1):30-37.
42. Larrosa-Garcia M, Baer MR. FLT3 Inhibitors in acute myeloid leukemia: current status & future directions. Mol Cancer Ther. 2017;16(6):991-1001.
43. Short NJ, Kantarjian H, Ravandi F, Daver N. Emerging treatment paradigms with FLT3 inhibitors in acute myeloid leukemia. Ther Adv Hematol. 2019;10:2040620719827310.

Networking for advanced molecular diagnosis in acute myeloid leukemia patients is possible: the PETHEMA NGS-AML project

Claudia Sargas,[1] Rosa Ayala,[2] María Carmen Chillón,[3] María J. Larráyoz,[4] Estrella Carrillo-Cruz,[5] Cristina Bilbao,[6] Manuel Yébenes-Ramírez,[7] Marta Llop,[1] Inmaculada Rapado,[2] Ramón García-Sanz,[3] Iria Vázquez,[4] Elena Soria,[5] Yanira Florido-Ortega,[6] Kamila Janusz,[7] Carmen Botella,[8] Josefina Serrano,[7] David Martínez-Cuadrón,[9,10] Juan Bergua,[11] Mari Luz Amigo,[12] Pilar Martínez-Sánchez,[2] Mar Tormo,[13] Teresa Bernal,[14] Pilar Herrera-Puente,[15] Raimundo García,[16] Lorenzo Algarra,[17] María J. Sayas,[18] Lisette Costilla-Barriga,[19] Esther Pérez-Santolalla,[20] Inmaculada Marchante,[21] Esperanza Lavilla-Rubira,[22] Víctor Noriega,[23] Juan M. Alonso-Domínguez,[24] Miguel Á. Sanz,[9,10] Joaquín Sánchez-Garcia,[7] María T. Gómez-Casares,[6] José A. Pérez-Simón,[5] María J. Calasanz,[4] Marcos González-Díaz,[3] Joaquín Martínez-López,[2] Eva Barragán,[1,10#] and Pau Montesinos,[9,10#] on behalf of the PETHEMA group

[1]Molecular Biology Unit, Hospital Universitari i Politècnic-IIS La Fe, Valencia; [2]Hematology Department, Hospital Universitario 12 de Octubre, CNIO, Complutense University, Madrid; [3]Hospital Universitario de Salamanca (HUS/IBSAL), CIBERONC and Center for Cancer Research-IBMCC (USAL/CSIC), Salamanca; [4]CIMA LAB Diagnostics-Universidad de Navarra, Pamplona; [5]Hospital Universitario Virgen del Rocío, Instituto de Biomedicina (IBIS/CSIC/CIBERONC), Universidad de Sevilla, Sevilla; [6]Hospital Universitario de Gran Canaria Dr. Negrín, Las Palmas de Gran Canaria; [7]IMIBIC, Hematology, Hospital Universitario Reina Sofía, UCO, Córdoba; [8]Hospital General Universitario de Alicante, Alicante; [9]Hematology Department, Hospital Universitari i Politécnic-IIS La Fe, Valencia; [10]CIBERONC Instituto de Salud Carlos III, Madrid; [11]Hospital Universitario San Pedro de Alcántara, Cáceres; [12]Hospital Universitario Morales Messeguer, Murcia; [13]Hematology Department, Hospital Clínico Universitario-INCLIVA, Valencia; [14]Hospital Universitario Central de Asturias, Oviedo; [15]Hospital Universitario Ramón y Cajal, Madrid; [16]Hospital Universitari General de Castelló, Castellón; [17]Hospital Universitario General de Albacete, Albacete; [18]Hospital Univerisitario Dr. Peset, Valencia; [19]Hospital Universitario Miguel Servet, Zaragoza; [20]Hospital de Donosti, San Sebastián; [21]Hospital Universitario Puerta del Mar, Cádiz; [22]Complexo Hospitalario Lucus Augusti, Lugo; [23]Complexo Hospitalario Universitario A Coruña, A Coruña and [24]Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain

[#]EB and PM contributed equally as co-senior authors.

 doi:10.3324/haematol.2020.263806

Received: June 24, 2020.

Accepted: November 3, 2020.

Pre-published: November 12, 2020.

Correspondence: *EVA BARRAGÁN GONZÁLEZ* - barragan_eva@gva.es

SUPPLEMENTARY MATERIAL:

Methods

Study design and reference laboratories

The overall population coverage was of 38.5 million habitants[1], with each laboratory receiving samples from institutions located at their assigned geographical areas, ranging from 2.2 to 8.9 million habitants. Bone marrow and peripheral blood samples from acute myeloid leukemia (AML) patients at diagnosis (DX) and at resistance (RS) or first and subsequent relapses (RP) were sent by courier and were isolated according to standardized methods.

Nucleic acid isolation

DNA from white blood cells was obtained in each center following previously established DNA isolation protocols. DNA quantification was assessed with Nanodrop (Thermo Fisher Scientific, Waltham, MA USA) and Qubit fluorometer (Thermo Fisher Scientific). DNA integrity was assessed with Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) or Tape Station 4100 (Agilent Technologies).

Cross-validation

Six of seven reference laboratories had already implemented next-generation sequencing (NGS) assays in a research context before starting the NGS standardization program. The remaining laboratory was included in the second cross-validation round.

Sequencing platforms and panels

The proposed strategy to select the sequencing platform and panel was aimed to allow each laboratory implementing their preferred panel fitting with their available facilities, as well as overlapping with the study of other myeloid neoplasms variants.

NGS was performed either with commercial or custom panels using Ion Torrent or Illumina platforms. Two laboratories implemented the Myeloid Solution panel (SOPHIA Genetics, Lausanne, Switzerland), including 30 genes involved in leukemia and other hematological disorders, such as myelodysplastic and chronic myeloproliferative syndromes (MDS and CMPS). Two laboratories used an extended version of this panel (Pan-Myeloid Panel, SOPHIA Genetics), including 48 genes involved in myeloid neoplasms. Two laboratories performed NGS with the Oncomine Myeloid Research Assay (Thermo Fisher Scientific), including 40 genes involved in diverse myeloid disorders (AML, MDS, and CMPS). One laboratory used a

custom AML panel already implemented in previous research projects that include genes involved in hematological disorders[2].

Table S1. Gene panels employed by central laboratories.

Gene	HULF, HUVR	H12O	HUDN, HURS	UNAV, HUS
	AML Oncomine Research (Ion Torrent)	Custom panel (Ion Torrent)	Myeloid Tumor Solution (Illumina)	Custom PanMyeloid (Illumina)
ASXL1	All exons	All exons	9, 11, 12	12
CEBPA	All exons	All exons	All exons	All exons
FLT3	8, 11 to 16, 20, 23, 24	All exons	13 to 15, 20	14 to 16, 20
IDH1	4	All exons	4	4
IDH2	4	All exons	4	4
NPM1	11	All exons	10, 11	10, 11
RUNX1	All exons	All exons	All exons	All exons
TP53	All exons	All exons	All exons	All exons
ABL1	4 to 9	-	4 to 9	-
BRAF	15	-	15	-
CALR	All exons	All exons	9	9
CBL	8, 9	All exons	8, 9	8, 9
CSF3R	14, 17	All exons	All exons	14 to 17
DNMT3A	11 to 23	All exons	All exons	All exons
ETV6	All exons	All exons	All exons	All exons
EZH2	All exons	All exons	All exons	All exons
GATA2	4, 5	-	-	2 to 6
HRAS	2, 3	-	2, 3	-
JAK2	12 to 15	All exons	All exons	12 to 15
KIT	2, 8 to 11, 13, 17, 18	All exons	2, 8 to 11, 13, 17, 18	2, 8 to 11, 13, 14, 17, 18
KRAS	2 to 6	All exons	2, 3	2 to 4
MPL	3 to 4, 10, 12	All exons	10	3 to 6, 10, 12
NRAS	2 to 4	All exons	2, 3	2 to 4
PTPN11	3, 12, 13	-	3, 7, 13	3, 7, 13
SETBP1	4	All exons	4	4
SF3B1	14 to 21	All exons	10 to 16	11 to 16
SRSF2	1	All exons	1	1
TET2	All exons	All exons	All exons	All exons
U2AF1	2, 6	All exons	2, 6	2, 6
WT1	7, 9	All exons	6 to 10	7, 9

HULF: Hospital Universitario La Fe, HUVR: Hospital Universitario Virgen del Rocío, H12O: Hospital Universitario 12 de Octubre, HUDN: Hospital Universitario de Gran Canaria Dr. Negrín, HURS: Hospital Universitario Reina Sofía, UNAV: CIMA LAB Diagnostics, HUS: Hospital Universitario de Salamanca.

Statistics

Continuous variables were summarized by using median, mean, range and standard deviation (SD). Categorical variables were summarized with relative and absolute frequencies. Association of categorical variables was assessed using the chi-square ($\chi 2$) test. Normal distribution of continuous variables was checked with Shapiro-Wilk test. Association of categorical with continuous variables was performed using Mann-Whitney´s U test (independent groups) or Wilcoxon Test (related groups). Exclusion patterns among genes were analyzed with the Mutually Exclusive Gene Sets (MEGS) analysis, an analytic framework based on a likelihood ratio test and a model selection procedure[3].

Results

Platform performance

Table S2. Cross-validation results comparing Ion Torrent and Illumina platforms.

			Illumina					Ion Torrent				
Gene	Coding	Protein	Detected	Included	Error Rate	Mean VAF	SD	Detected	Included	Error Rate	Mean VAF	SD
NPM1 (NM_002520)	c.860_863dupTCTG	p.Trp288Cysfs*12	3	3	0.00%	40.73%	26.61%	2	2	0.00%	43.11%	2.43%
IDH2 (NM_002168.3)	c.419G>A	p.Arg140Gln	3	3	0.00%	42.13%	1.63%	3	3	0.00%	47.32%	2.13%
DNMT3A (NM_022552)	c.2645G>A	p.Arg882His	3	3	0.00%	42.60%	0.78%	3	3	0.00%	44.94%	2.11%
STAG2 (NM_001042749.2)	c.2124del	p.Leu708Phefs*9	1	3	66.67%	NA	NA	0	0	NA	NA	NA
RUNX1 (NM_001754.4)	c.736A>C	p.Thr246Pro	1	3	66.67%	NA	NA	0	3	100.00%	NA	NA
ASXL1 (NM_015338.5)	c.1934dup	p.Gly646Trpfs*12	1	3	66.67%	NA	NA	0	3	100.00%	NA	NA
CEBPA (NM_004364.4)	c.68_78del	p.Pro23Glnfs*81	2	3	33.33%	56.15%	6.72%	2	2	0.00%	46.49%	3.97%
CEBPA (NM_004364.4)	c.895A>G	p.Ser299Gly	3	3	0.00%	43.77%	4.33%	2	2	0.00%	47.64%	2.07%
IDH2 (NM_002168.3)	c.419G>A	p.Arg140Gln	3	3	0.00%	51.67%	7.22%	3	3	0.00%	47.98%	2.38%
NRAS (NM_002524.4)	c.37G>C	p.Gly13Arg	3	3	0.00%	47.27%	2.38%	3	3	0.00%	45.38%	1.35%
EZH2 (NM_004456.4)	c.952del	p.Thr318Glnfs*3	3	3	0.00%	47.54%	2.16%	0	1	100.00%	NA	NA
EZH2 (NM_004456.4)	c.1321G>A	p.Glu441Lys	3	3	0.00%	50.08%	3.44%	1	1	0.00%	NA	NA
DNMT3A (NM_022552)	c.1961G>A	p.Gly654Asp	1	3	66.67%	NA	NA	0	3	100.00%	NA	NA
KMT2A (NM_001197104.1)	c.3253G>A	p.Val1085Met	1	3	66.67%	NA	NA	0	1	100.00%	NA	NA
GATA2 (NM_032638.4)	c.1084C>T	p.Arg362*	1	3	66.67%	NA	NA	0	2	100.00%	NA	NA
ASXL1 (NM_015338.5)	c.1934dup	p.Gly646Trpfs*12	2	3	33.33%	39.82%	4,41%	0	3	100.00%	NA	NA
DNMT3A (NM_022552)	c.2678G>C	p.Trp893Ser	3	3	0.00%	45.00%	3.49%	3	3	0.00%	44.08%	0.78%
TP53 (NM_000546.5)	c.652_670del	p.Val218fs	2	3	33.33%	75.75%	3.18%	3	3	0.00%	62.14%	25.26%
STAG2 (NM_001042749.2)	c.2858G>A	p.Arg953Gln	1	3	66.67%	NA	NA	0	0	NA	NA	NA
CUX1 (NM_181552.4)	c.1588A>C	p.Lys530Gln	1	3	66.67%	NA	NA	0	0	NA	NA	NA
ASXL1 (NM_015338.5)	c.1934dup	p.Gly646Trpfs*12	1	3	66.67%	NA	NA	0	3	100.00%	NA	NA
TP53 (NM_000546.5)	c.392A>T	p.Asn131Ile	3	3	0.00%	47.33%	2.02%	3	3	0.00%	47.33%	2.07%
EZH2 (NM_004456.4)	c.553G>C	p.Asp185His	1	3	66.67%	NA	NA	0	1	100.00%	NA	NA
ASXL1 (NM_015338.5)	c.1934dup	p.Gly646Trpfs*12	1	3	66.67%	NA	NA	0	3	100.00%	NA	NA
NPM1 (NM_002520)	c.863_864insCCTG	p.Trp288Cysfs*12	4	4	0.00%	33.29%	6.08%	3	3	0.00%	37.00%	12.17%
FLT3 (NM_004119.2)	c.1801_1802ins30	p.Asp600_Leu601ins10	4	4	0.00%	28.57%	11.75%	3	3	0.00%	31.03%	6.97%
FLT3 (NM_004119.2)	c.2505T>A	p.Asp835Glu	3	4	25.00%	2.15%	0.39%	2	3	33.33%	2.93%	0.31%
PHF6 (NM_032458.2)	c.548C>T	p.Ser183Phe	1	3	66.67%	NA	NA	3	3	0.00%	51.12%	1.93%
DNMT3A (NM_022552)	c.2264T>C	p.Phe755Ser	4	4	0.00%	42.12%	7.39%	3	3	0.00%	42.09%	4.57%
NRAS (NM_002524.4)	c.34G>A	p.Gly12Cys	3	4	25.00%	1.78%	0.37%	1	3	66.67%	NA	NA
RUNX1 (NM_001754.4)	c.1306dupT	p.Ser436Phefs*164	3	4	25.00%	40.69%	2.76%	3	3	0.00%	45.78%	4.45%
IDH1 (NM_005896.3)	c.394C>T	p.Arg132Cys	4	4	0.00%	16.64%	3.06%	3	3	0.00%	15.73%	1.16%
TET2 (NM_001127208.2)	c.3866G>T	p.Cys1289Phe	4	4	0.00%	40.83%	8.82%	3	3	0.00%	45.95%	0.48%
PHF6 (NM_032458.2)	c.346C>T	p.Arg116*	1	2	50.00%	NA	NA	3	3	0.00%	42.83%	1.85%
EZH2 (NM_004456.4)	c.2255G>C	p.*752Ser	4	4	0.00%	10.98%	1.95%	3	3	0.00%	9.60%	0.53%
SRSF2 (NM_003016.4)	c.161C>T	p.Ser54Phe	3	4	25.00%	5.85%	0.92%	3	3	0.00%	5.60%	0.43%
JAK2 (NM_004972.3)	c.1849G>T	p.Val617Phe	3	4	25.00%	2.35%	0.15%	3	3	0.00%	3.10%	0.80%
FLT3 (NM_004119.2)	c.2028C>G	p.Asn676Lys	2	4	50.00%	25.57%	3.56%	3	3	0.00%	19.91%	0.92%
FLT3 (NM_004119.2)	c.2504A>C	p.Asp835Ala	3	4	25.00%	5.21%	0.28%	3	3	0.00%	5.58%	0.88%
SH2B3 (NM_005475.2)	c.557G>T	p.Ser186Ile	0	0	NA	NA	NA	2	3	33.33%	56.40%	0.57%
PHF6 (NM_032458.2)	c.129_130insGG	p.Lys44Glyfs*38	1	2	50.00%	NA	NA	3	3	0.00%	51.12%	1.09%
EZH2 (NM_004456.4)	c.2212_2231del	p.Ala738Argfs*18	4	4	0.00%	26.68%	7.01%	2	3	33.33%	35.11%	0.47%
NRAS (NM_002524.4)	c.35G>A	p.Gly12Asp	4	4	0.00%	16.73%	2.38%	3	3	0.00%	17.54%	2.68%
EZH2 (NM_004456.4)	c.796G>A	p.Gly266Arg	1	4	75.00%	NA	NA	3	3	0.00%	4.40%	0.13%
ASXL1 (NM_015338.5)	c.1772dup	p.Tyr591*	4	4	0.00%	22.04%	1.58%	3	3	0.00%	21.23%	3.44%
ASXL1 (NM_015338.5)	c.1745_1758del	p.Pro582Argfs*32	3	4	25.00%	14.67%	3.79%	3	3	0.00%	13.26%	2.18%
TP53 (NM_000546.5)	c.916C>T	p.Arg306*	3	4	25.00%	4.31%	0.28%	3	3	0.00%	4.67%	0.16%
SF3B1 (NM_012433.3)	c.1873C>T	p.Arg625Cys	3	4	25.00%	7.99%	0.13%	3	3	0.00%	8.06%	1.67%
RUNX1 (NM_001754.4)	c.593A>G	p.Asp198Gly	4	4	0.00%	76.59%	2.58%	3	3	0.00%	79.85%	1.91%
ASXL1 (NM_015338.5)	c.2463_2478del	p.Asp821Glufs*12	4	4	0.00%	37.12%	10.03%	3	3	0.00%	44.93%	1.81%
ASXL1 (NM_015338.5)	c.2537G>A	p.Ser846Asn	4	4	0.00%	54.17%	6.94%	3	3	0.00%	55.40%	8.12%
SF3B1 (NM_012433.3)	c.2098A>G	p.Lys700Glu	3	4	25.00%	46.40%	1.40%	3	3	0.00%	48.53%	1.80%
CSF3R (NM_156039.3)	c.1853C>T	p.Thr618Ile	3	4	25.00%	46.23%	1.51%	3	3	0.00%	47.88%	1.12%
CSF3R (NM_156039.3)	c.2346dup	p.Ser783Glnfs*6	3	4	25.00%	37.77%	2.92%	0	3	100.00%	NA	NA

Detected: Number of centers which have detected the mutation; Included: Number of centers which include each variant in its NGS assay. Error Rate: Number of centers which failed to detect the variant regarding the total of centers. VAF: Variant allele frequency. SD: Standard deviation of VAF establishment among centers. NA: Not applicable; variants only were detected by one center

Mutation distribution

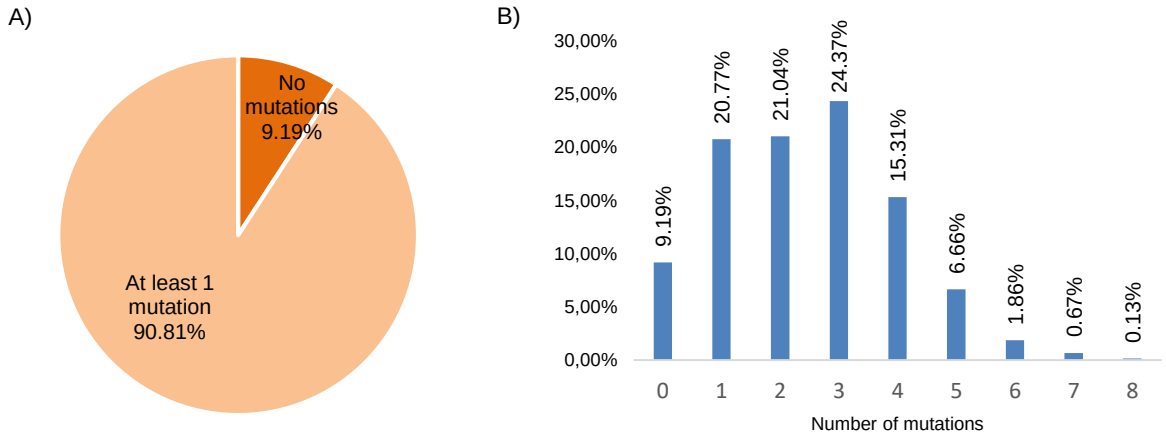


Figure S1. A) Percentage of patients according to the presence/absence of mutations. B) Samples distribution according to mutation number.

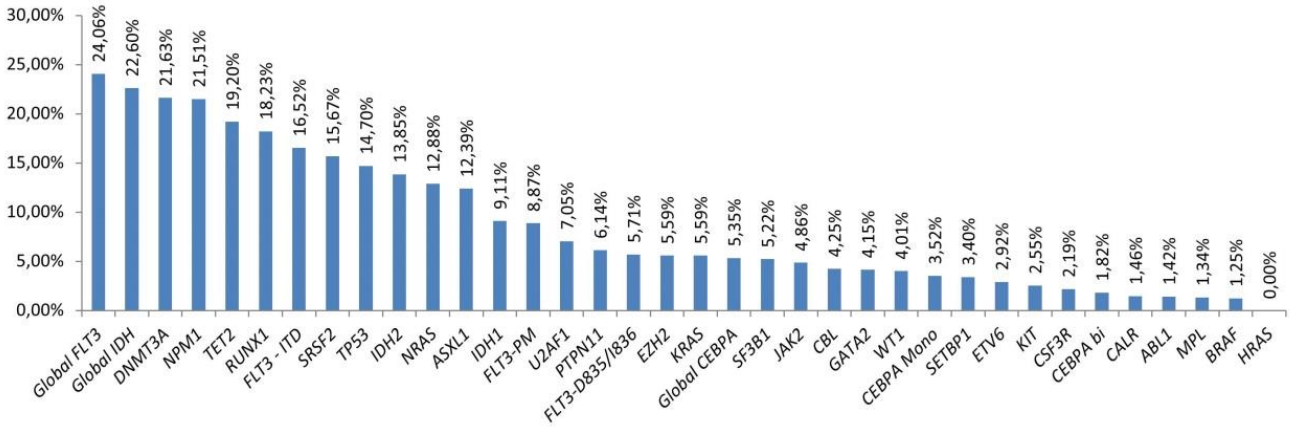


Figure S2. Mutational prevalence in 823 AML samples. Mono: monoallelic variant; bi: biallelic variant; PM: point mutations; ITD: internal tandem duplication. Global IDH: All variants detected in *IDH1* and *IDH2*. Global *FLT3*: All variants detected in *FLT3*.

Exclusion patterns. MEGS analysis

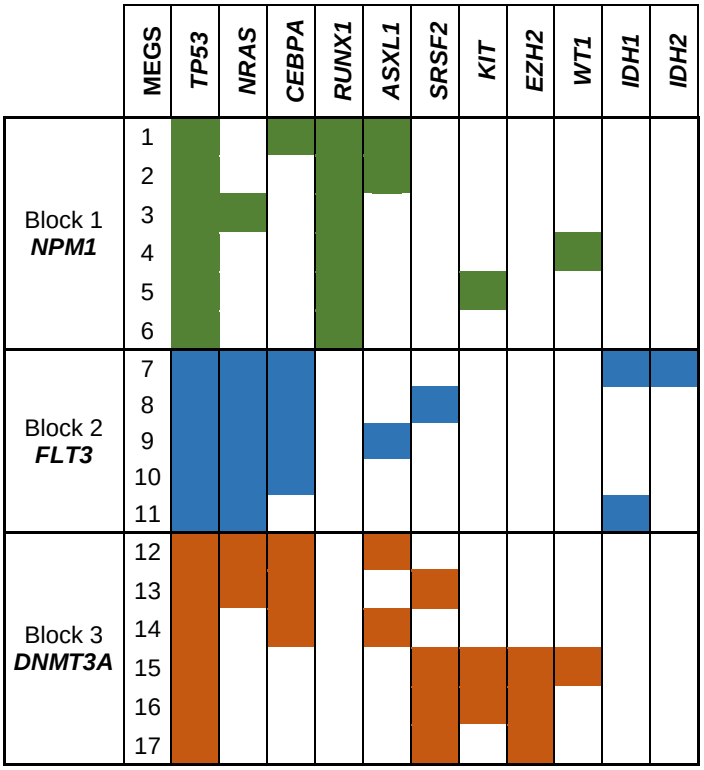


Figure S3. Schematic representation of gene exclusion patterns. 17 mutually exclusive gene sets (MEGS) are grouped in 3 blocks. All genes derived from MEGS analysis are shown in upper line. Colored squares indicate the included genes in each MEGS (line).

Table S3. Results from MEGS analysis. Exclusion patterns among genes.

	MEGS	Coverage	LRT	pNominal	pCorrected
1	*NPM1 TP53 RUNX1 CEBPA ASXL1*	0.63380282	74.7289156	2.70E-18	0
2	*NPM1 TP53 RUNX1 ASXL1*	0.60093897	69.5037372	3.81E-17	0
3	*NPM1 TP53 RUNX1*	0.53364632	100.110776	7.21E-24	0
4	Global *FLT3 TP53 NRAS CEBPA ASXL1*	0.61345853	64.7959538	4.15E-16	0
5	Global *FLT3 TP53 NRAS CEBPA*	0.53521127	57.9715794	1.33E-14	0
6	Global *FLT3 TP53 NRAS IDH1*	0.55399061	58.9706952	8.00E-15	0
7	Global *FLT3 TP53 NRAS CEBPA IDH2 IDH1*	0.66979656	82.5232132	5.22E-20	0
8	Global *FLT3 TP53 NRAS CEBPA SRSF2*	0.63380282	68.5592285	6.16E-17	0
9	*NPM1 TP53 RUNX1 KIT*	0.55242567	102.412508	2.25E-24	0
10	*DNMT3A TP53 CEBPA ASXL1*	0.50234742	47.0179694	3.52E-12	0
11	*DNMT3A TP53 SRSF2 EZH2 KIT*	0.55086072	68.2360199	7.25E-17	0
12	*DNMT3A TP53 SRSF2 EZH2 KIT WT1*	0.5743349	75.8696006	1.52E-18	0
13	*NPM1 TP53 RUNX1 WT1*	0.55555556	98.7308566	1.45E-23	0
14	*DNMT3A TP53 NRAS CEBPA ASXL1*	0.58685446	46.4554172	4.69E-12	0
15	*DNMT3A TP53 NRAS CEBPA SRSF2*	0.61032864	53.8452803	1.08E-13	0
16	*DNMT3A TP53 SRSF2 EZH2*	0.53051643	59.9203368	4.94E-15	0
17	*NPM1 TP53 RUNX1 NRAS*	0.61189358	72.6084052	7.91E-18	0

MEGS: mutually exclusive gene sets. Coverage: proportion of samples covered by the MEGS, LRT: likelihood ratio test for examining mutual exclusivity for a subset of genes

Age-related mutations

Table S4. Age-related mutational prevalence and p-values

	Diagnosis			Refractory AML			Relapsed AML		
	<65	≥65	p-value	<65	≥65	p-value	<65	≥65	p-value
ABL1	1.55%	0.97%	0.676	3.45%	3.03%	1.00	0.00%	3.03%	0.429
ASXL1	7.54%	15.95%	0.001	12.12%	15.15%	1.00	3.57%	20.00%	0.011
BRAF	1.04%	1.45%	1.00	0.00%	3.03%	1.00	0.00%	0.00%	NA
CALR	1.64%	1.23%	0.745	0.00%	3.03%	1.00	1.79%	2.22%	1.00
CBL	2.95%	5.83%	0.085	6.06%	3.03%	1.00	1.79%	4.44%	0.584
CEBPA mono	4.3%	3.1%	0.525	0.00%	3.03%	1.00	1.79%	8.90%	0.169
CEBPA bi	1.3%	1.8%	0.753	0.00%	3.03%	1.00	1.79%	4.44%	0.584
CEBPA	5.57%	4.91%	0.724	0.00%	6.06%	0.492	3.57%	13.33%	0.134
CSF3R	2.3%	2.76%	0.803	0.00%	0.00%	NA	3.57%	0.00%	0.501
DNMT3A	22.62%	19.33%	0.328	24.24%	12.12%	0.339	23.21%	26.67%	0.817
ETV6	1.97%	3.99%	0.165	0.00%	3.03%	1.00	1.79%	6.67%	0.321
EZH2	2.62%	7.98%	0.040	6.06%	9.09%	1.00	5.36%	6.67%	1.00
FLT3-PM	10.49%	7.06%	0.158	9.09%	15.15%	0.708	10.71%	6.67%	0.727
FLT3-ITD	19.34%	12.88%	0.030	15.15%	18.18%	1.00	21.43%	15.56%	0.610
GATA2	2.2%	4.74%	0.202	6.67%	3.33%	1.00	6.12%	8.33%	0.695
HRAS	0.0%	0.0%	NA	0.00%	0.00%	NA	0.00%	0.00%	NA
IDH1	7.54%	8.59%	0.663	12.12%	12.12%	1.00	8.93%	22.22%	0.090
IDH2	9.84%	16.34%	0.042	12.12%	15.15%	1.00	16.07%	26.67%	0.223
Global *IDH*	16.7%	23.9%	0.030	24.20%	27.30%	1.00	25.00%	46.70%	0.035
JAK2	2.3%	7.36%	0.003	3.03%	6.06%	1.00	5.36%	6.67%	1.00
KIT	3.28%	1.84%	0.314	3.03%	3.03%	1.00	3.57%	0.00%	0.501
KRAS	4.92%	6.44%	0.493	6.06%	12.12%	0.672	3.57%	0.00%	0.501
MPL	1.64%	1.84%	1.00	0.00%	0.00%	NA	0.00%	0.00%	NA
NPM1	29.18%	17.18%	0.001	9.09%	12.12%	1.00	17.86%	24.44%	0.466
NRAS	14.75%	12.27%	0.414	9.09%	15.15%	0.708	15.50%	8.89%	0.750
PTPN11	7.49%	5.54%	0.386	6.06%	15.15%	0.427	0.00%	2.63%	0.422
RUNX1	13.77%	19.02%	0.086	24.24%	30.30%	0.783	14.29%	24.44%	0.211
SETBP1	1.64%	3.99%	0.095	3.03%	9.09%	0.613	3.57%	6.67%	0.654
SF3B1	1.97%	6.44%	0.006	6.06%	12.12%	0.672	10.71%	4.44%	0.293
SRSF2	7.54%	22.09%	0.000	12.12%	30.30%	0.130	8.93%	22.22%	0.090
TET2	11.8%	27.91%	0.000	6.06%	24.24%	0.082	12.50%	24.44%	0.190
TP53	11.48%	19.33%	0.008	15.15%	9.09%	0.708	10.71%	15.56%	0.556
U2AF1	4.26%	8.9%	0.025	9.09%	12.12%	1.00	5.36%	4.44%	1.00
WT1	3.93%	2.45%	0.365	15.15%	0.00%	0.053	10.71%	4.44%	0.293

Mono: monoallelic variant; bi: biallelic variant; PM: point mutations; ITD: internal tandem duplication. Global IDH: All variants detected in *IDH1* and *IDH2*. NA: Not applicable. Shaded results are statistically significant.

Mutational stability in paired samples

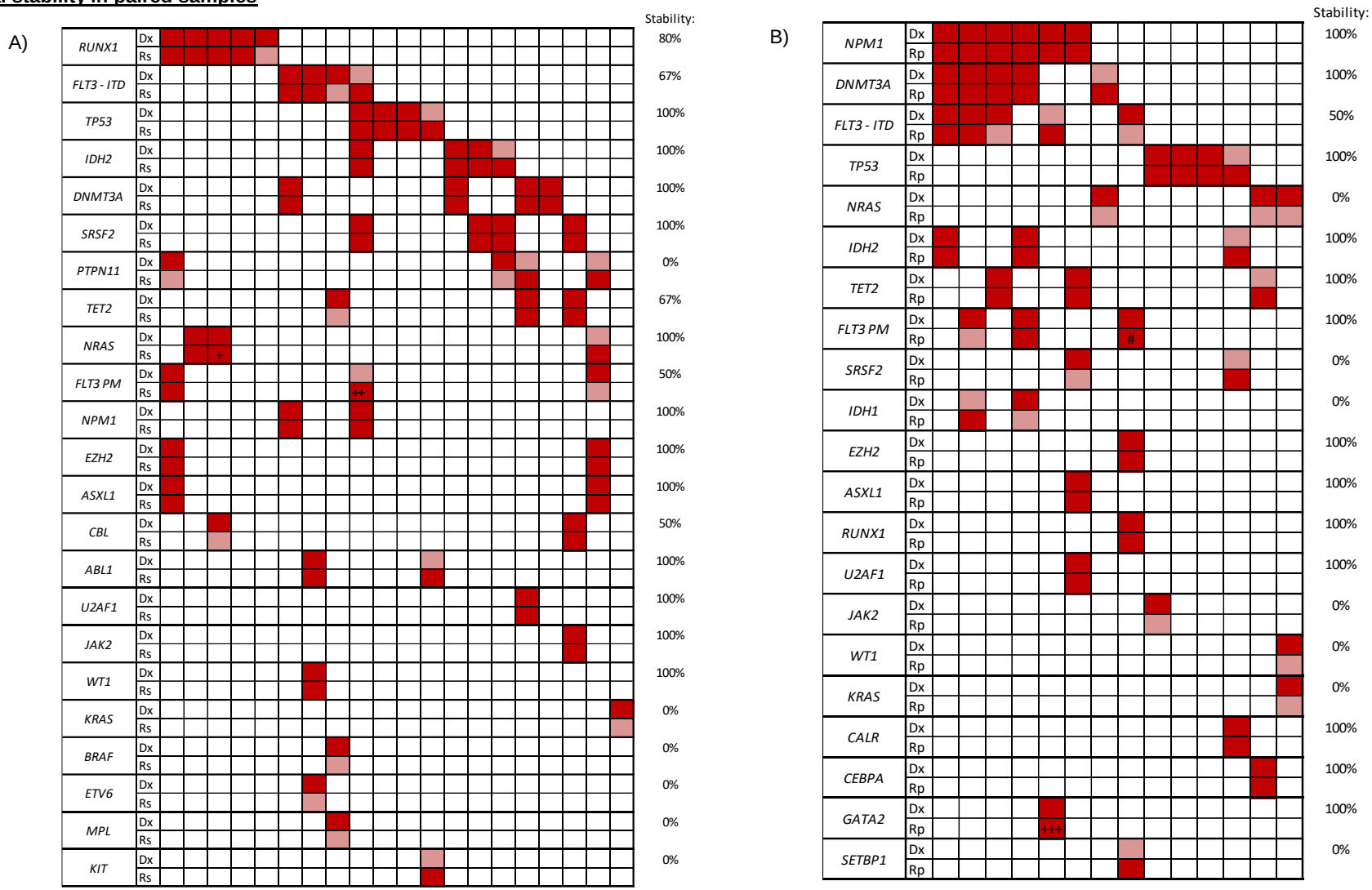


Figure S4. Mutation stability in diagnosis (DX) /refractory (RS) AML (panel A) and DX/relapse (RP) (Panel B). Dark red: mutated gene; Light red: lack of mutation detected in other moment disease; White: wild-type. #: one diagnosis mutation is lost at relapse. +: A second mutation is acquired. ++: Two new mutations are acquired. +++: keep the diagnosis mutation and acquires two new. ITD: Internal tandem duplication. PM: Point mutations.

Clinically relevant mutations

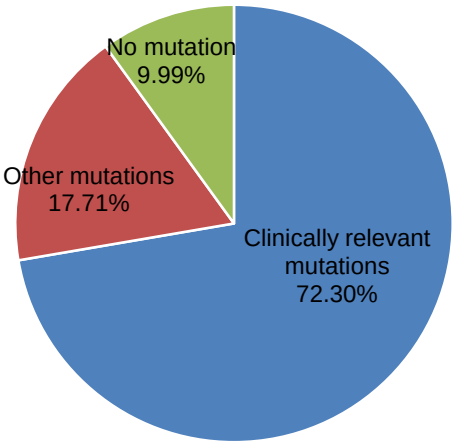


Figure S5. Percentage of patients with clinically relevant mutations.

***NPM1* mutations**

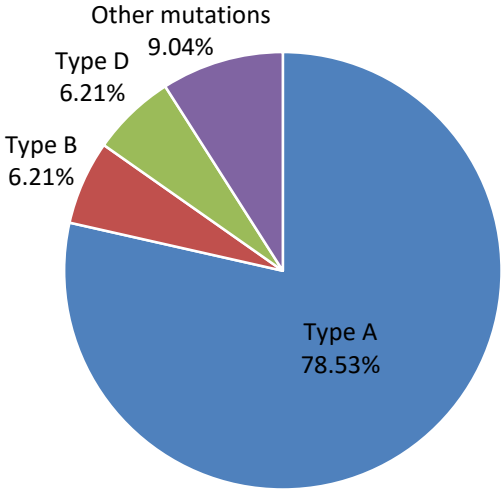


Figure S6. *NPM1* mutation type distribution.

Table S5. *NPM1* mutations. Coding sequence, mutation type and percentage are shown.

Coding	Protein	Type	N	%
c.860_863dupTCTG	p.Trp288Cysfs*12	A	139	78.53%
c.863_864insCATG	p.Trp288Cysfs*12	B	11	6.21%
c.863_864insCCTG	p.Trp288Cysfs*12	D	11	6.21%
c.863_864insTATG	p.Trp288Cysfs*12	R	2	1.13%
c.863_864insCAGA	p.Trp288Cysfs*12	ZM	2	1.13%
c.863_864insTCGC	p.Trp288Cysfs*12	YJ	2	1.13%
c.863_864insCCAG	p.Trp288Cysfs*12	K	1	0.56%
c.863_864insCAGG	p.Trp288Cysfs*12	G	1	0.56%
c.863_864insCCGG	p.Trp288Cysfs*12	J	1	0.56%
c.863_864insCTTG	p.Trp288Cysfs*12	I	1	0.56%
c.863_864insTAGG	p.Trp288Cysfs*12	ZA	1	0.56%
c.863_864insCGCG	p.Trp288Cysfs*12	-	1	0.56%
c.863_864insCTGC	p.Trp288Cysfs*12	-	1	0.56%
c.863_864insTGTA	p.Trp288Cysfs*12	-	1	0.56%
c.499G>A	p.Asp167Asn	-	1	0.56%
c.868_869delinsCGGTTC	p.Trp290Argfs*10	-	1	0.56%

N: number of positive samples; %: percentage of each mutation type.

***FLT3* mutations**

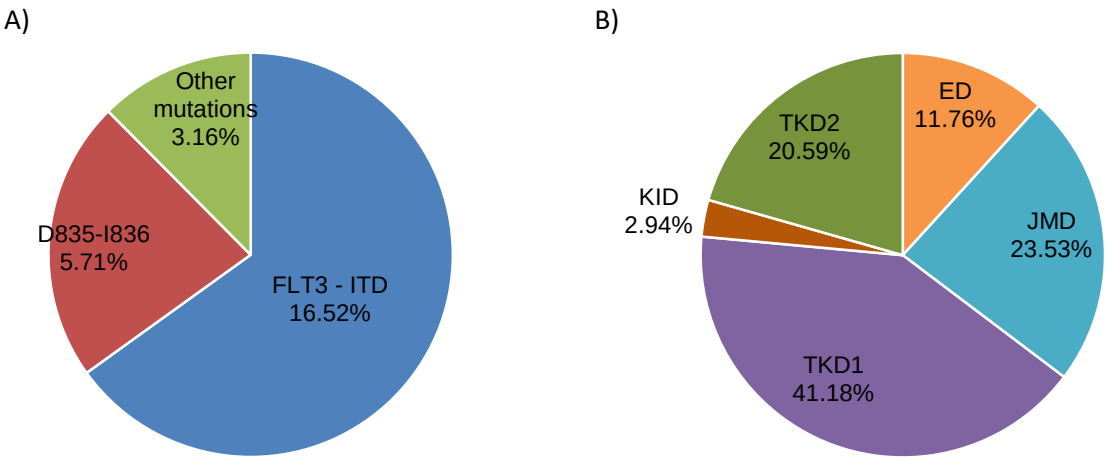


Figure S7. A) Percentage of samples with *FLT3*-internal tandem duplications (ITD), D835/I836 and other *FLT3* mutations. B) Location of other *FLT3* mutations (*FLT3*-ITD and FLT3-D835/I836 excluded). ED: extracellular domain, JMD: juxtamembrane domain, TKD1: tyrosine kinase 1 domain, TKD2: tyrosine kinase 2 domain, KID: kinase insert domain

***CEBPA* mutations**

Table S6. Biallelic mutations in *CEBPA*.

ID	Coding	Protein	VAF (%)
1	c.934_936dup	p.Gln312dup	37.57
	c.139_155delinsAGGC	p.Ala47Argfs*109	42.10
2	c.224del	p.Asp75Alafs*85	19.30
	c.92dup	p.Gly32Argfs*76	14.30
3	c.296_302del	p.Gly99Alafs*59	25.90
	c.542_543insTT	p.Gln182Serfs*137	26.30
4	c.112_116del	p.Gly38Argfs*68	41.67
	c.916_917delins21	p.Arg306delins8	45.02
5	c.341_350del	p.Gly114Alafs*43	37.80
	c.949_950insGTC	p.Glu316_Leu317insArg	41.40
6	c.949_951dup	p.Leu317dup	42.76
	c.120_121del	p.Gln41Alafs*66	39.17
7	c.476dup	p.Ile160Aspfs*10	45.81
	c.68_78del	p.Pro23Glnfs*81	44.98
8	c.45_56delinsCC	p.Met15Ilefs*142	19.31
	c.971T>C	p.Leu324Pro	16.41
9	c.917_918insTTG	p.Arg306_Asn307insCys	49.12
	c.247del	p.Gln83Serfs*77	47.18
10	c.997_1003del	p.Arg333Trpfs*87	30.48
	c.198_201dup	p.Ile68Leufs*41	32.03
11	c.1070_1071insTTGGGG	p.Cys357_Ala358insTrpGly	20.67
	c.4G>T	p.Glu2*	17.56
12	c.78_87dup	p.Ala30Glnfs*81	10.49
	c.74_84dup	p.Ala29Argfs*135	9.27
13	c.955_971del	p.Ser319Alafs*78	50.69
	c.971T>C	p.Leu324Pro	46.07
14	c.245_249dup	p.His84Serfs*78	43.65
	c.815dup	p.Lys273Glnfs*48	42.55
15	c.247del	p.Gln83Serfs*77	50.22
	c.917_918insTTG	p.Arg306_Asn307insCys	44.49

VAF: Variant allele frequency

***IDH1* and *IDH2* mutations**

Table S7. Protein and coding change for mutations in A) *IDH1* and *IDH2* and B) patients with mutations in both genes.

	Gene	Coding	Protein	N	%
A)	*IDH1*	c.394C>T	p.Arg132Cys	39	52.00%
		c.395G>A	p.Arg132His	23	30.67%
		c.394C>G	p.Arg132Gly	8	10.67%
		c.394C>A	p.Arg132Ser	3	4.00%
		c.395G>T	p.Arg132Leu	2	2.67%
	IDH2	c.419G>A	p.Arg140Gln	91	79.82%
		c.515G>A	p.Arg172Lys	18	15.79%
		c.418C>T	p.Arg140Trp	3	2.63%
		c.419G>T	p.Arg140Leu	2	1.75%

	Gene	Coding	Protein	VAF (%)	ID
B)	*IDH1*	c.395G>A	p.Arg132His	26.77	1
	IDH2	c.419G>A	p.Arg140Gln	8.15	
	IDH1	c.395G>A	p.Arg132His	7.22	2
	IDH2	c.419G>A	p.Arg140Gln	22.10	
	IDH1	c.395G>A	p.Arg132His	40.50	3
	IDH2	c.418C>T	p.Arg140Trp	2.00	

N: number of positive samples; %: percentage of each protein change; VAF: Variant allele frequency

References

1. INE. Instituto Nacional de Estadística. https://ine.es/ (accessed May 5, 2020).
2. Onecha E, Linares M, Rapado I, et al. A novel deep targeted sequencing method for minimal residual disease monitoring in acute myeloid leukemia. Haematologica 2019;104(2):288–296.
3. Hua X, Hyland PL, Huang J, et al. MEGSA: A Powerful and Flexible Framework for Analyzing Mutual Exclusivity of Tumor Mutations. Am J Hum Genet 2016;98(3):442–455.

Appendix

Institutions and clinicians participating in the PETHEMA epidemiologic registry of acute myeloid leukemia and acute promyelocytic leukemia:

Argentina (Grupo Argentino para el Tratamiento de la Leucemia Aguda - **GATLA**)— Hospital de Clínicas, Buenos Aires: F. Rojas; H. Longoni; Fundaleu, Buenos Aires: G. Milone, I. Fernández, Clínica Conciencia, Neuquén: R. Ramirez; Hospital Rossi ,La Plata : C. Canepa, S. Saba, G. Balladares, Hospital General San Martin, Parana : G. Milone, C. Ventiurini, R. Mariano, P. Negri; Hospital Italiano de La Plata, La Plata: M. V. Prates, J. Milone; Hospital General San Martín, La Plata:P. Fazio, M. Gelemur; Hospital Clemente Alvarez, Rosario: G. Milone, S. Ciarlo, F. Bezares; Hospital de Córdoba, Córdoba: L. López , Hospital Privado de Córdoba, Córdoba: J. J.García; Instituto Privado Hematología, Paraná: P. Negri, M. Giunta, G. Milone; Hospital Teodoro Alvarez, Buenos Aires: M. Kruss; Hospital Tornú, Buenos Aires: D. Lafalse, G. Milone; Hospital Gobernador Centeno, La Pampa: E. Marquesoni, M. F. Casale; Hospital Italiano de Buenos Aires, Buenos Aires: A. Gimenez , E. B. Brulc, M. A. Perusini; Complejo Médico Policía Federal, La Plata: G. Milone, L. Palmer; **Colombia** (Asociación Colombian de Hematología y Oncología - **ACHO**)— Clínica La Estancia, Popayán: M. E. Correa; Fundación Valle del Lili , Cauca: F.J. Jaramillo, J. Rosales; Instituto FOSCAL, Bucaramanga: C. Sossa, J. C. Herrera ; Hospital Pablo Tobón Uribe, Antioquia: M. Arango; **Poland** (Polish Adult Leukemia Group - **PALG**)—City Hospital Legnica, Baja Silesia: J. Holojda; IHIT Hematology and transfusiology institute, Warszawa: A. Golos, A. Ejduk; Wojewódzki Szpital Specjalistyczny w Olsztynie, Olsztyn: B. Ochrem; WIM (Military Institute of Medicine in Warsaw), Warszawa: G. Małgorzata; Poland Medical University of Warsaw Banacha, Warszawa:A. Waszczuk-Gajda, J. Drozd-Sokolowska, M. Czemerska, M. Paluszewska; Medical University School Gdansk, Gdansk: E. Zarzycka; Wojewódzki Szpital Specjalistyczny im. Św.. Jadwigi Śląskiej, Opole: A. Masternak; Hospital Brzozow, Brzozow: Dr. Hawrylecka; Medical University Lublin, Lublin: M. Podhoreka, K. Giannopoulos, T. Gromek; Medical University Bialystok, Bialystok: J. Oleksiuk; Silesian Medical University Katowice, Katowice:bA. Armatys, G. Helbig; Universitary Hospital Wroclaw ,Wroclaw: M. Sobas; Poznan University of Medical Sciences, Pozna: A. Szczepaniak; Rydigier City Hospital Krakow, Krakow: E. Rzenno, M. Rodzaj; Collegium Medicum Jagiellonian University Krakow, Krakow: B. Piatkowska-Jakubas; City Hospital Rzeszów,Rzeszów: A. Skret; Medical University Lodz, Lodz: A. Pluta, M. Czemerska; Center of Oncology Kielce, Kielce: E. Barańska; Medical University of Warsaw , Warsaw : M. Paluszewska; **Portugal**—Hospital de Santa Maria-Lisboa, Lisboa:G. Vasconcelos, J. Brioso; IPOFG Lisboa, Lisboa: A. Nunes, I. Bogalho; Centro Hospitalar e Universitário de Coimbra, Coimbra: A. Espadana, M. Coucelo, S. Marini, J. Azevedo, A. I. Crisostomo, L. Ribeiro, V. Pereira; Centro Hospitalar

de Lisboa Central E. P. E, Lisboa: A. Botelho; Instituto Português Oncologia do Porto Francisco Gentil, Porto: J. M. Mariz; Centro Hospitalar São João, Porto: J. E. Guimaraes, E. Aguiar; Centro Hospitalar do Porto E.P.E. Porto: J. Coutinho; **Spain** (Programa Español de Tratamiento de las Hemopatías Malignas, **PETHEMA**)—Complejo Hospitalario Universitario A Coruña, A Coruña: V. Noriega, L. García, C. Varela, G. Debén, M. R. González; Hospital Clínico Universitario de Santiago, A Coruña: M. Encinas, A. Bendaña, S. González, J.L. Bello, M. Albors; Hospital General de Albacete, Albacete: L. Algarra, J.R.Romero, J.S.Bermon, M.J. Varo; Hospital Vinalopó, Alicante: V. López, E. López; Hospital Virgen de los Lirios, Alcoy: C. Mora, C. Amorós; Hospital General Elche, Alicante: E. López, A. Romero; Hospital Torrevieja Salud, Alicante: A. Jaramillo, N. Valdez, I. Molina, A. Fernández, B. Sánchez; Hospital de la Marina Baixa Villajoyosa, Alicante: A. García; Hospital General de Elda, Alicante: V. Castaño, T. López, J. Bernabeu; Hospital de Denia-Marina Salud, Alicante: M.J. Sánchez; Hospital de la Vega Baja de Orihuela, Alicante: C. Fernández; Hospital General de Alicante, Alicante: C. Gil, C. Botella, P. Fernández, M. Pacheco, F. Tarín; J.J. Verdú; Complejo Hospitalario Torrecardenas, Almeria: M.J. García, A. Mellado, M.C. García, J. González; Hospital Central de Asturias, Asturias: T. Castillo, E. Colado, S. Alonso; Complejo Asistencial Ávila, Ávila: I. Recio, M. Cabezudo, J. Davila, M. J. Rodríguez, A. Barez, B. Díaz; Hospital Don Benito-Villanueva, Badajoz: J. Prieto; Institut Catala d'Oncologia L´Hospitalet, Barcelona: M. Arnan, C. Marín, M. Mansilla; Hospital de Cruces, Bizkaia: A. Balaberdi, M. E. Amutio, R. A. del Orbe, I. Ancin, J. C. Ruíz; Hospital Galdakao-Usansolo, Bizkaia: M. Olivalres,C. Gómez, I. gonzález, M. Celis, K. Atutxa, T. Carrascosa, T. Artola, M. Lizuain; Basurtuko Ospitalea, Bizkaia: J .I. Rodriguez, O. Arce, J. A. Márquez, J. Atuch, F. Marco de Lucas, Z. Díez, B. Dávila; Hospital Santos Reyes, Burgos: R. Cantalejo, M. Díaz; Hospital Universitario de Burgos, Burgos: J. Labrador, F. Serra, G. Hermida, F. J. Díaz, P. de Vicente, R. Álvarez: Hospital Santiago Apóstol, Burgos: C. Alonso, Hospital San Pedro de Alcántara, Cáceres: J. M. Bergua; Hospital Campo Arañuelo, Cáceres: N. Ugalde; Hospital Virgen del Puerto, Cáceres: E. Pardal; Hospital General Jerez de la Frontera, Cádiz: R. Saldaña, F. Rodríguez, E. Martín, L. Hermosín; Hospital Universitario Puerta del Mar, Cádiz: M. P. Garrastazul, I. Marchante, J. A. Raposo, F. J. Capote; Hospital U. Marqués de Valdecilla, Cantabria: M. Colorado, A. Batlle, L. Yañez, S. García, P. González, E. M. Ocio, M. Briz, A. Bermúdez, S. García; Consorcio Hospitalario Provincial de Castellón, Castellón: C. Jiménez, S. Beltrán; Hospital de Vinaroz: M. Montagud; Hospital Universitario de La Plana, Castellón: I. Castillo; Hospital General de Castellón, Castellon: R. García, A. Gascón, J. Clavel, A. Lancharro, L. Lnares; Hospital Santa Bárbara , Ciudad Real: M. M. Herráez, A. Milena; Hospital Virgen de Altagracia, Ciudad Real: M. J. Romero, Hospital General de Ciudad Real, Ciudad Real: B. Hernández, C. Calle, R. Benegas; Hospital Gutierrez Ortega de Valdepeñas, Ciudad Real: Dr. Bolívar; Hospital General La Mancha Centro, Ciudad Real: M. A. Pozas; Hospital Reina Sofia, Córdoba: J. Serrano, F. J. Dorado, J. Sánchez, M. C. Martínez; Hospital Virgen de la Luz, Cuenca: C. J. Cerveró, M. J. Busto; Hospitales HUVN-HC San Cecilio

de Ganada, Granada: M. Bernal, E. López, L. Moratalla, Z. Mesa, M. Jurado, A. Romero, P. González; Complejo Hospitalario Universitario Granada, Granada: L. Moratalla, A. Romero, L. López; Hospital Universitario de Guadalajara, Guadalajara: M. Díaz, D. De Miguel, A. B. Santos, J. Arbeteta; Hospital Donostia, Donosti: E. Pérez, N. Caminos, N. Uresandi, N. Argoitiaituart, T. Artola, J. Swen, A. Uranga, I. Olazaba, M. Lizuain, E. Gainza, P. Romero; Hospital Juan Ramón Jimenez Huelva, Huelva: E. Gil, A. J. Palma, K. G. Gómez, M. Solé, J. N. Rodríguez; Hospital San Jorge, Huesca: I. M. Murillo, J. Marco, J. Serena, V. Marco; Hospital de Barbastro, Huesca: M. Perella, L. Costilla; Hospital General Ciudad de Jaen, Jaén: J. A. López, A. Baena, P. Almagro; Hospital San Pedro de Logroño, La Rioja: M. Hermosilla, A. Esteban, B. A. Campeny, M. J. Nájera, P. Herrra; Hospital Insular de Las Palmas, Las Palmas: R. Fernández, J. D. González, L. Torres; Hospital Dr. Negrín, Las Palmas: S. Jiménez; M. T. Gómez, C. Bilbao, C. Rodríguez; Hospital Doctor José Molina Orosa, Las Palmas: A. Hong, Y. Ramos de Laón, V. Afonso; Hospital Universitario de León, León: F. Ramos, M. Fuertes; Hospital Comarcal del Bierzo, León: E. de Cabo, C. Aguilera, M. Megido; Hospital Universitari Arnau de Vilanova de Lleida, Leida: T. García; Hospital Lucus Augusti, Lugo: E. Lavilla, M. Varela, S. Ferrero, M. J. Sánchez, L. López, J. Arias, L. Vizcaya; Hospital Infanta Sofía, Madrid: A. Roldán, A. Vilches, M. J. Penalva, J. Vázquez; Hospital Central de la Defensa Gómez Ulla, Madrid: M. T. Calderón, A. Matilla, C. Serí, M. J. Otero, N. García, E. Sandoval; Hospital de Fuenlabrada, Madrid: C. Franco, R. Flores, P. Bravo, A. López; Hospital Fundación Jiménez Díaz, Madrid: J. L. López, C. Blas, A. Díez, J. M. Alonso, C. Soto, A. Arenas; Hospital U. Príncipe de Asturias, Madrid: J. García, Y. Martín, P. S. Villafuerte, E. Magro; Hospital Puerta de Hierro, Madrid: G. Bautista; A. De Laiglesia; Hospital Gregorio Marañón, Madrid: G. Rodríguez, L. Solán, M. Chicano, P. Balsalobre, S. Monsalvo, P. Font, D. Carbonell, C. Martínez; Hospital U. La Paz, Madrid: K. Humala, A. E. Kerguelen, D. Hernández, M. Gasior, P. Gómez, I. Sánchez; Hospital Madrid Norte Sanchinarro, Madrid: S. Redondo, L. Llorente, M. Bengochea, J. Pérez; Hospital Sanitas Torrejón, Madrid: A. Sebrango, M. santero, A. Morales; Hospital La Princesa, Madrid: A. Figuera, P. Villafuerte, A. Alegre, E. Fernández; Hospital Ruber Internacional, Madrid: A. Alonso; Hospital 12 de Octubre, Madrid: M. P. Martínez, J. Martínez, M. T. Cedena, L. Moreno; MD Anderson Cancer Center, Madrid: A. De la Fuente; Hospital Sanitas La Zarzuela, Madrid: D. García; Hospital Universitario Quiron, Madrid: C. Chamorro, V. Pradillo, E. Martí, J. M. Sánchez, I. Delgado, A. Alonso; Hospital Rey Juan Carlos, Madrid: B. Rosado, A. Velasco, C. Miranda, G. Salvatierra, J. M. Alonso,, J. L. López; Hospital Infanta Leonor, Madrid: M. Foncillas, J. A. Hernández; Hospital Universitario de Getafe, Madrid: C. Escolano, L. García, I. Delgado; Hospital Clínico San Carlos, Madrid: C. Benabente, R. Martínez, M. Polo, E. Anguita; Hospital Universitario Severo Ochoa, Madrid: R. Riaza, G. Amores, M. J. Requena; Hospital Universitario Fundación Alcorcón, Madrid: F. Javier, L. Villalón; Hospital Universitario Moncloa, Madrid: C. Aláez, V. Pradillo, S. Nistal, B. Navas; Hospital Universitario de Móstoles, Madrid: J. Sánchez, M. A. Andreu; Hospital Ramon y Cajal, Madrid: P. Herrera,

J. López; Hospital U. Virgen de la Victoria, Málaga: M. García, M. J. Moreno, A. Fernández, M. P. Queipo; Hospital Quirón salud Málaga, Málaga: A. Hernández; Hospital Regional de Málaga, Málaga: M. Barrios, A. Heiniger, A. Jiménez, A. Contento, F. López, M. Alcalá; Hospital Vithas Xanit Internacional, Málaga: S. Lorente, M. González, E. M. Morales, J. Gutierrez; Hospital Virgen del Castillo, Murcia: M. J. Serna, V. Beltrán; Hospital Santa Lucía de Cartagena, Murcia: M. Romera, M. Berenguer, A. Martínez, A. Tejedor; Hospital Morales Meseguer, Murcia: M. L. Amigo, F. Ortuño, L. García, A. Jerez, O. López; Hospital U. Virgen de la Arrixaca, Murcia: J. M. Moraleda, P. Rosique, J. Gómez, M. C. Garay; Hospital Los Arcos Mar Menor, Murcia: P. Cerezuela, C. Martínez, A. B. MArtínez, A. González; Hospital STª Mª del Rosell, Murcia: J. Ibáñez; Clínica San Miguel, Navarra: M. J. Alfaro; Complejo Hospitalario de Navarra, Navarra: M. Mateos, M. A. Goñi, M. A. Araiz, A. Gorosquieta, M. Zudaire, M. Viguria, A. Zabala, M. Alvarellos, I. Quispe, M. P. Sánchez, G. Hurtado, M. Pérez, Y. Burguete, N. Areizaga, T. Galicia; Clínica Universitaria de Navarra, Navarra: J. Rifón, A. Alfonso, F. Prósper,M. Marcos, L. E. Tamariz, V. Riego. A. Manubens, M. J. Larrayoz, M. J. Calasanz, A. Mañú, B. Paiva, I. Vázquez, L. Burgos; Complejo Hospitalario de Ourense (CHOU), Ourense: M. Pereiro, M. Rodríguez, M. C. Pastoriza, J. A. Mendez, J. L. Sastre, M. Iglesias, C. Ulibarrena, F. Campoy; Hospital Valdeorras, Ourense: D. Jaimes; Hospital Rio Carrión, Palencia: J. M. Alonso, B. Albarrán, J. Solano, A. Silvestre; Complexo Hospitalario Universitario de Vigo, Vigo: C. Albo, S. Suarez, C. Loureiro, I. Figueroa, M. Rodríguez, M. A. Fernández, A. Martínez, C. Poderós, J. Vazquez, L. Iglesias, A. Nieto, T. Torrado, A. M. Martínez; Hospital Provincial de Pontevedra, Pontevedra: M.L. Amador, P. Oubiña, E. Feijó, A. Dios, I. Loyola, R. Roreno; Hospital POVISA, Pontevedra: A. Simiele, L. Álvarez, V. Turcu; Hospital U. Salamanca, Salamanca: B. Vidriales, M. González, R. García, A. Avendaño, C. Chillón, E. Pérez, V. González; Hospital General La Palma, Santa Cruz de Tenerife: J. V. Govantes, S. Rubio, M. Tapia; Hospital General de Segovia, Segovia: C. Olivier, J. A. Queizán; Hospital U. Virgen Macarena, Sevilla: : O. Pérez, J. A. Vera, C. Muñoz, A. rodriguez, N. González; Hospital U. Virgen del Rocio, Sevilla: J. A. Pérez, E. Soria, I.Espigado, J. Falantes, I. Montero, P. García, E. Rodríguez, E. Carrillo, T. Caballero, C. García; Hospital Virgen de Valme, Sevilla: C. Couto, I. Simón, M. Gómez; Hospital Virgen del Mirón de Soria, Soria: C. Aguilar; Hospital Universitario Canarias, Tenerife: B. J. González, S. Lakhwani, A. Bienert, B. González; Hospital Universitario Nuestra Señora de Candelaria, Tenrife: A. Cabello, A. Y. Oliva, H. González; Hospital Obispo Polanco, Teruel: N. González, Hospital de Alcañiz, Teruel: L. Sancho, M. Paricio, L. Perdiguer; Hospital General Nuestra Señora del Prado, Toledo: F. Solano, A. Lerma, M. D. Martínez; Hospital Virgen de la Salud de Toledo, Toledo: M. I. Gómez, A. Yeguas; Hospital U. La Fe, Valencia: P. Montesinos, E. Barragán, C. Sargas, R. Amigo, D. Martinez, B. Boluda, R. Rodríguez, E. Acuña, I. Cano; Hospital de Requena, Valencia: A. Escrivá, M. Pedreño; Hospital de Lluis Alcanyis de Xativa, Valencia: R. Renart; IVO (Instituto Valenciano de Oncología), Valencia: A. Navalón; Hospital de Sagunto, Valencia: I. Castillo, M. Orts; Hospital Dr. Peset,

Valencia: M. J. Sayas, M. J. Fernández, M. L. Juan, E. Gómez, M. Gimeno, E. Donato, M. Cejalvo, J. Marco; Hospital Clínico Universitario, Valencia: M. Tormo, M. Calabuig, B. Navarro, I. Martin, E. Villamont, A. Miralles; Hospital de La Ribera, Valencia: R. Lluch; Hospital Casa de la Salud, Valencia: J. García; Hospital de Gandía, Valencia: M. Moragues, M. A. Ruiz; Hospital Arnau de Vilanova, Valencia: A. López, C. Benet, M. Valero; Hospital General de Valencia, Valencia: M. Linares, R. Collado, M. Orero, P. Ibañez, M. J. Lis, P. L. Pérez, M. Roig, M. López, A. V. Mena; Hospital Manises, Valencia: I. Picón, V. Cánovas, A. Palacios, E. Martí; Hospital Clínico de Valladolid, Valladolid: R. Cuello, J. Borrego, M. burgois; Hospital Rio Hortega, Valladolid: A. Cantalapiedra, O. Norberto, E. Angomas, B. Cidoncha; Hospital Universitario Araba, Victoria: L. Cuevas, D. Robles, A. Mendiazabal, I. Oiartzabal, J. M. Guinea de Castro; Hospital Virgen de la Concha, Zamora: C. Montes, M. Pérez, L. García; Hospital Royo Villanova, Zaragoza: V. Carrasco, A. Pérez, L. López, J. J. Moneva; Hospital Clínico U. Lozano Blesa, Zaragoza: M. Olave, E. Bonafonte, L. Mayor, G. Azaceta, L. Palomera; Hospital Ernest Lluch Martin, Zaragoza: M. Malo, M. J. Escobar; Hospital Quiron Salud Zaragoza, Zaragoza: J. M. Grasa; Hospital Miguel Servet, Zaragoza: B. De Rueda, A. Aulés, C. Salvador, V. Ansó, A. Iborra, P. Delagado, A. Rubio; **Uruguay** —Hospital de Clínicas, Montevideo: M. Stevenazzi, I. Alpire, V. Irigoin, L. Díaz, C. Guillermo, R. Guadagna, S. Grille, C. Oliver, M. Boada, V. Vales; Hospital Maciel, Montevideo: A. I. Prado; COMERO (Cooperativa Medica de Rocha), Rocha: A. P. De los Santos.

cancers

MDPI

Article

Molecular Landscape and Validation of New Genomic Classification in 2668 Adult AML Patients: Real Life Data from the PETHEMA Registry

Claudia Sargas [1], Rosa Ayala [2], María José Larráyoz [3], María Carmen Chillón [4], Estrella Carrillo-Cruz [5], Cristina Bilbao-Sieyro [6], Esther Prados de la Torre [7], David Martínez-Cuadrón [8,9], Rebeca Rodríguez-Veiga [8], Blanca Boluda [8], Cristina Gil [10], Teresa Bernal [11], Juan Miguel Bergua [12], Lorenzo Algarra [13], Mar Tormo [14], Pilar Martínez-Sánchez [2], Elena Soria [5], Josefina Serrano [7], Juan Manuel Alonso-Domínguez [15], Raimundo García-Boyero [16], María Luz Amigo [17], Pilar Herrera-Puente [18], María José Sayas [19], Esperanza Lavilla-Rubira [20], Joaquín Martínez-López [2], María José Calasanz [3], Ramón García-Sanz [4], José Antonio Pérez-Simón [5], María Teresa Gómez-Casares [6], Joaquín Sánchez-García [7], Eva Barragán [10,21,*,†], Pau Montesinos [8,10,†] and on behalf of PETHEMA group [‡]

1 Grupo Acreditado de Investigación en Hematología, Instituto de Investigación Sanitaria La Fe (IIS La Fe), 46026 Valencia, Spain
2 Hospital Universitario 12 de Octubre, National Cancer Research Center, Complutense University, 28041 Madrid, Spain
3 CIMA LAB Diagnostics, Departamento de Bioquímica y Genética, Universidad de Navarra, 31008 Pamplona, Spain
4 Servicio de Hematología, Hospital Universitario de Salamanca (HUS/IBSAL), CIBERONC, Centro de Investigación del Cáncer–IBMCC (USAL–CSIC), 37007 Salamanca, Spain
5 Hospital Universitario Virgen del Rocío, Instituto de Biomedicina (IBIS/CSIC/CIBERONC), Universidad de Sevilla, 41013 Sevilla, Spain
6 Hospital Universitario de Gran Canaria Dr. Negrín, 35010 Las Palmas de Gran Canaria, Spain
7 Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Hospital Universitario Reina Sofía, Universidad de Córdoba (UCO), 14004 Córdoba, Spain
8 Servicio de Hematología, Grupo Acreditado de Investigación en Hematología, Hospital Universitario y Politécnico La Fe, Instituto de Investigación Sanitaria La Fe (IIS La Fe), 46026 Valencia, Spain
9 Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Instituto de Investigación Sanitaria La Fe (IIS La Fe), 46026 Valencia, Spain
10 Hospital General Universitario de Alicante, 03010 Alicante, Spain
11 Hospital Universitario Central de Asturias, Instituto Universitario (IUOPA), Instituto de Investigación del Principado de Asturias (ISPA), 33011 Oviedo, Spain
12 Hospital Universitario San Pedro de Alcántara, 10003 Cáceres, Spain
13 Hospital Universitario General de Albacete, 02006 Albacete, Spain
14 Hospital Clínico Universitario–INCLIVA, 46010 Valencia, Spain
15 Hospital Universitario Fundación Jiménez Díaz, 28040 Madrid, Spain
16 Hospital Universitario General de Castellón, 12004 Castellón de la Plana, Spain
17 Hospital Universitario Morales Messeguer, 30008 Murcia, Spain
18 Hospital Universitario Ramón y Cajal, 28034 Madrid, Spain
19 Hospital Universitari Dr. Peset, 46017 Valencia, Spain
20 Complexo Hospitalario Lucus Augusti, 27003 Lugo, Spain
21 Servicio Análisis Clínicos, Grupo Acreditado de Investigación en Hematología, Hospital Universitario y Politécnico La Fe, Instituto de Investigación Sanitaria La Fe (IIS La Fe), 46026 Valencia, Spain
* Correspondence: barragan_eva@gva.es; Tel.: +34-961-244589
† These authors contributed equally to this work.
‡ The consortium members are listed in Appendix A.

check for updates

Citation: Sargas, C.; Ayala, R.; Larráyoz, M.J.; Chillón, M.C.; Carrillo-Cruz, E.; Bilbao-Sieyro, C.; Prados de la Torre, E.; Martínez-Cuadrón, D.; Rodríguez-Veiga, R.; Boluda, B.; et al. Molecular Landscape and Validation of New Genomic Classification in 2668 Adult AML Patients: Real Life Data from the PETHEMA Registry. *Cancers* **2023**, *15*, 438. https://doi.org/10.3390/cancers15020438

Academic Editor: Sibaji Sarkar

Received: 22 November 2022
Revised: 30 December 2022
Accepted: 7 January 2023
Published: 10 January 2023

Simple Summary: Next–Generation Sequencing (NGS) has provided a deeper genetic understanding of acute myeloid leukemia (AML) that has been recently incorporated into AML classification and risk–stratification guidelines. Single molecular analysis has become inefficient and molecular testing based on NGS is emerging as an irreplaceable diagnostic tool in clinical settings. The

PETHEMA cooperative group has constituted a nationwide NGS network with centralized analysis in seven high–skilled laboratories. The study of molecular profiles in the "real–life" PETHEMA cohort supports the increasing role of NGS on the clinical management of AML patients.

Abstract: Next–Generation Sequencing (NGS) implementation to perform accurate diagnosis in acute myeloid leukemia (AML) represents a major challenge for molecular laboratories in terms of specialization, standardization, costs and logistical support. In this context, the PETHEMA cooperative group has established the first nationwide diagnostic network of seven reference laboratories to provide standardized NGS studies for AML patients. Cross–validation (CV) rounds are regularly performed to ensure the quality of NGS studies and to keep updated clinically relevant genes recommended for NGS study. The molecular characterization of 2856 samples (1631 derived from the NGS–AML project; NCT03311815) with standardized NGS of consensus genes (*ABL1*, *ASXL1*, *BRAF*, *CALR*, *CBL*, *CEBPA*, *CSF3R*, *DNMT3A*, *ETV6*, *EZH2*, *FLT3*, *GATA2*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NPM1*, *NRAS*, *PTPN11*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, *U2AF1* and *WT1*) showed 97% of patients having at least one mutation. The mutational profile was highly variable according to moment of disease, age and sex, and several co–occurring and exclusion relations were detected. Molecular testing based on NGS allowed accurate diagnosis and reliable prognosis stratification of 954 AML patients according to new genomic classification proposed by Tazi et al. Novel molecular subgroups, such as mutated *WT1* and mutations in at least two myelodysplasia–related genes, have been associated with an adverse prognosis in our cohort. In this way, the PETHEMA cooperative group efficiently provides an extensive molecular characterization for AML diagnosis and risk stratification, ensuring technical quality and equity in access to NGS studies.

Keywords: acute myeloid leukemia; Next–Generation Sequencing; cross–validations; mutational profile; genomic classification; clinical validation

1. Introduction

Introduction of Next–Generation Sequencing (NGS) into routine molecular diagnosis has provided deep molecular knowledge of acute myeloid leukemia (AML). These findings have allowed for the refinement of classification and risk stratification systems based on recurrent genetic abnormalities.

In 2016, Papaemmanuil et al. proposed the first genomic classification of AML that identifies 11 molecular classes, each with distinct diagnostic features and clinical outcomes [1]. This classification has been recently revised and updated in Tazi et al., 2022, proposing 16 molecular classes based on cytogenetics and the mutational status of 32 genes [2]. The importance of genomic characterization has also been reflected in the recently revised World Health Organization (WHO) Classification [3], new International Consensus Classification (ICC) [4] and European LeukemiaNet (ELN) risk stratification [5], which prioritize genetic abnormalities to establish diagnosis and prognosis to evaluate measurable residual disease (MRD) and to select treatment.

In this situation, molecular analysis by single–gene techniques has become inefficient in order to provide a complete characterization of AML. In contrast, NGS represents a more sensitive tool to capture all the relevant molecular markers in one assay and is widely recommended to study the molecular landscape of this disease [6].

NGS implementation to perform accurate diagnosis in AML is currently demanded by physicians and patients. However, introduction of NGS into clinical routine faces novel challenges [7]. NGS requires large batches of samples in order to be cost–effective, workflows are time–consuming, and interpretation needs highly qualified specialists. Moreover, the diversity of NGS panels, platforms and quality control criteria might prevent the success of the approach [8]. Hence, to efficiently introduce NGS into routine

molecular diagnostics, it is necessary to establish quality requirements and to standardize gene panels and variant reporting.

In order to provide comprehensive NGS studies to AML patients and to guarantee equity of access, the PETHEMA cooperative group (Programa Español de Tratamientos en Hematología) has established a nationwide network of central laboratories aimed to harmonize NGS results under consensual criteria in newly diagnosed and relapsed/refractory AML patients [9].

This study summarized the NGS–AML project (NCT03311815), reporting quality control assays and the molecular profile of 2668 AML patients reported in the PETHEMA AML registry. We show co–occurring and mutual exclusion relationships among genes and distinct molecular profiles according to disease stage, age and sex and genomic classification in the "real–life PETHEMA cohort".

2. Materials and Methods

2.1. Development of the Diagnostic Platform

Implementing NGS studies in the routine molecular diagnosis of AML patients requires specialization, budgetary stability and logistical support. The PETHEMA cooperative group established a nationwide network of NGS studies for fast and standardized molecular diagnosis of AML. This strategy aims to provide coverage of NGS studies to 38.5 million inhabitants distributed in geographical areas ranging from 2.2 to 8.9 million habitants. For this purpose, seven centers with logistical and technical capacity for the management of a high number of samples were designed as reference laboratories for the centralization of samples submitted by PETHEMA institutions in each area. In this way, a large territory and population was covered. The platform was supported by PETHEMA in logistical management, as well as, closer and well–established relationships between the sample referral institution and the assigned central laboratory. The designated reference centers for NGS analysis concentrate a large number of AML samples, allowing for the rapid completion of the sequencing runs and their management by highly specialized staff.

2.2. Study Design and Reference Laboratories

We show a prospective, multi–center, non–interventional and translational biomedical research, performed in seven Spanish PETHEMA reference laboratories: Hospital Universitario La Fe (HULF, Valencia, Spain), Hospital Universitario de Salamanca (HUS, Salamanca, Spain), Hospital Universitario 12 de Octubre (H12O, Madrid, Spain), Hospital Universitario Virgen del Rocío (HUVR, Sevilla, Spain), Hospital Universitario Reina Sofía (HURS, Córdoba, Spain), Hospital Universitario de Gran Canaria Dr. Negrín (HUDN, Las Palmas de Gran Canaria, Spain), CIMA LAB Diagnostics (UNAV, Pamplona, Spain).

2.3. Consensus Genes Establishment

The development of the diagnostic platform required several meetings to coordinate criteria on which genes should be analyzed based on their clinical relevance in AML. After extensive bibliographic revision of current molecular basis of AML, all reference laboratories should assess by NGS the mutational status of genes that define the diagnosis and prognosis as well as guide treatment options (*ASXL1*, *BCOR*, *CEBPA*, *EZH2*, *FLT3*, *IDH1*, *IDH2*, *NPM1*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, *ZRSR2* and *TP53*). Moreover, there was a recommendation for the study of other genes with proven evidence on their relevance in AML pathogenesis (*ABL1, BRAF, CALR, CBL, CSF3R, DNMT3A, ETV6, GATA2, HRAS, JAK2, KIT, KRAS, MPL, NRAS, PTPN11, SETBP1, TET2* and *WT1*).

The establishment of consensus genes has enabled laboratories to work with the NGS platforms and panels according to their individual requirements, which have largely enabled the development and maintenance of the diagnostic platform. NGS panels and platforms used by each center are described in Supplementary Materials Tables S1 and S2.

2.4. NGS Standardization Procedures and Cross–Validation Rounds

The diagnostic platform established a quality control assay by exchanging control samples among reference laboratories every 9–12 months. To date, three cross validation (CV) rounds have been performed. As previously reported, in the first and second CV rounds, minimum quality parameters [uniformity (>85%) and mean read depth of 1000X] and consensus recommendations for variant report [All centers should report: (1) mutations in relevant genes for clinical guidelines, targeted therapy and risk stratification and (2) all pathogenic variants detected with VAF >5% excepting those described at hotspot regions which will be reported up to 1% VAF] were stablished. For both CV rounds, 10 samples harboring 54 variants were distributed. In first CV round, VAF for all variants was >5% while in the second CV round, 5 from 30 total variants had low VAF ranging from 1.8–4.9%. In the third CV round, to explore the accuracy and reliability of low VAF variants (<5%) detection, 4 samples harboring 32 variants (11 VAF < 5%) were analyzed, according to previously established criteria [9].

2.5. Patients and Inclusion Criteria

All adult patients (≥18 years) with newly diagnosed or relapsed/refractory AML (excluding acute promyelocytic leukemia) according to the World Health Organization criteria (2008 and 2016), regardless of the treatment received, were eligible for mutational profile study by NGS. The Institutional Ethics Committee for Clinical Research of each institution approved this study. Written informed consent in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki, and institutional regulations were obtained from all patients.

2.6. Clinical Validation

Clinical validation was performed based on the new genomic classification which proposes unified framework for disease classification and risk–stratification in AML based on cytogenetic analysis and an NGS–panel of 32 genes [2]. Molecular class defining genes were: ***NPM1***, ***TP53***, ***WT1***, ***CEBPA***, ***DNMT3A***, ***IDH1***, ***IDH2***, *ZRSR2*, ***U2AF1***, ***SRSF2***, ***SF3B1***, ***ASXL1***, *STAG2*, *BCOR*, ***RUNX1***, ***EZH2***, *MLL*, *PHF6*, *SF1*, *NF1*, *CUX1*, ***SETBP1***, ***FLT3*** and ***TET2***. * Bold genes represent PETHEMA consensus genes.

This new classification categorizes AML in 16 molecular classes with different prognostic values and encompass the established WHO entities: "WHO2016 set 1" [inv(16), t(8;21) and *NPM1*] and "WHO2016 set 2" [t(11;x), t(6;9), inv(3) and *CEBPA*bi]; and also novel categories: "*TP53* and complex karyotype (CK)", "sAML1" (Mutated *SRSF2*, *SF3B1*, *U2AF1*, *ASXL1*, *EZH2*, *RUNX1* or *SETBP1*), "sAML2" (More than one mutations in sAML1 genes including *DNMT3A* and *TET2*), "*WT1*", "Trisomies", "*DNMT3A* + *IDH1/2*", "Not class defining mutations (mNOS)" and "No events" category. Since our study excludes acute promyelocytic leukemia, category "t(15;17)" is not applicable.

This classification also proposes an integrated risk score based on the 16 molecular classes: "*NPM1*", "inv(16)", "t(8;21)", "*CEBPA*bi" and "No events" define the favorable risk group; "sAML1", "t(6;9)", "*WT1*", "mNOS", "t(11;X)", "*DNMT3A–IDH1/2*" and "trisomies" the intermediate and "*TP53*–CK", "sAML2" and "inv(3)" the adverse risk group. Genomic groups and sub–classifications are summarized in Supplementary Table S3. Internal tandem duplications (ITD) in *FLT3* are the only genetic alterations with independent prognosis value from class membership. These mutations were not considered as "class defining" alterations as they are represented in all classes but modulate risk groups classification as follows: In the favorable risk group, patients with mutated *NPM1* who also harbored a *FLT3*–ITD mutation were reclassified to the intermediate risk group. Similarly, a *FLT3*–ITD mutation reclassifies intermediate risk patients to an adverse risk group. Moreover, in order to assess the prognostic impact of *TP53* configurations, we classified patients according *TP53* mono–allelic or multi–hit as described by Tazi et al.: Mono–allelic: One *TP53* mutation with VAF ≤ 65%; and multi–hit: Two *TP53* mutations or one *TP53* mutation with VAF > 65% or one *TP53* mutation + del(17).

2.7. Statistics

All statistics were performed using SPSS version 22 (IBM, Armonk, NY, USA) and GraphPad Prism 4 (GraphPad, La Jolla, CA, USA) software programs. Chi square test was used to assess associations between categorical variables. Survival analyses were performed using the Kaplan–Meier method and the log–rank test. The Cox proportional–hazards model was used to evaluate the risk of death among groups. p–value (p) < 0.05 was considered as a statistically significant test.

3. Results

3.1. Third Cross Validation Round

In the third CV round, the error rate (ER) for variants with VAF > 5% decreased from previous rounds: 1st: 39%, 2nd: 14.4% and 3rd: 4.76%. However, the ER in variants with VAF < 5% increased from 28.6% (five variants with mean VAF 3.3%) to 59.6% (11 variants with mean VAF 1.2%). ER, mean VAF and standard deviation (SD) for the last CV round are summarized in Supplementary Table S4 and Figure S1. Therefore, the diagnostic platform maintained: (1) the cut–off of VAF > 5% to report clinically relevant variants and (2) the criteria to report only low VAF variants (<5%) in hotspot regions with strong clinical evidence, suggesting variant confirmation in an additional sample.

3.2. Baseline Demographics and Molecular Profile in NGS–AML Protocol Cohort

From October 2017 to October 2019, NGS analyses were performed in 1631 samples from 1471 AML patients enrolled in the NGS–AML protocol (NCT03311815), with available clinical date (i.e., treatment approach). Disease status at samples collection was: 1268 diagnosis (DX), 204 relapse (REL) and 159 refractoriness (RES) (Table 1).

Table 1. Diagnosis cohort (N = 1268). Demographic and baseline characteristics.

Characteristic	Mean	Median	Range	N	(%)
Age, years	64.9	67.7	18–98	1268	100
<65				540	42.6
≥65				728	57.4
Sex				1268	100
Male				712	56.2
Female				556	43.8
ECOG				1075	100
0				420	39.1
1				452	42.0
2				135	12.6
3				53	4.9
4				15	1.4
Not available				193	
WBC ($\times 10^9$/L)	32.8	8.8	0.24–407	1118	
BM blast cells, %	53.4	52.0	0–100	1026	
Creatinine, mg/dL	1.1	0.90	0.28–10.3	1071	
MRC cytogenetic profile				1011	100
Favorable				57	5.6
Intermediate				178	17.6
Unfavorable				269	26.6
Normal karyotype				507	50.1
Not available				257	
AML FAB subtype				715	100
M0				88	12.3
M1				144	20.1
M2				126	17.6
M4				173	24.2
M5				144	20.1
M6				31	4.3
M7				9	1.3
Not available				553	
Therapeutic approach				1268	100
Intensive				695	54.8
Non–intensive				513	40.5
Supportive care only				60	4.7
Type of AML				1268	100
De novo				920	72.6
Secondary				348	27.4

An additional cohort of 1225 samples analyzed by NGS between November 2019–May 2021 has been included only for molecular characterization of AML: 1166 (DX), 47 (REL) and 12 (RES). Only those samples that met NGS quality requirements established by the diagnostic platform were included in the study. Overall, 2856 samples were analyzed (2434 DX, 251 REL, and 171 RES).

3.3. Summary Mutation Profile

In the global cohort (N = 2856 samples), 7768 variants were detected. A total of 96.5% of samples showed at least 1 mutation, mean 2.7 mutations/sample (range 0–9). Most patients had three variants (21.1%), followed by patients with two (20.9%) and four (17.8%). *FLT3* (24.6%), *DNMT3A* (24.3%), *NPM1* (22.4%) and *TET2* (21.6%) were the most frequently mutated genes (Supplementary Figure S2). According to ELN–2022 [5], 85.3% of patients at diagnosis showed at least one mutation in clinically relevant genes to establish the diagnosis, prognosis or to select treatment.

3.4. Co–Mutations and Exclusivity Patterns

NPM1, *FLT3* and *DNMT3A* were significantly co–mutated for all combinations ($p < 0.001$). *PTPN11* and *NPM1* showed a strong association ($p < 0.001$) as well as *PTPN11–DNMT3A* ($p = 0.0017$) and *PTPN11–FLT3* ($p = 0.024$). Mutations in *NPM1* were highly associated with mutated *IDH1* ($p < 0.001$) but were exclusive with R172–*IDH2* ($p < 0.001$). In contrast, *DNMT3A* mutations were highly associated with both mutated *IDH1* and *IDH2* ($p < 0.001$). *CEBPA* was frequently co–mutated with *GATA2* ($p < 0.001$); and *ASXL1*, *RUNX1* and *SRSF2* were strongly associated with each other ($p < 0.001$).

On the other hand, mutations in *TP53* were the most exclusive with all the analyzed genes ($p < 0.05$). Mutations in *IDH1* and *IDH2* were also mutually exclusive of each other ($p < 0.001$). Mutated *NPM1* was highly exclusive with mutations in: *RUNX1* ($p < 0.001$), *SRSF2* ($p < 0.001$) and *ASXL1* ($p < 0.001$). The main association and exclusivity patterns for all genes are shown in Figure 1.

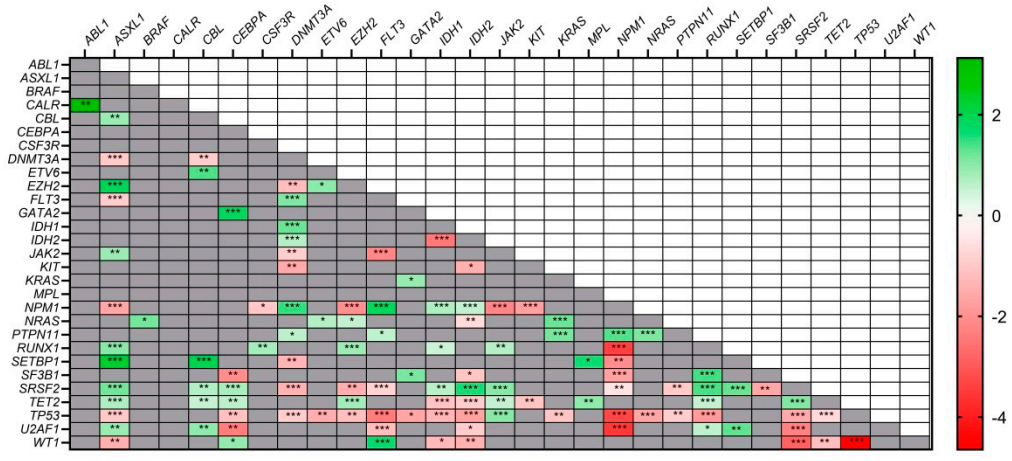


Figure 1. Co–occurrence and mutual exclusivity patterns among genes. Red: exclusive relationship; Green: co–occurring relationship. Higher color intensity indicates stronger association: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

We also identified co–occurring and exclusivity patterns according to functional categories of AML [10]. Genes were grouped into nine categories based on their biological function (Supplementary Table S5). We identified commonly co–occurring events between "transcription–factor (TF) fusions" and "activating signaling genes" ($p < 0.001$). Associations were also found between "*NPM1*" with "DNA–methylation genes" ($p < 0.001$) and "activating signaling genes" ($p < 0.001$)" as well as co–occurring events between "Spliceosome–genes" with "myeloid TFs" ($p < 0.001$), "chromatin modifiers" ($p < 0.01$) and "DNA–methylation genes" ($p < 0.001$). On the other hand, several mutually exclusive

relationships were observed: "*NPM1*" mutations were highly exclusive with "myeloid TFs" ($p < 0.001$), "Spliceosome–genes" ($p < 0.001$), and "chromatin–modifying" ($p < 0.001$); mutations in "Spliceosome–genes" were highly exclusive with "TF fusions" ($p < 0.001$) and remarkably, the "Tumor suppressor–genes" category was highly exclusive with all other functional categories ($p < 0.001$) (Figure 2).

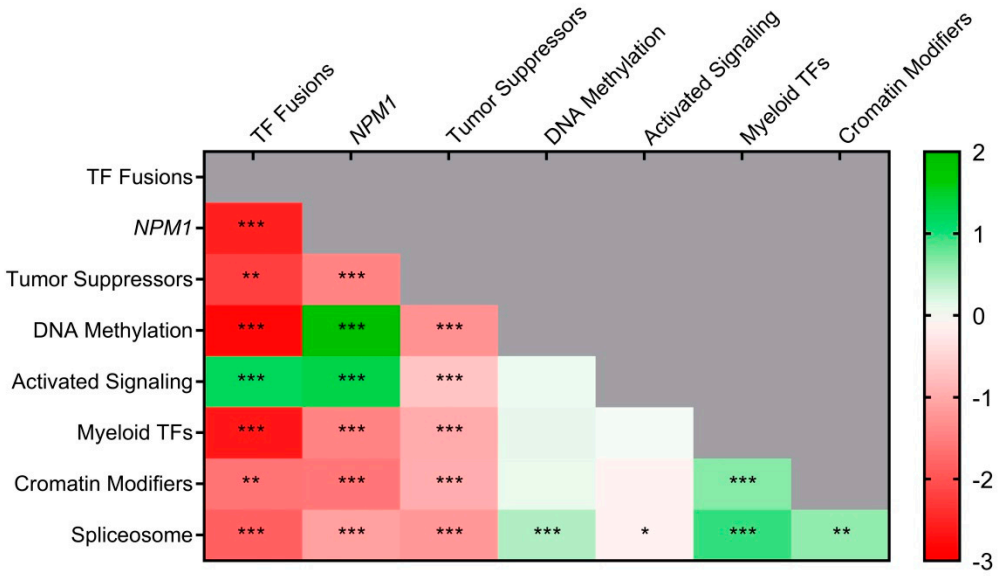


Figure 2. Heatmap of association and exclusivity patterns among functional categories. Red: exclusivity relationship; Green: co–occurring relationship. Higher color intensity indicates stronger association. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5. Disease Stage Mutational Profile

Subgroup analyses based on disease stage (DX or REL or RES) showed statistically significant results: *NPM1* ($p < 0.001$) and signaling pathway genes such as *KRAS* ($p = 0.007$) and *NRAS* ($p < 0.001$) were more frequently mutated at diagnosis. In refractory AML, *WT1* ($p < 0.001$) was more frequent, meanwhile relapse AML exhibited more mutations in *RUNX1* ($p = 0.037$), *DNMT3A* ($p = 0.018$), *IDH1* ($p = 0.017$) and *WT1* ($p < 0.001$). Mutational frequency and *p*–values for each correlation are described in Supplementary Table S6A, Figure 3.

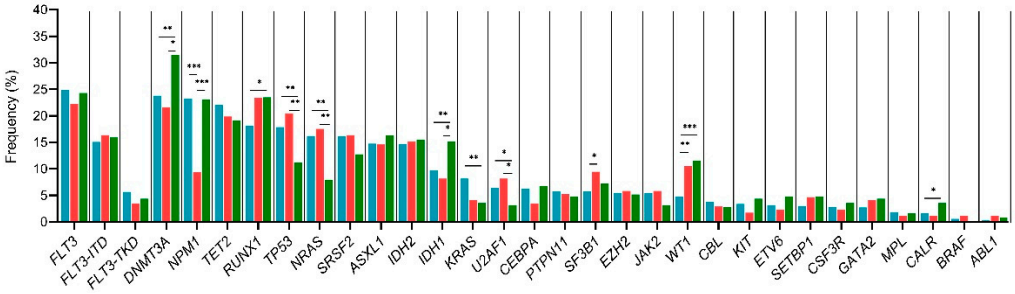


Figure 3. Mutational frequency according to disease stage. Blue bars: Diagnosis, green bars: Relapse and red bars: refractoriness. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ITD: internal tandem duplication; TKD: tyrosine kinase domain.

3.6. Age–Related Mutational Profile

NGS studies revealed distinct mutational profile in young (<65 years–old) and elderly (≥65 years–old) AML patients. The mean number of gene mutations at diagnosis was higher in older patients than younger (2.9 ± 0.04 vs. 2.5 ± 0.04; $p < 0.001$). Older patients also had a higher frequency of *TET2* ($p < 0.001$), *RUNX1* ($p < 0.001$), *TP53* ($p < 0.001$), *IDH2* ($p < 0.01$), *ASXL1* ($p < 0.001$), *SRSF2* ($p < 0.001$), *U2AF1* ($p < 0.01$), *SF3B1* ($p = 0.028$), *JAK2* ($p < 0.001$) and

EZH2 ($p < 0.001$). In contrast *FLT3* ($p < 0.001$), *NPM1* ($p < 0.001$), *DNMT3A* ($p = 0.032$), *NRAS* ($p < 0.01$), *PTNP11* ($p < 0.001$) and *WT1* ($p < 0.001$), were frequently mutated in young AML. Mutational frequencies and *p*–values are shown in Supplementary Table S6B, Figure 4.

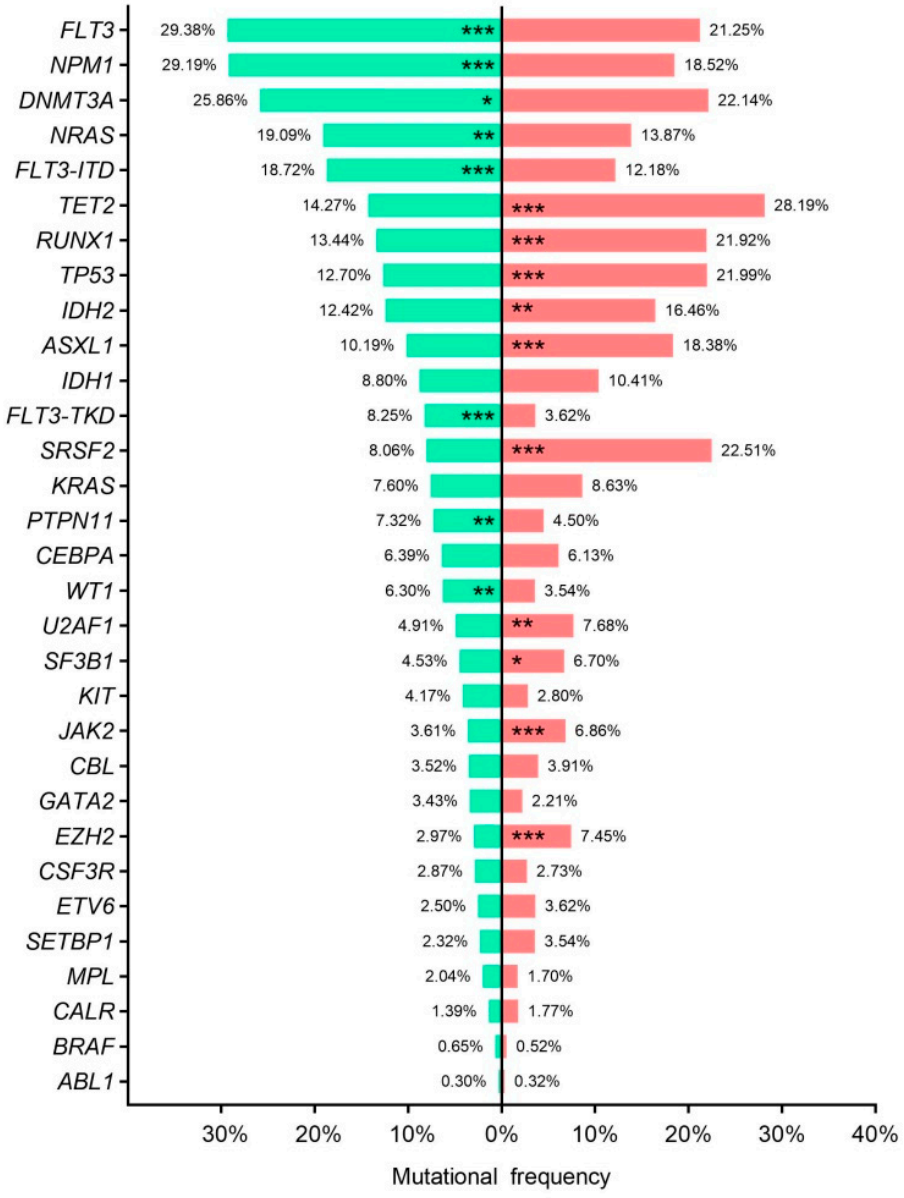


Figure 4. Age–related mutational profile. Bar chart representing mutational frequencies according to age at diagnosis. Green; <65 years old, red; ≥65 years old. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ITD: internal tandem duplication; TKD: tyrosine kinase domain.

3.7. Sex–Related Mutational Profile

Our cohort showed a well–balanced distribution between male (56.2%) and female (43.8%) patients, similar to that described in previous AML cohorts [1,10]. Sex–specific mutational profiles were observed. Females harbored lower number of mutations than male patients (2.8 ± 0.04 vs. 2.6 ± 0.04; $p < 0.01$). Mutations in *DNMT3A* ($p < 0.001$), *FLT3* ($p < 0.01$) and *NPM1* ($p < 0.001$) were overrepresented in females and *TET2* ($p = 0.014$), *RUNX1* ($p < 0.001$), *ASXL1* ($p < 0.001$), *SRSF2* ($p < 0.001$), *EZH2* ($p < 0.001$), *U2AF1* ($p < 0.001$), *JAK2* ($p = 0.01$) and *CBL* ($p = 0.025$) mutations in male patients (Supplementary Table S6C, Figure 5).

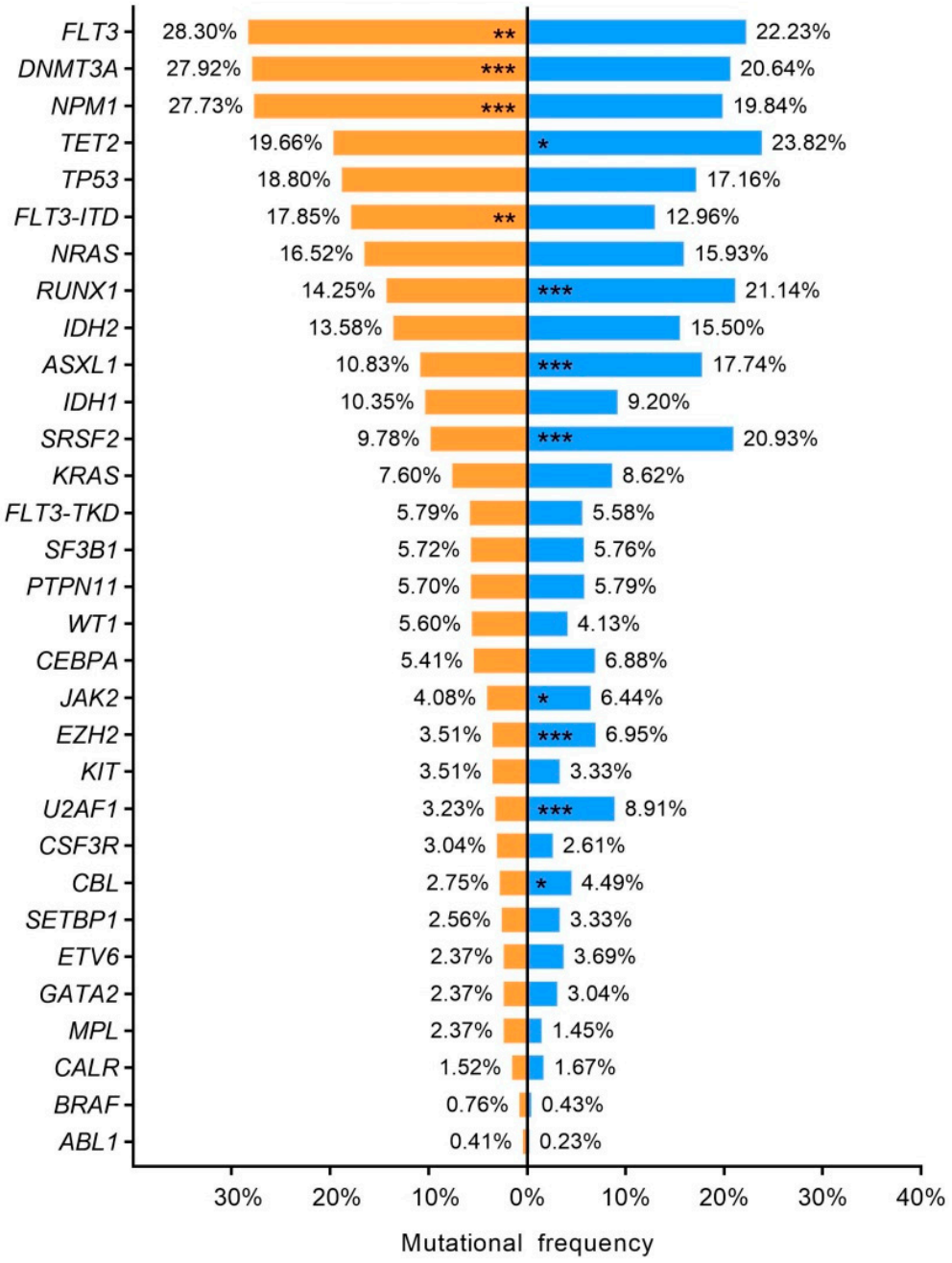


Figure 5. Sex–related mutational profile. Bar chart representing mutational frequencies. Orange; female, blue; male. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ITD: internal tandem duplication; TKD: tyrosine kinase domain.

3.8. Paired Samples and Mutation Stability

Molecular profiling of paired samples was conducted in 97 patients at diagnosis–relapse (DX–REL) and 59 patients at diagnosis–refractoriness (DX–RES). In DX–REL comparison, loss events were more frequently observed than gain events (43.7% vs. 26.4%) in relapse samples. Interestingly, 17.2% of patients showed simultaneous mutation loss and gain events, while 24.1% maintain the diagnosis' mutational profile. The most stable mutated genes were *TP53*, *WT1* and *NPM1*, with stability rates of 81.3%, 80% and 77.8%, respectively. In contrast, signaling activating genes were found to be highly unstable: *KIT*, *FLT3*–ITD and *FLT3*–TKD mutations, *NRAS*, *KRAS* and *PTPN11* showed stability rates below 50%. Moreover, while mutations in *KIT*, *FLT3*–TKD, *KRAS* and *PTPN11* were almost equally lost and acquired, mutations in *NRAS* and *FLT3*–ITD were predominantly lost (Supplementary Figure S3).

In refractory AML, 49.2% of patients retained the mutational status found at diagnosis. *NPM1* mutations were also the most stable gene at refractory AML (83.3%). Signaling activating genes were highly unstable: *NRAS*, *KRAS*, *PTPN11* and *FLT3*–TKD showed stability rates below 45%, being more frequent the loss of these mutations (Supplementary Figure S4).

3.9. New Genomic Classification Applied to PETHEMA–NGS Cohort

Based on the availability of clinical, cytogenetic and mutational data, 954 patients were eligible to assess the updated genomic classification by Tazi et al. [2]. The most frequently mutated classes were: "sAML2" (25.4%), "*NPM1*" (23.9%), and "*TP53*–CK" (19.2%). Other less frequent classes were: "sAML1" (8.7%) and "Not class defining mutations" (8.3%). Molecular classes' distribution is shown in Figure 6. The "*CEBPA*bi" category was underrepresented in the PETHEMA cohort (0.8%). Therefore, based on novel diagnosis and prognosis classifications of AML, patients with in–frame mutations in basic leucine zipper domain of *CEBPA* (*CEBPA* bZIP) were assessed as a biological AML subgroup (1.2%). In this regard, WHO entities [inv(16), t(8;21), *NPM1*, *CEBPA* bZIP, t(11;x), t(6;9) and inv(3)] represented the 34% of PETHEMA–cohort.

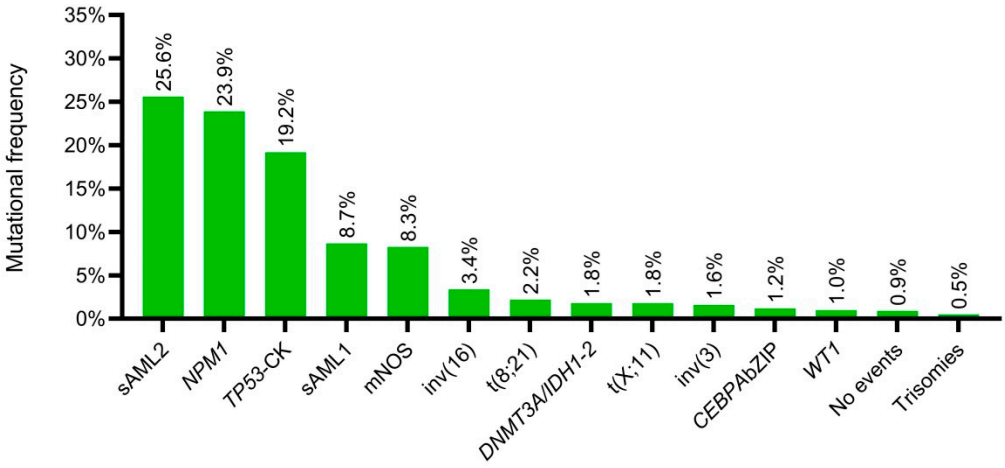


Figure 6. Molecular classes' distribution according to the Tazi et al., 2022 genomic classification. CK: Complex karytotype. mNOS: Not class defining mutations.

3.9.1. Prognosis Value of Molecular Classes

Molecular classes have also been associated with different prognostic values. The previously established WHO categories, "inv(16)" (Median OS not reached at 42 months), "*CEBPA* bZIP" (Median OS not reached at 32 months) and "*NPM1*" [29.0 months (95%CI 19.9–38.0)] had the best outcomes while inv(3) had the worst prognostic value [4.9 months (95%CI 0.8–9.1)]. Among new molecular classes, those patients without driver mutations "No events" (Median OS not reached at 33 months) or "Not class defining mutations" [23.3 months (95%CI 11.0–35.6)] showed the best prognostic value while "*TP53*/CK" [5.3 months (95%CI 2.9–7.6)], "sAML2" [12.1 months (95%CI 9.9–14.2)] and *WT1* [4.0 months (95%CI 0.0–18.4)] classes had the worst OS ($p < 0.001$) (Table 2; Supplementary Figure S5). When we evaluated the prognostic value according to the mono–allelic (N = 47; 32.9%) or multi–hit (N = 96; 67.1%) status of *TP53* mutations, we did not find a different outcome between both groups [mono–allelic: 5.4 months (95%CI 0.007–10.7); multi–hit: 4.1 months (95%CI 2.9–5.3) ($p = 0.088$)] (Supplementary Figure S6).

Table 2. Median overall survival and 95% CI for molecular AML classes.

Molecular Classes	OS	(95% CI)		*p*
		Lower IC	Upper IC	
inv(16)	NR			<0.001
CEBPA bZIP	NR			
No events	NR			
NPM1	29.0	19.9	38.0	
Not class defining mutations	23.3	11.0	35.6	
DNMT3A/IDH1–2	18.5	1.7	35.3	
sAML1	18.1	12.5	23.7	
t(8;21)	17.5	3.7	31.3	
Trisomies	14.4			
t(X;11)	13.2	0.0	31.3	
sAML2	12.1	9.9	14.2	
TP53–CK	5.3	2.9	7.6	
inv(3)	4.9	0.8	9.1	
WT1	4.0	0.0	18.4	

NR: Mean overall survival not reached at: "inv(16)": 42 months; "No events": 33 months and "*CEBPA* bZIP": 31 months.

In terms of risk of death, the molecular classes "inv(3)" [3.9 (95%CI 2.1–7.2) ($p < 0.001$)] and "*TP53*/CK" [3.5 (95%CI 2.6–4.6) ($p < 0.001$)] showed the highest risk of death compared to "*NPM1*" class. Remarkably, the new established "sAML2" [2.1 (95%CI 1.6–2.7) ($p < 0.001$)] and "*WT1*" [2.3 (95%CI 1.1–5) ($p < 0.05$)] categories were the next with higher risk of death (Figure 7A, Supplementary Table S7A).

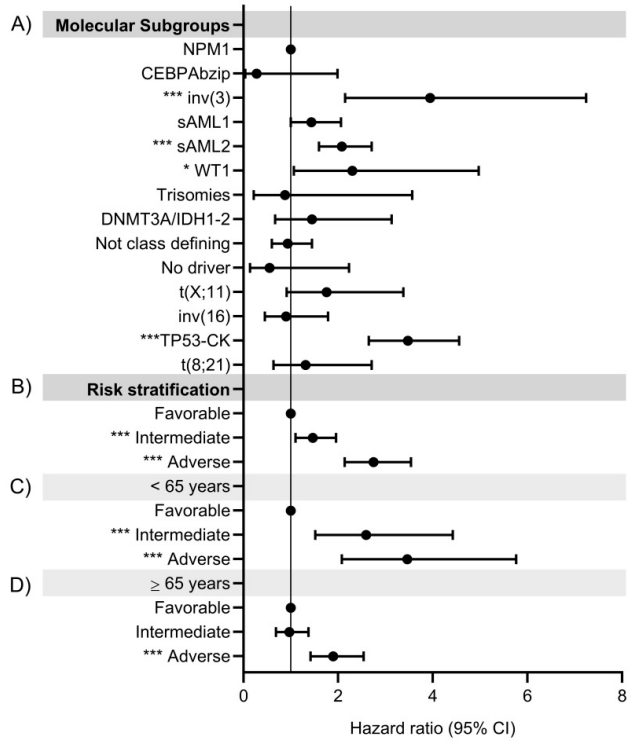


Figure 7. Hazard ratio for death according to (**A**) New molecular subgroups in the global cohort; and new genomic risk score for: (**B**) Global cohort, (**C**) Patients < 65 years and (**D**) Patients ≥65 years. * $p < 0.05$, *** $p < 0.001$.

3.9.2. Integrated Risk Score

We also assessed the integrated risk score based on cytogenetic and gene mutations proposed in the new genomic classification [2]. In the evaluable cohort (N = 954), 23.8% of patients were classified to a favorable risk group, 27.1% were included in the intermediate risk group and 49.1% in the adverse risk group. We also evaluated the impact of age at diagnosis in the new genomic classification. Risk stratification distribution was significantly different between age groups: <65 years (intensive = 416; non–intensive = 10) vs. ≥65 years (intensive = 150; non–intensive = 378). Young patients showed homogeneous distribution of the different risk groups (Favorable: 31.2%, Intermediate: 32.4% and adverse: 36.4%), while older patients were predominantly included in the adverse risk group (Favorable: 17.8%, Intermediate: 22.9% and adverse: 59.3%; $p < 0.001$).

In terms of outcomes, median OS was 30.8 months (95%CI NR) in the favorable risk group, 18.5 months (95%CI 14.3–22.7) in the intermediate group and 9.4 months (95%CI 7.8–11.00) in the adverse risk group ($p < 0.001$) (Figure 8A). Intermediate and adverse risk patients had 1.5 (95%CI 1.1–2.0; $p < 0.01$) and 2.7 (95%CI 2.1–3.5; $p < 0.001$) increased risk of death relative to favorable risk group (Figure 7B, Supplementary Table S7B).

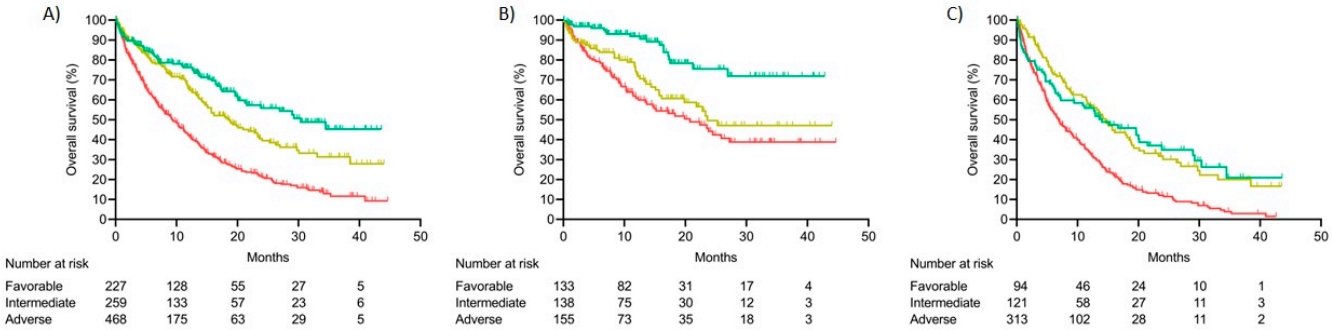


Figure 8. Overall survival according to new genomic risk score: (**A**) Global cohort, (**B**) Patients < 65 years, (**C**) Patients ≥ 65 years.

We also found differences in terms of OS in both age groups. Median OS in favorable–risk group was not reached at 42.8 months in patients < 65 years, while intermediate and adverse risk groups reached a median OS of 23.6 (95%CI NR) months and 20.8 (95%CI 13.2–28.3), respectively ($p < 0.001$) (Figure 8B). In contrast, median OS of older patients was significantly decreased for all risk groups: Favorable (14.0; 95%CI 6.3–21.8), intermediate (14.6; 95%CI 11.9–17.4) and adverse (6.9; 95%CI 5.6–8.3) ($p < 0.001$) (Figure 8C).

Regarding risk score, young patients classified in the intermediate and adverse risk group showed 2.6 (95%CI 1.5–4.4; $p < 0.001$) and 3.5 (95%CI 2.1–5.8; $p < 0.001$) higher risk of death relative to those patients classified in the favorable risk group (Figure 7C, Supplementary Table S7C). On the other hand, patients >65 years classified in the adverse risk group showed an increased risk of death of 1.9 (95%CI 1.4–2.5; $p < 0.001$) compared to those classified as favorable risk. No statistically significant results were found in the risk of death of intermediate risk patients: 1.0 (95%CI 0.7–1.4; $p = 0.864$) (Figure 7D, Supplementary Table S7D). In general, patients >65 years had a dismal prognosis and higher risk of death compared to younger ones for all risk groups: Favorable: 4.7 (95%CI 2.8–8.0; $p < 0.001$), intermediate: 1.8 (95%CI 1.2–2.6; $p < 0.01$) and adverse: 2.6 (95%CI 2.0–3.5; $p < 0.001$).

Comparison between the Integrated Risk Score and 2022 ELN Risk Classification

According to current 2022 ELN risk stratification, we selected 546 fit patients for a tentative comparison with the AML genomic classification risk score. We did not find a distinct OS (2022 ELN vs. Tazi et al.) for favorable (Median OS not reached in both groups; $p = 0.839$), intermediate (Median OS not reached vs. 25.3 months; $p = 0.336$) or adverse–risk patients (Median OS 15.2 months vs. 14.7 months; $p = 0.786$) according to both classifications (Figure 9).

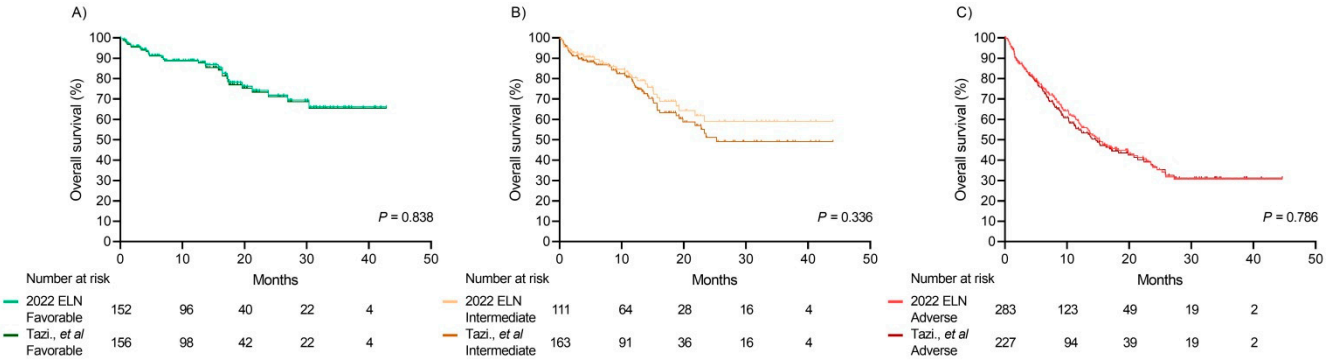


Figure 9. Overall survival curves according to 2022 ELN risk classification and Tazi et al. [2] genomic AML classification: (**A**) Favorable, (**B**) Intermediate and (**C**) Adverse risk groups.

4. Discussion

NGS has become the preferred technology to capture the heterogeneous molecular landscape of AML [2–5]. These approaches have been rapidly adopted as a potential routine tool for molecular diagnosis in AML patients [11]. However, its translation into clinical practice is hampered by specific requirements, such as the necessity of highly skilled laboratories, the increased cost compared to single–gene assays and the expected high turnaround time [7,12]. In this scenario, our results show that an NGS diagnostic platform, established by the PETHEMA cooperative group, was able to provide NGS reliable results with all relevant molecular data currently required for diagnosis and prognosis stratification and therapy choice. In addition, this kind of cooperative study allows for the assessment of the genetic heterogeneity of AML in large cohorts of patients and provides an extensive quality molecular data to evaluate current genomic knowledge in "real–life" AML cohorts.

Due to the rapid NGS implementation into routine molecular diagnosis of AML, NGS workflows and quality specifications are heavily reliant on laboratory specific procedures [13,14]. Therefore, current NGS analyses are characterized by the lack of standardized procedures, the diversity of quality metrics criteria, and the high variability of the assessed genes and variant reporting criteria [15]. To address the need for harmonization procedures, the PETHEMA cooperative group implemented the first Spanish nationwide NGS testing strategy. Regular rounds of cross–validation were planned in order to identify weaknesses and to establish consensus quality metrics criteria for variant reporting among seven central laboratories in AML molecular diagnosis.

Networking for NGS studies allowed us to identify challenges in its clinical implementation [9]. The first CV round revealed the absence of AML key genes in some NGS approaches, and consequently a consensus set of relevant genes for the clinical management was defined. Next steps of the diagnostic platform enabled us to unify variant reporting according to the role of genes in diagnostic classification, prognostic utility and targeted therapy. Cooperative studies have also allowed us to address technical challenges. Comparison of results between centers facilitates discrimination of polymorphic variants and sequencing artifacts from real AML–related variants [7].

Our results demonstrated that NGS standardization in the context of a cooperative group is possible with a concordance of 96.6% in variant detection (VAF > 5%). Noteworthy, the detection of low VAF (<5%) variants (concordance 41.2%) was also consistent with previous studies, which report that accurate detection of low VAF variants by NGS could be compromised. CV rounds results reflected the improvement of the diagnostic platform performance as the error rate decreased in variants VAF > 5% as a result of the experience gained in NGS studies. Although the second CV round included 5 variants with a mean VAF of 3.3%, in the third CV round we aim to explore the performance of NGS studies in very low VAF variants. For this purpose, we included 11 variants with a mean VAF of 1.2%. Data analysis revealed an increase of the ER between both CV rounds due to the very low VAF variants of the third CV round. Consequently, we established VAF 5% cut–off for

variant reporting, although according to other specific studies, variants in hotspot regions were reported up to 1% VAF even at borderline technical quality [16–18].

Our results reflect that a comprehensive NGS approach is suitable for defining the molecular profile of AML as over 96% of patients of the "real–life" PETHEMA cohort harbored at least one mutated gene. Our study also reflects the genomic heterogeneity that encompasses AML (13,14) as several gene–to–gene interactions but also co–occurrence and mutual exclusivity patterns across functional categories were described. Recently, published studies have highlighted the impact of co–mutations in modulating treatment response [19,20].

Similarly, distinct mutational profiles were detected among disease stages, reflecting the clonal evolution of AML [1,21]. In fact, when compared to the diagnosis' molecular profile we detected fewer mutational changes in resistance to induction therapy (51%) than relapse AML patients (87%) which may suggest different mechanisms underlying both moments [22–25]. Clonal evolution of the disease could be especially relevant for treatment response [26,27], and in relapsed AML patients, our results supported that molecular testing should be conducted again in order to identify targetable abnormalities such as *FLT3* mutations, which showed a stability rate of 43.3% in our cohort [28].

In addition to mutational changes according to disease stages, specific mutational profiles have been associated to patient's clinical characteristics, such as sex and age, with significant impact on prognosis, therapeutic allocation and disease monitoring [29]. In fact, we report that young patients show a mutational profile very similar to female AML patients, which allows them to benefit more from targeted therapies due to higher frequency of *FLT3* mutations [30–33]. In contrast, male and elderly patients show a molecular profile with a higher frequency of adverse risk genetic abnormalities and limited targeted therapy opportunities [34–36]. Indeed, some studies suggest that these features should be considered as an essential variable in clinical trials to deepen understanding of the disease and to identify new treatment opportunities [37,38].

Molecular diagnosis of AML has shifted towards a comprehensive mutational study driven by NGS, yielding large amounts of data. Updated diagnostic (WHO and ICC) and prognostic (ELN) classifications [3–5] include an increasing number of genetic abnormalities, which may challenge its applicability in many countries and institutions who cannot afford molecular data integration. However, comprehensive molecular analyses, as the recent revision of the genomic classification of AML are needed to understand the clinical relevance of molecular biomarkers to define novel clusters with prognostic value. In our comparison between genomic AML classification and 2022 ELN risk stratification we did not find differences in OS for favorable, intermediate and adverse risk groups. However, for a comprehensive comparison it would be necessary to evaluate the prognosis impact of individual genetic abnormalities in larger cohorts. Mutations in sAML genes are considered in both risk classification proposals with different prognostic impact [2,5]. Although ELN guidelines associate mutations in "myelodisplasia–related genes" as adverse risk regardless of the number of mutations, our results found significant differences in the OS between sAML1 and sAML2 patients, as described by Tazi et al., ($p = 0.018$). In this sense, further studies are needed in order to certainly clarify the impact of sAML mutations on prognosis.

Regarding *TP53* mutations, in the PETHEMA cohort, 47 patients were included in the mono–allelic group (32.9%), while 96 were included in the multi–hit group (67.1%). Similar results were described in the Tazi et al. cohort: mono–allelic (31.1%) vs. multi–hit: (68.9%). We did not find a distinct outcome between these groups, although the sample size was limited to draw solid conclusions. Our results are concordant with Tazi et al., who concluded that the allelic state of *TP53* (mono allelic or multi–hit) provide no further prognostic information in AML.

On the other hand, recent studies reported that instead of biallelic mutations, only in–frame mutations in the bZIP domain of *CEBPA* should be considered as a favorable prognosis marker [39]. In our cohort, the percentage of patients with *CEBPA* bZIP mutations is similar to the *CEBPA*bi subgroup reported by Tazi et al., (1.2% vs. 1.8%) and in both

cases, OS analysis includes them within a favorable risk subgroup. However, *CEBPA*bi and *CEBPA* bZIP may be overlapping categories in some patients as several studies suggest that *CEBPA* double mutated frequently includes mutations in the C–terminal region which allocated bZIP domain [40]. Moreover, we believe that the recent recognition of in–frame mutations in the bZIP domain of *CEBPA* as a prognostic biomarker may allow for more homogeneous analysis by reducing the variability in the interpretation of the biallelic character of these mutations [41].

5. Conclusions

This report reflects the efforts of the PETHEMA cooperative scientific group to adopt a nationwide strategy network of reliable and consistent NGS analyses. The establishment of consensus subset genes and the periodic CV rounds have strengthened the diagnostic network by unifying analysis criteria and decreasing reporting variability. Molecular analyses through NGS are routinely performed for AML patients and a comprehensive molecular profile of the disease is offered to clinicians in order to individualize the therapeutic strategy. Moreover, NGS results have provided a large amount of molecular data that has revealed the molecular complexity of the disease. In this cohort, mutual exclusion and mutational co–occurrences among genes and functional categories have been deciphered and a distinct molecular profile between age groups at diagnosis and sex has been detected. Moreover, clinical validation of the current genomic classification in the "real–life" PETHEMA cohort has demonstrated the correlation of the molecular subgroups with clinical prognosis, reflecting the utility of the cooperative NGS studies in routine molecular diagnostics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15020438/s1, Table S1: Description of the sequencing technology, NGS panel and NGS platform used by each reference laboratory; Table S2: Detailed gene panel composition; Table S3: Genomic groups and integrated risk score based on 16 molecular classes defined by Tazi et al., 2022 in the updated genomic classification of AML; Table S4: Third Cross–validation round results; Table S5: Molecular alterations pertaining to described functional categories. Table S6: Mutational frequency distribution according to moment disease, age and sex; Table S7: Hazard ratio for (A) molecular classes, (B) Risk score in global cohort, (C) Risk score in <65 years–old, (D) Risk score in ≥65 years–old; Figure S1: Third Cross–validation round results; Figure S2: Mutational frequency of PETHEMA consensus genes in the global cohort; Figure S3: Stability rates in diagnosis–relapse samples. Figure S4: Stability rates in diagnosis–refractoriness samples; Figure S5: Overall survival curves according to AML molecular classes; Figure S6: Overall survival curves of *TP53* mutated patients according to mono–allelic vs. multi–hit configurations.

Author Contributions: Conceptualization, C.S., E.B. and P.M.; methodology: C.S., R.A., M.J.L., M.C.C., E.C.-C. and C.B.-S.; formal analysis, C.S. and E.B.; data curation, C.S., E.B., D.M.-C., B.B. and P.M.; investigation, C.S., R.A., M.J.L., M.C.C., E.C.-C., C.B.-S., E.P.d.l.T., D.M.-C., R.R.-V., B.B., C.G., T.B., J.M.B., L.A., M.T., P.M.-S., E.S., J.S., J.M.A.-D., R.G.-B., M.L.A., P.H.-P., M.J.S., E.L.-R., J.M.-L., M.J.C., R.G.-S., J.A.P.-S., M.T.G.-C., J.S.-G., E.B. and P.M.; writing—original draft preparation, C.S.; writing—review and editing, E.B. and P.M. visualization, C.S.; supervision, E.B. and P.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by Bristol-Myers Squibb/Celgene: PETHEMA-NGS-LMA; Ministry of Economy and Competitiveness | Instituto de Salud Carlos III, Spain, Spain: PI18/01340, PI19/00730, PI19/01518, FI19/00059.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Instituto de Investigación Sanitaria La Fe (2017/00304/PI; 13/09/2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank María D. García, Carlos Pastorini, Rafael Vianney, Yolanda Mendizabal and Alvaro Fernández for data collection and management.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

Institutions and clinicians participating in the PETHEMA epidemiologic registry of acute myeloid leukemia and acute promyelocytic leukemia.

Argentina (Grupo Argentino para el Tratamiento de la Leucemia Aguda—GATLA)—Hospital de Clínicas, Buenos Aires: F. Rojas; H. Longoni; Fundaleu, Buenos Aires: G. Milone, I. Fernández, Clínica Conciencia, Neuquén: R. Ramirez; Hospital Rossi, La Plata: C. Canepa, S. Saba, G. Balladares, Hospital General San Martin, Parana: G. Milone, C. Ventiurini, R. Mariano, P. Negri; Hospital Italiano de La Plata, La Plata: M. V. Prates, J. Milone; Hospital General San Martín, La Plata:P. Fazio, M. Gelemur; Hospital Clemente Alvarez, Rosario: G. Milone, S. Ciarlo, F. Bezares; Hospital de Córdoba, Córdoba: L. López, Hospital Privado de Córdoba, Córdoba: J. J.García; Instituto Privado Hematología, Paraná: P. Negri, M. Giunta, G. Milone; Hospital Teodoro Alvarez, Buenos Aires: M. Kruss; Hospital Tornú, Buenos Aires: D. Lafalse, G. Milone; Hospital Gobernador Centeno, La Pampa: E. Marquesoni, M. F. Casale; Hospital Italiano de Buenos Aires, Buenos Aires: A. Gimenez, E. B. Brulc, M. A. Perusini; Complejo Médico Policía Federal, La Plata: G. Milone, L. Palmer; Colombia (Asociación Colombian de Hematología y Oncología—ACHO)—Clínica La Estancia, Popayán: M. E. Correa; Fundación Valle del Lili, Cauca: F.J. Jaramillo, J. Rosales; Instituto FOSCAL, Bucaramanga: C. Sossa, J. C. Herrera; Hospital Pablo Tobón Uribe, Antioquia: M. Arango; Poland (Polish Adult Leukemia Group—PALG)—City Hospital Legnica, Baja Silesia: J. Holojda; IHIT Hematology and transfusiology institute, Warszawa: A. Golos, A. Ejduk; Wojewódzki Szpital Specjalistyczny w Olsztynie, Olsztyn: B. Ochrem; WIM (Military Institute of Medicine in Warsaw), Warszawa: G. Małgorzata; Poland Medical University of Warsaw Banacha, Warszawa:A. Waszczuk-Gajda, J. Drozd-Sokolowska, M. Czemerska, M. Paluszewska; Medical University School Gdansk, Gdansk: E. Zarzycka; Wojewódzki Szpital Specjalistyczny im. Św.. Jadwigi Śląskiej, Opole: A. Masternak; Hospital Brzozow, Brzozow: Dr. Hawrylecka; Medical University Lublin, Lublin: M. Podhoreka, K. Giannopoulos, T. Gromek; Medical University Bialystok, Bialystok: J. Oleksiuk; Silesian Medical University Katowice, Katowice:bA. Armatys, G. Helbig; Universitary Hospital Wroclaw, Wroclaw: M. Sobas; Poznan University of Medical Sciences, Pozna: A. Szczepaniak; Rydigier City Hospital Krakow, Krakow: E. Rzenno, M. Rodzaj; Collegium Medicum Jagiellonian University Krakow, Krakow: B. Piatkowska-Jakubas; City Hospital Rzeszów, Rzeszów: A. Skret; Medical University Lodz, Lodz: A. Pluta, M. Czemerska; Center of Oncology Kielce, Kielce: E. Barańska; Medical University of Warsaw, Warsaw: M. Paluszewska; Portugal—Hospital de Santa Maria-Lisboa, Lisboa:G. Vasconcelos, J. Brioso; IPOFG Lisboa, Lisboa: A. Nunes, I. Bogalho; Centro Hospitalar e Universitário de Coimbra, Coimbra: A. Espadana, M. Coucelo, S. Marini, J. Azevedo, A. I. Crisostomo, L. Ribeiro, V. Pereira; Centro Hospitalar de Lisboa Central E. P. E, Lisboa: A. Botelho; Instituto Português Oncologia do Porto Francisco Gentil, Porto: J. M. Mariz; Centro Hospitalar São João, Porto: J. E. Guimaraes, E. Aguiar; Centro Hospitalar do Porto E.P.E. Porto: J. Coutinho; Spain (Programa Español de Tratamiento de las Hemopatías Malignas, PETHEMA)—Complejo Hospitalario Universitario A Coruña, A Coruña: V. Noriega, L. García, C. Varela, G. Debén, M. R. González; Hospital Clínico Universitario de Santiago, A Coruña: M. Encinas, A. Bendaña, S. González, J.L. Bello, M. Albors; Hospital General de Albacete, Albacete: L. Algarra, J.R.Romero, J.S.Bermon, M.J. Varo; Hospital Vinalopó, Alicante: V. López, E. López; Hospital Virgen de los Lirios, Alcoy: C. Mora, C. Amorós; Hospital General Elche, Alicante: E. López, A. Romero; Hospital Torrevieja Salud, Alicante: A. Jaramillo, N. Valdez, I. Molina, A. Fernández, B.

Sánchez; Hospital de la Marina Baixa Villajoyosa, Alicante: A. García; Hospital General de Elda, Alicante: V. Castaño, T. López, J. Bernabeu; Hospital de Denia-Marina Salud, Alicante: M.J. Sánchez; Hospital de la Vega Baja de Orihuela, Alicante: C. Fernández; Hospital General de Alicante, Alicante: C. Gil, C. Botella, P. Fernández, M. Pacheco, F. Tarín; J.J. Verdú; Complejo Hospitalario Torrecardenas, Almeria: M.J. García, A. Mellado, M.C. García, J. González; Hospital Central de Asturias, Asturias: T. Castillo, E. Colado, S. Alonso; Complejo Asistencial Ávila, Ávila: I. Recio, M. Cabezudo, J. Davila, M. J. Rodríguez, A. Barez, B. Díaz; Hospital Don Benito-Villanueva, Badajoz: J. Prieto; Institut Catala d'Oncologia L'Hospitalet, Barcelona: M. Arnan, C. Marín, M. Mansilla; Hospital de Cruces, Bizkaia: A. Balaberdi, M. E. Amutio, R. A. del Orbe, I. Ancin, J. C. Ruíz; Hospital Galdakao-Usansolo, Bizkaia: M. Olivalres, C. Gómez, I. gonzález, M. Celis, K. Atutxa, T. Carrascosa, T. Artola, M. Lizuain; Basurtuko Ospitalea, Bizkaia: J.I. Rodriguez, O. Arce, J. A. Márquez, J. Atuch, F. Marco de Lucas, Z. Díez, B. Dávila; Hospital Santos Reyes, Burgos: R. Cantalejo, M. Díaz; Hospital Universitario de Burgos, Burgos: J. Labrador, F. Serra, G. Hermida, F. J. Díaz, P. de Vicente, R. Álvarez: Hospital Santiago Apóstol, Burgos: C. Alonso, Hospital San Pedro de Alcántara, Cáceres: J. M. Bergua; Hospital Campo Arañuelo, Cáceres: N. Ugalde; Hospital Virgen del Puerto, Cáceres: E. Pardal; Hospital General Jerez de la Frontera, Cádiz: R. Saldaña, F. Rodríguez, E. Martín, L. Hermosín; Hospital Universitario Puerta del Mar, Cádiz: M. P. Garrastazul, I. Marchante, J. A. Raposo, F. J. Capote; Hospital U. Marqués de Valdecilla, Cantabria: M. Colorado, A. Batlle, L. Yañez, S. García, P. González, E. M. Ocio, M. Briz, A. Bermúdez, S. García; Consorcio Hospitalario Provincial de Castellón, Castellón: C. Jiménez, S. Beltrán; Hospital de Vinaroz: M. Montagud; Hospital Universitario de La Plana, Castellón: I. Castillo; Hospital General de Castellón, Castellon: R. García, A. Gascón, J. Clavel, A. Lancharro, L. Lnares; Hospital Santa Bárbara, Ciudad Real: M. M. Herráez, A. Milena; Hospital Virgen de Altagracia, Ciudad Real: M. J. Romero, Hospital General de Ciudad Real, Ciudad Real: B. Hernández, C. Calle, R. Benegas; Hospital Gutierrez Ortega de Valdepeñas, Ciudad Real: Dr. Bolívar; Hospital General La Mancha Centro, Ciudad Real: M. A. Pozas; Hospital Reina Sofia, Córdoba: J. Serrano, F. J. Dorado, J. Sánchez, M. C. Martínez; Hospital Virgen de la Luz, Cuenca: C. J. Cerveró, M. J. Busto; Hospitales HUVN-HC San Cecilio de Ganada, Granada: M. Bernal, E. López, L. Moratalla, Z. Mesa, M. Jurado, A. Romero, P. González; Complejo Hospitalario Universitario Granada, Granada: L. Moratalla, A. Romero, L. López; Hospital Universitario de Guadalajara, Guadalajara: M. Díaz, D. De Miguel, A. B. Santos, J. Arbeteta; Hospital Donostia, Donosti: E. Pérez, N. Caminos, N. Uresandi, N. Argoitiaituart, T. Artola, J. Swen, A. Uranga, I. Olazaba, M. Lizuain, E. Gainza, P. Romero; Hospital Juan Ramón Jimenez Huelva, Huelva: E. Gil, A. J. Palma, K. G. Gómez, M. Solé, J. N. Rodríguez; Hospital San Jorge, Huesca: I. M. Murillo, J. Marco, J. Serena, V. Marco; Hospital de Barbastro, Huesca: M. Perella, L. Costilla; Hospital General Ciudad de Jaen, Jaén: J. A. López, A. Baena, P. Almagro; Hospital San Pedro de Logroño, La Rioja: M. Hermosilla, A. Esteban, B. A. Campeny, M. J. Nájera, P. Herrra; Hospital Insular de Las Palmas, Las Palmas: R. Fernández, J. D. González, L. Torres; Hospital Dr. Negrín, Las Palmas: S. Jiménez; M. T. Gómez, C. Bilbao, C. Rodríguez; Hospital Doctor José Molina Orosa, Las Palmas: A. Hong, Y. Ramos de Laón, V. Afonso; Hospital Universitario de León, León: F. Ramos, M. Fuertes; Hospital Comarcal del Bierzo, León: E. de Cabo, C. Aguilera, M. Megido; Hospital Universitari Arnau de Vilanova de Lleida, Leida: T. García; Hospital Lucus Augusti, Lugo: E. Lavilla, M. Varela, S. Ferrero, M. J. Sánchez, L. López, J. Arias, L. Vizcaya; Hospital Infanta Sofía, Madrid: A. Roldán, A. Vilches, M. J. Penalva, J. Vázquez; Hospital Central de la Defensa Gómez Ulla, Madrid: M. T. Calderón, A. Matilla, C. Serí, M. J. Otero, N. García, E. Sandoval; Hospital de Fuenlabrada, Madrid: C. Franco, R. Flores, P. Bravo, A. López; Hospital Fundación Jiménez Díaz, Madrid: J. L. López, C. Blas, A. Díez, J. M. Alonso, C. Soto, A. Arenas; Hospital U. Príncipe de Asturias, Madrid: J. García, Y. Martín, P. S. Villafuerte, E. Magro; Hospital Puerta de Hierro, Madrid: G. Bautista; A. De Laiglesia; Hospital Gregorio Marañón, Madrid: G.

Rodríguez, L. Solán, M. Chicano, P. Balsalobre, S. Monsalvo, P. Font, D. Carbonell, C. Martínez; Hospital U. La Paz, Madrid: K. Humala, A. E. Kerguelen, D. Hernández, M. Gasior, P. Gómez, I. Sánchez; Hospital Madrid Norte Sanchinarro, Madrid: S. Redondo, L. Llorente, M. Bengochea, J. Pérez; Hospital Sanitas Torrejón, Madrid: A. Sebrango, M. santero, A. Morales; Hospital La Princesa, Madrid: A. Figuera, P. Villafuerte, A. Alegre, E. Fernández; Hospital Ruber Internacional, Madrid: A. Alonso; Hospital 12 de Octubre, Madrid: M. P. Martínez, J. Martínez, M. T. Cedena, L. Moreno; MD Anderson Cancer Center, Madrid: A. De la Fuente; Hospital Sanitas La Zarzuela, Madrid: D. García; Hospital Universitario Quiron, Madrid: C. Chamorro, V. Pradillo, E. Martí, J. M. Sánchez, I. Delgado, A. Alonso; Hospital Rey Juan Carlos, Madrid: B. Rosado, A. Velasco, C. Miranda, G. Salvatierra, J. M. Alonso,, J. L. López; Hospital Infanta Leonor, Madrid: M. Foncillas, J. A. Hernández; Hospital Universitario de Getafe, Madrid: C. Escolano, L. García, I. Delgado; Hospital Clínico San Carlos, Madrid: C. Benabente, R. Martínez, M. Polo, E. Anguita; Hospital Universitario Severo Ochoa, Madrid: R. Riaza, G. Amores, M. J. Requena; Hospital Universitario Fundación Alcorcón, Madrid: F. Javier, L. Villaloón; Hospital Universitario Moncloa, Madrid: C. Aláez, V. Pradillo, S. Nistal, B. Navas; Hospital Universitario de Móstoles, Madrid: J. Sánchez, M. A. Andreu; Hospital Ramon y Cajal, Madrid: P. Herrera, J. López; Hospital U. Virgen de la Victoria, Málaga: M. García, M. J. Moreno, A. Fernández, M. P. Queipo; Hospital Quirónsalud Málaga, Málaga: A. Hernández; Hospital Regional de Málaga, Málaga: M. Barrios, A. Heiniger, A. Jiménez, A. Contento, F. López, M. Alcalá; Hospital Vithas Xanit Internacional, Málaga: S. Lorente, M. González, E. M. Morales, J. Gutierrez; Hospital Virgen del Castillo, Murcia: M. J. Serna, V. Beltrán; Hospital Santa Lucía de Cartagena, Murcia: M. Romera, M. Berenguer, A. Martínez, A. Tejedor; Hospital Morales Meseguer, Murcia: M. L. Amigo, F. Ortuño, L. García, A. Jerez, O. López; Hospital U. Virgen de la Arrixaca, Murcia: J. M. Moraleda, P. Rosique, J. Gómez, M. C. Garay; Hospital Los Arcos Mar Menor, Murcia: P. Cerezuela, C. Martínez, A. B. MArtínez, A. González; Hospital ST^{a} M^{a} del Rosell, Murcia: J. Ibáñez; Clínica San Miguel, Navarra: M. J. Alfaro; Complejo Hospitalario de Navarra, Navarra: M. Mateos, M. A. Goñi, M. A. Araiz, A. Gorosquieta, M. Zudaire, M. Viguria, A. Zabala, M. Alvarellos, I. Quispe, M. P. Sánchez, G. Hurtado, M. Pérez, Y. Burguete, N. Areizaga, T. Galicia; Clínica Universitaria de Navarra, Navarra: J. Rifón, A. Alfonso, F. Prósper, M. Marcos, L. E. Tamariz, V. Riego. A. Manubens, M. J. Larrayoz, M. J. Calasanz, A. Mañú, B. Paiva, I. Vázquez, L. Burgos; Complejo Hospitalario de Ourense (CHOU), Ourense: M. Pereiro, M. Rodríguez, M. C. Pastoriza, J. A. Mendez, J. L. Sastre, M. Iglesias, C. Ulibarrena, F. Campoy; Hospital Valdeorras, Ourense: D. Jaimes; Hospital Rio Carrión, Palencia: J. M. Alonso, B. Albarrán, J. Solano, A. Silvestre; Complexo Hospitalario Universitario de Vigo, Vigo: C. Albo, S. Suarez, C. Loureiro, I. Figueroa, M. Rodríguez, M. A. Fernández, A. Martínez, C. Poderós, J. Vazquez, L. Iglesias, A. Nieto, T. Torrado, A. M. Martínez; Hospital Provincial de Pontevedra, Pontevedra: M.L. Amador, P. Oubiña, E. Feijó, A. Dios, I. Loyola, R. Roreno; Hospital POVISA, Pontevedra: A. Simiele, L. Álvarez, V. Turcu; Hospital U. Salamanca, Salamanca: B. Vidriales, M. González, R. García, A. Avendaño, C. Chillón, E. Pérez, V. González; Hospital General La Palma, Santa Cruz de Tenerife: J. V. Govantes, S. Rubio, M. Tapia; Hospital General de Segovia, Segovia: C. Olivier, J. A. Queizán; Hospital U. Virgen Macarena, Sevilla: O. Pérez, J. A. Vera, C. Muñoz, A. rodriguez, N. González; Hospital U. Virgen del Rocio, Sevilla: J. A. Pérez, E. Soria, I.Espigado, J. Falantes, I. Montero, P. García, E. Rodríguez, E. Carrillo, T. Caballero, C. García; Hospital Virgen de Valme, Sevilla: C. Couto, I. Simón, M. Gómez; Hospital Virgen del Mirón de Soria, Soria: C. Aguilar; Hospital Universitario Canarias, Tenerife: B. J. González, S. Lakhwani, A. Bienert, B. González; Hospital Universitario Nuestra Señora de Candelaria, Tenrife: A. Cabello, A. Y. Oliva, H. González; Hospital Obispo Polanco, Teruel: N. González, Hospital de Alcañiz, Teruel: L. Sancho, M. Paricio, L. Perdiguer; Hospital General Nuestra Señora del Prado, Toledo: F. Solano, A. Lerma, M. D. Martínez; Hospital Virgen de la Salud de Toledo, Toledo: M. I. Gómez, A. Yeguas; Hospital U. La Fe, Valencia: P.

Montesinos, E. Barragán, C. Sargas, R. Amigo, D. Martinez, B. Boluda, R. Rodríguez, E. Acuña, I. Cano; Hospital de Requena, Valencia: A. Escrivá, M. Pedreño; Hospital de Lluis Alcanyis de Xativa, Valencia: R. Renart; IVO (Instituto Valenciano de Oncología), Valencia: A. Navalón; Hospital de Sagunto, Valencia: I. Castillo, M. Orts; Hospital Dr. Peset, Valencia: M. J. Sayas, M. J. Fernández, M. L. Juan, E. Gómez, M. Gimeno, E. Donato, M. Cejalvo, J. Marco; Hospital Clínico Universitario, Valencia: M. Tormo, M. Calabuig, B. Navarro, I. Martin, E. Villamont, A. Miralles; Hospital de La Ribera, Valencia: R. Lluch; Hospital Casa de la Salud, Valencia: J. García; Hospital de Gandía, Valencia: M. Moragues, M. A. Ruiz; Hospital Arnau de Vilanova, Valencia: A. López, C. Benet, M. Valero; Hospital General de Valencia, Valencia: M. Linares, R. Collado, M. Orero, P. Ibañez, M. J. Lis, P. L. Pérez, M. Roig, M. López, A. V. Mena; Hospital Manises, Valencia: I. Picón, V. Cánovas, A. Palacios, E. Martí; Hospital Clínico de Valladolid, Valladolid: R. Cuello, J. Borrego, M. burgois; Hospital Rio Hortega, Valladolid: A. Cantalapiedra, O. Norberto, E. Angomas, B. Cidoncha; Hospital Universitario Araba, Victoria: L. Cuevas, D. Robles, A. Mendiazabal, I. Oiartzabal, J. M. Guinea de Castro; Hospital Virgen de la Concha, Zamora: C. Montes, M. Pérez, L. García; Hospital Royo Villanova, Zaragoza: V. Carrasco, A. Pérez, L. López, J. J. Moneva; Hospital Clínico U. Lozano Blesa, Zaragoza: M. Olave, E. Bonafonte, L. Mayor, G. Azaceta, L. Palomera; Hospital Ernest Lluch Martin, Zaragoza: M. Malo, M. J. Escobar; Hospital Quiron Salud Zaragoza, Zaragoza: J. M. Grasa; Hospital Miguel Servet, Zaragoza: B. De Rueda, A. Aulés, C. Salvador, V. Ansó, A. Iborra, P. Delagado, A. Rubio; Uruguay—Hospital de Clínicas, Montevideo: M. Stevenazzi, I. Alpire, V. Irigoin, L. Díaz, C. Guillermo, R. Guadagna, S. Grille, C. Oliver, M. Boada, V. Vales; Hospital Maciel, Montevideo: A. I. Prado; COMERO (Cooperativa Medica de Rocha), Rocha: A. P. De los Santos.

References

1. Papaemmanuil, E.; Gerstung, M.; Bullinger, L.; Gaidzik, V.I.; Paschka, P.; Roberts, N.D.; Potter, N.E.; Heuser, M.; Thol, F.; Bolli, N.; et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N. Engl. J. Med.* **2016**, *374*, 2209–2221. [CrossRef] [PubMed]
2. Tazi, Y.; Arango-Ossa, J.E.; Zhou, Y.; Bernard, E.; Thomas, I.; Gilkes, A.; Freeman, S.; Pradat, Y.; Johnson, S.J.; Hills, R.; et al. Unified Classification and Risk-Stratification in Acute Myeloid Leukemia. *medRxiv* **2022**, 2022.03.09.22271087. [CrossRef] [PubMed]
3. Khoury, J.D.; Solary, E.; Abla, O.; Akkari, Y.; Alaggio, R.; Apperley, J.F.; Bejar, R.; Berti, E.; Busque, L.; Chan, J.K.C.; et al. The 5th Edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. *Leukemia* **2022**, *36*, 1703–1719. [CrossRef] [PubMed]
4. Arber, D.A.; Orazi, A.; Hasserjian, R.P.; Borowitz, M.J.; Calvo, K.R.; Kvasnicka, H.M.; Wang, S.A.; Bagg, A.; Barbui, T.; Branford, S.; et al. International Consensus Classification of Myeloid Neoplasms and Acute Leukemia: Integrating Morphological, Clinical, and Genomic Data. *Blood* **2022**, *140*, 1200–1228. [CrossRef] [PubMed]
5. Döhner, H.; Wei, A.H.; Appelbaum, F.R.; Craddock, C.; DiNardo, C.D.; Dombret, H.; Ebert, B.L.; Fenaux, P.; Godley, L.A.; Hasserjian, R.P.; et al. Diagnosis and Management of AML in Adults: 2022 ELN Recommendations from an International Expert Panel. *Blood* **2022**, *129*, 424–447. [CrossRef]
6. Levine, R.L.; Valk, P.J.M. Next-Generation Sequencing in the Diagnosis and Minimal Residual Disease Assessment of Acute Myeloid Leukemia. *Haematologica* **2019**, *104*, 868. [CrossRef] [PubMed]
7. Bacher, U.; Shumilov, E.; Flach, J.; Porret, N.; Joncourt, R.; Wiedemann, G.; Fiedler, M.; Novak, U.; Amstutz, U.; Pabst, T. Challenges in the Introduction of Next-Generation Sequencing (NGS) for Diagnostics of Myeloid Malignancies into Clinical Routine Use. *Blood Cancer J.* **2018**, *8*, 113. [CrossRef]
8. Haferlach, T. Advancing Leukemia Diagnostics: Role of Next Generation Sequencing (NGS) in Acute Myeloid Leukemia. *Hematol. Rep.* **2020**, *12*, 8957. [CrossRef]
9. Sargas, C.; Ayala, R.; Chillón, M.C.; Larráyoz, M.J.; Carrillo-Cruz, E.; Bilbao, C.; Yébenes-Ramírez, M.; Llop, M.; Rapado, I.; García-Sanz, R.; et al. Networking for Advanced Molecular Diagnosis in Acute Myeloid Leukemia Patients Is Possible: The PETHEMA NGS-AML Project. *Haematologica* **2021**, *106*, 3079–3089. [CrossRef]
10. Ley, T.J.; Miller, C.; Ding, L.; Raphael, B.J.; Mungall, A.J.; Robertson, G.; Hoadley, K.; Triche, T.J.; Laird, P.W.; Baty, J.D.; et al. Genomic and Epigenomic Landscapes of Adult de Novo Acute Myeloid Leukemia. *N. Engl. J. Med.* **2013**, *368*, 2059–2074. [CrossRef]
11. Leisch, M.; Jansko, B.; Zaborsky, N.; Greil, R.; Pleyer, L. Next Generation Sequencing in AML-On the Way to Becoming a New Standard for Treatment Initiation and/or Modulation? *Cancers* **2019**, *11*, 252. [CrossRef] [PubMed]

12. Luthra, R.; Patel, K.P.; Routbort, M.J.; Broaddus, R.R.; Yau, J.; Simien, C.; Chen, W.; Hatfield, D.Z.; Medeiros, L.J.; Singh, R.R. A Targeted High-Throughput Next-Generation Sequencing Panel for Clinical Screening of Mutations, Gene Amplifications, and Fusions in Solid Tumors. *J. Mol. Diagn.* **2017**, *19*, 255–264. [CrossRef] [PubMed]
13. Petrackova, A.; Vasinek, M.; Sedlarikova, L.; Dyskova, T.; Schneiderova, P.; Novosad, T.; Papajik, T.; Kriegova, E. Standardization of Sequencing Coverage Depth in NGS: Recommendation for Detection of Clonal and Subclonal Mutations in Cancer Diagnostics. *Front. Oncol.* **2019**, *9*, 851. [CrossRef] [PubMed]
14. Endrullat, C.; Glökler, J.; Franke, P.; Frohme, M. Standardization and Quality Management in Next-Generation Sequencing. *Appl. Transl. Genom.* **2016**, *10*, 2–9. [CrossRef]
15. Pandzic, T.; Ladenvall, C.; Engvall, M.; Mattsson, M.; Hermanson, M.; Cavelier, L.; Ljungström, V.; Baliakas, P. Five Percent Variant Allele Frequency Is a Reliable Reporting Threshold for TP53 Variants Detected by Next Generation Sequencing in Chronic Lymphocytic Leukemia in the Clinical Setting. *Hemasphere* **2022**, *6*, e761. [CrossRef]
16. Jennings, L.J.; Arcila, M.E.; Corless, C.; Kamel-Reid, S.; Lubin, I.M.; Pfeifer, J.; Temple-Smolkin, R.L.; Voelkerding, K.V.; Nikiforova, M.N. Guidelines for Validation of Next-Generation Sequencing-Based Oncology Panels: A Joint Consensus Recommendation of the Association for Molecular Pathology and College of American Pathologists. *J. Mol. Diagn.* **2017**, *19*, 341–365. [CrossRef]
17. Guillermin, Y.; Lopez, J.; Chabane, K.; Hayette, S.; Bardel, C.; Salles, G.; Sujobert, P.; Huet, S. What Does This Mutation Mean? The Tools and Pitfalls of Variant Interpretation in Lymphoid Malignancies. *Int. J. Mol. Sci.* **2018**, *19*, 1251. [CrossRef]
18. Strom, S.P. Current Practices and Guidelines for Clinical Next-Generation Sequencing Oncology Testing. *Cancer Biol. Med.* **2016**, *13*, 3–11. [CrossRef]
19. Qin, G.; Ilya, S.; Kim, T.-K.; Tercan, B.; Martins, T.J.; Dai, J.; Chien, S.; Carson, A.; Patay, B.; Estey, E.H.; et al. Co-Occurring Mutation Clusters Predict Drug Sensitivity in Acute Myeloid Leukemia. *Blood* **2020**, *136*, 12–13. [CrossRef]
20. Lachowiez, C.A.; Reville, P.K.; Kantarjian, H.; Jabbour, E.; Borthakur, G.; Daver, N.; Issa, G.; Furudate, K.; Tanaka, T.; Pierce, S.; et al. Contemporary Outcomes in IDH-Mutated Acute Myeloid Leukemia: The Impact of Co-Occurring NPM1 Mutations and Venetoclax-Based Treatment. *Am. J. Hematol.* **2022**, *97*, 1443–1452. [CrossRef]
21. Bullinger, L.; Döhner, K.; Dohner, H. Genomics of Acute Myeloid Leukemia Diagnosis and Pathways. *J. Clin. Oncol.* **2017**, *35*, 934–946. [CrossRef] [PubMed]
22. Ding, L.; Ley, T.J.; Larson, D.E.; Miller, C.A.; Koboldt, D.C.; Welch, J.S.; Ritchey, J.K.; Young, M.A.; Lamprecht, T.; McLellan, M.D.; et al. Clonal Evolution in Relapsed Acute Myeloid Leukaemia Revealed by Whole-Genome Sequencing. *Nature* **2012**, *481*, 506–510. [CrossRef] [PubMed]
23. Niu, J.; Peng, D.; Liu, L. Drug Resistance Mechanisms of Acute Myeloid Leukemia Stem Cells. *Front. Oncol.* **2022**, *12*, 896426. [CrossRef] [PubMed]
24. van Gils, N.; Denkers, F.; Smit, L. Escape From Treatment; the Different Faces of Leukemic Stem Cells and Therapy Resistance in Acute Myeloid Leukemia. *Front. Oncol.* **2021**, *11*, 659253. [CrossRef] [PubMed]
25. Zhang, J.; Gu, Y.; Chen, B. Mechanisms of Drug Resistance in Acute Myeloid Leukemia. *Onco. Targets Ther.* **2019**, *12*, 1937–1945. [CrossRef]
26. Benard, B.A.; Leak, L.B.; Azizi, A.; Thomas, D.; Gentles, A.J.; Majeti, R. Clonal Architecture Predicts Clinical Outcomes and Drug Sensitivity in Acute Myeloid Leukemia. *Nat. Commun.* **2021**, *12*, 7244. [CrossRef]
27. Thol, F.; Heuser, M. Treatment for Relapsed/Refractory Acute Myeloid Leukemia. *Hemasphere* **2021**, *5*, E572. [CrossRef]
28. Daver, N.; Schlenk, R.F.; Russell, N.H.; Levis, M.J. Targeting FLT3 Mutations in AML: Review of Current Knowledge and Evidence. *Leukemia* **2019**, *33*, 299–312. [CrossRef]
29. Tarlock, K.; Zhong, S.; He, Y.; Ries, R.; Severson, E.; Bailey, M.; Morley, S.; Balasubramanian, S.; Erlich, R.; Lipson, D.; et al. Distinct Age-Associated Molecular Profiles in Acute Myeloid Leukemia Defined by Comprehensive Clinical Genomic Profiling. *Oncotarget* **2018**, *9*, 26417–26430. [CrossRef]
30. Grimwade, D.; Ivey, A.; Huntly, B.J.P. Molecular Landscape of Acute Myeloid Leukemia in Younger Adults and Its Clinical Relevance. *Blood* **2016**, *127*, 29. [CrossRef]
31. di Nardo, C.D.; Cortes, J.E. Mutations in AML: Prognostic and Therapeutic Implications. *Hematol. Am. Soc. Hematol. Educ. Program* **2016**, *2016*, 348. [CrossRef]
32. Yu, J.; Jiang, P.Y.Z.; Sun, H.; Zhang, X.; Jiang, Z.; Li, Y.; Song, Y. Advances in Targeted Therapy for Acute Myeloid Leukemia. *Biomark. Res.* **2020**, *8*, 17. [CrossRef]
33. Ranieri, R.; Pianigiani, G.; Sciabolacci, S.; Perriello, V.M.; Marra, A.; Cardinali, V.; Pierangeli, S.; Milano, F.; Gionfriddo, I.; Brunetti, L.; et al. Current Status and Future Perspectives in Targeted Therapy of NPM1-Mutated AML. *Leukemia* **2022**, *36*, 2351–2367. [CrossRef] [PubMed]
34. Ayala, R.; Rapado, I.; Onecha, E.; Martínez-Cuadrón, D.; Carreño-Tarragona, G.; Bergua, J.M.; Vives, S.; Algarra, J.L.; Tormo, M.; Martinez, P.; et al. The Mutational Landscape of Acute Myeloid Leukaemia Predicts Responses and Outcomes in Elderly Patients from the PETHEMA-FLUGAZA Phase 3 Clinical Trial. *Cancers* **2021**, *13*, 2458. [CrossRef] [PubMed]
35. Prassek, V.V.; Rothenberg-Thurley, M.; Sauerland, M.C.; Herold, T.; Janke, H.; Ksienzyk, B.; Konstandin, N.P.; Goerlich, D.; Krug, U.; Faldum, A.; et al. Genetics of Acute Myeloid Leukemia in the Elderly: Mutation Spectrum and Clinical Impact in Intensively Treated Patients Aged 75 Years or Older. *Haematologica* **2018**, *103*, 1853. [CrossRef] [PubMed]

36. Ni, J.; Hong, J.; Long, Z.; Li, Q.; Xia, R.; Zeng, Q. Mutation Profile and Prognostic Relevance in Elderly Patients with de Novo Acute Myeloid Leukemia Treated with Decitabine-Based Chemotherapy. *Int. J. Lab. Hematol.* **2020**, *42*, 849–857. [CrossRef] [PubMed]
37. Hellesøy, M.; Engen, C.; Grob, T.; Löwenberg, B.; Valk, P.J.M.; Gjertsen, B.T. Sex Disparity in Acute Myeloid Leukaemia with FLT3 Internal Tandem Duplication Mutations: Implications for Prognosis. *Mol. Oncol.* **2021**, *15*, 2285–2299. [CrossRef]
38. De-Morgan, A.; Meggendorfer, M.; Haferlach, C.; Shlush, L. Male Predominance in AML Is Associated with Specific Preleukemic Mutations. *Leukemia* **2021**, *35*, 867–870. [CrossRef]
39. Bullinger, L. CEBPA Mutations in AML: Site Matters. *Blood* **2022**, *139*, 6–7. [CrossRef]
40. Wakita, S.; Sakaguchi, M.; Oh, I.; Kako, S.; Toya, T.; Najima, Y.; Doki, N.; Kanda, J.; Kuroda, J.; Mori, S.; et al. Prognostic Impact of CEBPA BZIP Domain Mutation in Acute Myeloid Leukemia. *Blood Adv.* **2022**, *6*, 238–247. [CrossRef]
41. Mannelli, F.; Ponziani, V.; Bencini, S.; Bonetti, M.I.; Benelli, M.; Cutini, I.; Gianfaldoni, G.; Scappini, B.; Pancani, F.; Piccini, M.; et al. CEBPA–Double-Mutated Acute Myeloid Leukemia Displays a Unique Phenotypic Profile: A Reliable Screening Method and Insight into Biological Features. *Haematologica* **2017**, *102*, 529–540. [CrossRef] [PubMed]

SUPPLEMENTARY MATERIAL:

Table S1. Description of the sequencing technology, NGS panel and NGS platform used by each reference laboratory.

Centers	Amplicon/Capture	Commercial or custom panel	Platform
HULF	Amplicon	Comercial: Oncomine Myeloid Reserach Assay (Thermo Fisher)	Ion S5 System (Thermo Fisher)
HUVR	Amplicon	Comercial: Oncomine Myeloid Reserach Assay (Thermo Fisher)	Ion GeneStudio S5 Prime (Thermo Fisher)
H12O	Amplicon	Custom: Ampliseq (Thermo Fisher)	Ion S5 System (Thermo Fisher)
HURS	Capture	Comercial: Myeloid Tumor Solution (SOPHiA GENETICS)	MiSeq System (Illumina)
HUDN	Capture	Comercial: Myeloid Tumor Solution (SOPHiA GENETICS)	MiSeq System (Illumina)
HUS	Capture	Custom: PanMyeloid (SOPHiA GENETICS)	MiSeq System (Illumina)
UNAV	Capture	Custom: PanMyeloid (SOPHiA GENETICS)	MiSeq System (Illumina)

HULF: Hospital Universitario La Fe, HUVR: Hospital Universitario Virgen del Rocío, H12O: Hospital Universitario 12 de Octubre, HURS: Hospital Universitario Reina Sofía, HUDN: Hospital Universitario de Gran Canaria Dr. Negrín, HUS: Hospital Universitario de Salamanca, UNAV: CIMA LAB Diagnostics.

Table S2. Detailed gene panel composition

Genes	HULF, HUVR	HURS, HUDN	H12O	HUS, UNAV
ABL1	4 to 9	4 to 9	-	-
ASXL1	All exons	10, 12, 13	All exons	12
BCOR	All exons	-	All exons	All exons
BRAF	2 to 4, 6, 8, 11, 15, 17 and 18	15	-	-
CALR	All exons	9	All exons	9
CBL	8, 9	8, 9	All exons	8, 9
CEBPA	All exons	All exons	All exons	All exons
CSF3R	14, 17	All exons	All exons	14 to 17
DNMT3A	11 to 23	All exons	All exons	All exons
ETV6	All exons	All exons	All exons	All exons
EZH2	All exons	All exons	All exons	All exons
FLT3	8, 11, 13 to 16, 20, 23, 24	13 to 15 and 20	All exons	14 to 16 and 20
GATA2	4, 5	-	-	2 to 6
HRAS	2, 3	2, 3	-	-
IDH1	4	4	All exons	4
IDH2	4	4	All exons	4
JAK2	12 to 15	All exons	All exons	12 to 15
KIT	1, 2, 8 to 11, 13, 17	2, 8 to 11, 13, 17 and 18	All exons	2, 8 to 11, 13, 14, 17
KRAS	2 to 6	2, 3	All exons	2 to 4
MPL	3, 4, 10, 12	10	All exons	3 to 6, 10, 12
NPM1	11	10, 11	All exons	10, 11
NRAS	2 to 4	2, 3	All exons	2 to 4
PTPN11	3, 12, 13	3, 7 to 13	-	3, 7, 13
RUNX1	All exons	All exons	All exons	All exons
SETBP1	4	4	All exons	4
SF3B1	14 to 21	10 to 16	All exons	11 to 16
SRSF2	1	1	All exons	1
STAG2	All exons	-	All exons	All exons
TET2	All exons	All exons	All exons	All exons
TP53	All exons	All exons	All exons	All exons
U2AF1	2, 6	2, 6	All exons	2, 6
WT1	7, 9	6 to 10	All exons	7, 9
ZRSR2	All exons	All exons	All exons	All exons

HULF: Hospital Universitario La Fe, HUVR: Hospital Universitario Virgen del Rocío, H12O: Hospital Universitario 12 de Octubre, HURS: Hospital Universitario Reina Sofía, HUDN: Hospital Universitario de Gran Canaria Dr. Negrín, HUS: Hospital Universitario de Salamanca, UNAV: CIMA LAB Diagnostics.

Table S3. Genomic groups and integrated risk score based on 16 molecular classes defined by Tazi *et al*., 2022 in the updated genomic classification of AML.

		Risk groups		
Hierarchical model	**Molecular classes**	**Favorable**	**Intermediate**	**Adverse**
1. WHO 2016 Set 1	*NPM1*	■		
	t(8;21)	■		
	inv(16)	■		
2. *TP53* and/or complex karyotype (CK)	*TP53* ±CK			■
3. WHO 2016 Set 2	*CEBPA* bi	■		
	t(11;x)		■	
	t(6;9)		■	
	inv(3)			■
4. sAML1: Single mutation in one of the following genes: *SRSF2, SF3B1, U2AF1, ASXL1, EZH2, RUNX1* and *SETBP1*	sAML1		■	
5. sAML2: Two or more mutations in sAML1 defining genes. *In this subset, *DNMT3A* and *TET2* account as a second hit	sAML2			■
6. *WT1*	*WT1*		■	
7. Trisomies: Presence of ≥1 trisomies	Trisomies		■	
8. *DNMT3A + IDH1/2*	*DNMT3A + IDH1/2*		■	
9. Not class defining mutations	mNOS		■	
10. No events	No events	■		

Table S4. Third Cross-validation round results

Cross-Validation Round									
VAF≥5% variants									
Variant ID	**Gene**	**NM**	**Coding**	**Protein**	**Detected**	**Included**	**Error Rate**	**Mean VAF**	**SD**
1	*JAK2*	(NM_004972)	c.1849G>T	p.(Val617Phe)	8	8	0,0%	80,9%	2,0%
2	*TP53*	(NM_000546.5)	c.833C>T	p.(Pro278Leu)	8	8	0,0%	80,3%	2,5%
3	*RUNX1*	(NM_001754.4)	c.592G>A	p.(Asp198Asn)	8	8	0,0%	63,2%	2,0%
4	*SRSF2*	(NM_003016.4)	c.284C>A	p.(Pro95His)	8	8	0,0%	49,6%	3,8%
5	*IDH2*	(NM_002168.3)	c.419G>A	p.(Arg140Gln)	8	8	0,0%	46,8%	1,8%
6	*DNMT3A*	(NM_022552.4)	c.2644C>T	p.(Arg882Cys)	8	8	0,0%	44,1%	1,5%
7	*ASXL1*	(NM_015338)	c.1934dup	p.(Gly646Trpfs*12)	5	8	37.5%	43,5%	4,5%
8	*IDH1*	(NM_005896.3)	c.395G>A	p.(Arg132His)	8	8	0,0%	41,3%	1,9%
9	*NPM1*	(NM_002520.6)	c.860_863dup	p.(Trp288Cysfs*12)	8	8	0,0%	41,1%	7,9%
10	*NPM1*	(NM_002520.6)	c.860_863dup	p.(Trp288Cysfs*12)	8	8	0,0%	37,3%	4,4%
11	*TET2*	(NM_001127208.2)	c.3814G>C	p.(Ala1272Pro)	7	8	12,5%	31,3%	5,8%
12	*FLT3*	(NM_004119.2)	c.2027A>C	p.(Asn676Thr)	6	8	25,0%	22,8%	1,9%
13	*CSF3R*	(NM_156039.3)	c.2308C>T	p.(Gln770*)	6	8	12,5%	15,7%	0,8%
14	*FLT3*	(NM_004119.2)	c.2504A>T	p.(Asp835Val)	8	8	0,0%	14,1%	0,9%
15	*FLT3*	(NM_004119.2)	c.1770_1796dup	p.(Tyr591_Tyr599dup)	7	8	12,5%	13,2%	3,5%
16	*KRAS*	(NM_033360.3)	c.182A>C	p.(Gln61Pro)	8	8	0,0%	13,1%	2,4%
17	*NRAS*	(NM_002524.4)	c.35G>A	p.(Gly12Asp)	8	8	0,0%	10,3%	1,1%
18	*PTPN11*	(NM_002834)	c.218C>T	p.(Thr73Ile)	7	8	12,5%	7,5%	1,0%
19	*SETBP1*	(NM_015559.2)	c.2602G>A	p.(Asp868Asn)	8	8	0,0%	7,3%	0,5%
20	*CSF3R*	(NM_156039.3)	c.2296C>T	p.(Gln766*)	6	8	12,5%	5,6%	0,7%
21	*NRAS*	(NM_002524.4)	c.35G>A	p.(Gly12Asp)	8	8	0,0%	5,0%	0,9%
VAF<5% variants									
22	*FLT3*	(NM_004119.2)	c.2522A>T	p.(Asn841Ile)	7	8	12,5%	3,7%	0,7%
23	*TP53*	(NM_000546.5)	c.742C>G	p.(Arg248Gly)	6	8	25,0%	2,4%	0,5%
24	*FLT3*	(NM_004119.2)	c.2505T>A	p.(Asp835Glu)	4	8	50,0%	1,6%	0,2%
25	*KRAS*	(NM_033360.3)	c.38G>A	p.(Gly13Asp)	3	8	62,5%	1,9%	0,8%
26	*JAK2*	(NM_004972)	c.1849G>T	p.(Val617Phe)	1	8	87,5%	1,4%	N/A
27	*NRAS*	(NM_002524.4)	c.35G>A	p.(Gly12Asp)	4	8	50,0%	1,4%	0,2%
28	*PTPN11*	(NM_002834)	c.1505C>T	p.(Ser502Leu)	3	8	62,5%	1,1%	0,1%
29	*PTPN11*	(NM_002834)	c.213T>G	p.(Phe71Leu)	1	8	87,5%	1,0%	N/A
30	*FLT3*	(NM_004119.2)	c.1812_1813ins30	p.(Glu604_605ins10)	4	8	50,0%	1,0%	0,2%
31	*PTPN11*	(NM_002834)	c.227A>G	p.(Glu76Gly)	1	8	87,5%	1,0%	N/A
32	*FLT3*	(NM_004119.2)	c.1740_1788dup	p.(Tyr597Glyfs*18)	5	8	37,5%	0,8%	0,4%

Detected: number of centers which have detected the variant; Included: number of centers which include variant region in its next-generation sequencing assay; Error Rate: percentage of centers which failed variant detection; VAF: variant allele frequency; SD: VAF standard deviation; NA: not applicable: variants only were detected by one center.

Table S5. Molecular alterations pertaining to described functional categories.

Functional categories	Molecular alterations
Transcription factor (TF) fusions	-
NPM1	*NPM1*
Tumor suppresors	*TP53* and *WT1*
DNA methylation	*DNMT3A*, *IDH1*, *IDH2* and *TET2*
Activating signaling	*ABL1*, *BRAF*, *FLT3*, *HRAS*, *JAK2*, *KIT*, *KRAS*, *NRAS* and *PTPN11*
Myeloid TF	*CEBPA*, *ETV6*, *GATA2* and *RUNX1*
Chromatin modifiers	*ASXL1* and *EZH2*
Spliceosome	*SF3B1*, *SRSF2* and *U2AF1*
Cohesin	-

Table S6. Mutational frequency distribution according to moment disease, age and sex. Bold format means statistically significant values.

Genes	A) Disease status				B) Age			C) Sex		
	Diagnosis	Relapse	Refractoriness	*P*	≤65 years	>65 years	*P*	Male	Female	*P*
	%	%	%		%	%		%	%	
ABL1	0.3%	0.9%	1.2%		0.3%	0.3%		0.2%	0.4%	
ASXL1	14.7%	16.3%	14.6%		**10.2%**	**18.4%**	**<0.001**	**17.7%**	**10.8%**	**<0.001**
BRAF	0.6%	0.0%	1.2%		0.6%	0.5%		0.4%	0.8%	
CALR	1.6%	3.6%	1.2%		1.4%	1.8%		1.7%	1.5%	
CBL	3.7%	2.8%	2.9%		3.5%	3.9%		**4.5%**	**2.8%**	**0.025**
CEBPA	6.2%	6.8%	3.5%		6.4%	6.1%		6.9%	5.4%	
CSF3R	2.8%	3.6%	2.3%		2.9%	2.7%		2.6%	3.0%	
DNMT3A	**23.8%**	**31.5%**	**21.6%**	**0.018**	**25.9%**	**22.1%**	**0.032**	**20.6%**	**27.9%**	**<0.001**
ETV6	3.1%	4.8%	2.3%		2.5%	3.6%		3.7%	2.4%	
EZH2	5.5%	5.2%	5.8%		**3.0%**	**7.5%**	**<0.001**	**7.0%**	**3.5%**	**<0.001**
FLT3	24.9%	24.3%	22.2%		**29.4%**	**21.3%**	**<0.001**	**22.2%**	**28.3%**	**<0.001**
GATA2	2.8%	4.4%	4.1%		3.4%	2.2%		3.0%	2.4%	
IDH1	**9.7%**	**15.1%**	**8.2%**	**0.017**	8.8%	10.4%		9.2%	10.4%	
IDH2	14.7%	15.5%	15.2%		**12.4%**	**16.5%**	**<0.001**	15.5%	13.6%	
JAK2	5.4%	3.2%	5.8%		**3.6%**	**6.9%**	**<0.001**	**6.4%**	**4.1%**	**0.01**
KIT	3.4%	4.4%	1.8%		4.2%	2.8%		3.3%	3.5%	
KRAS	**8.2%**	**3.6%**	**4.1%**	**0.007**	7.6%	8.6%		8.6%	7.6%	
MPL	1.8%	1.6%	1.2%		2.0%	1.7%		1.4%	2.4%	
NPM1	**23.3%**	**23.1%**	**9.4%**	**<0.001**	**29.2%**	**18.5%**	**<0.001**	**19.8%**	**27.7%**	**<0.001**
NRAS	**16.2%**	**8.0%**	**17.5%**	**<0.001**	**19.1%**	**13.9%**	**<0.001**	15.9%	16.5%	
PTPN11	5.8%	4.8%	5.3%		**7.3%**	**4.5%**	**<0.001**	5.8%	5.7%	
RUNX1	**18.2%**	**23.5%**	**23.4%**	**0.037**	**13.4%**	**21.9%**	**<0.001**	**21.1%**	**14.2%**	**<0.001**
SETBP1	3.0%	4.8%	4.7%		2.3%	3.5%		3.3%	2.6%	
SF3B1	5.7%	7.2%	9.5%		**4.5%**	**6.7%**	**0.028**	5.8%	5.7%	
SRSF2	16.1%	12.7%	16.4%		**8.1%**	**22.5%**	**<0.001**	**20.9%**	**9.8%**	**<0.001**
TET2	22.0%	19.1%	19.9%		**14.3%**	**28.2%**	**<0.001**	**23.8%**	**19.7%**	**0.014**
TP53	**17.9%**	**11.2%**	**20.5%**	**0.016**	**12.7%**	**22.0%**	**<0.001**	17.2%	18.8%	
U2AF1	6.5%	3.2%	8.2%		**4.9%**	**7.7%**	**<0.001**	**8.9%**	**3.2%**	**<0.001**
WT1	**4.8%**	**11.6%**	**10.5%**	**<0.001**	**6.3%**	**3.5%**	**<0.001**	4.1%	5.6%	

Table S7. Hazard ratio for A) molecular classes, B) Risk score in global cohort, C) Risk score in <65 years-old, D) Risk score in ≥65 years-old.

		Hazard ratio	(95% CI)		*P*
			Lower IC	Upper IC	
A)	**Molecular classes**				
	NPM1	Reference			-
	CEBPA bZIP	0,3	0,0	2,0	0.201
	inv(3)	3,9	2,1	7,2	<0.001
	sAML1	1,4	1,0	2,1	0.051
	sAML2	2,1	1,6	2,7	<0.001
	WT1	2,3	1,1	5,0	<0.05
	Trisomies	0,9	0,2	3,6	0.855
	DNMT3A/IDH1-2	1,4	0,7	3,1	0.348
	Not class defining mutations	0,9	0,6	1,4	0.753
	No events	0,5	0,1	2,2	0.402
	t(X;11)	1,8	0,9	3,4	0.093
	inv(16)	0,9	0,5	1,8	0.758
	TP53-CK	3,5	2,6	4,6	<0.001
	t(8;21)	1,3	0,6	2,7	0.465
B)	**Risk score in global cohort**				
	Favorable	Reference			-
	Intermediate	1,5	1,1	2,0	<0.01
	Adverse	2,7	2,1	3,5	<0.001
C)	**Risk classification in <65 years**				
	Favorable	Reference			-
	Intermediate	2,6	1,5	4,4	<0.001
	Adverse	3,5	2,1	5,8	<0.001
D)	**Risk classification in ≥65 years**				
	Favorable	Reference			-
	Intermediate	1,0	0,7	1,4	0.864
	Adverse	1,9	1,4	2,5	<0.001

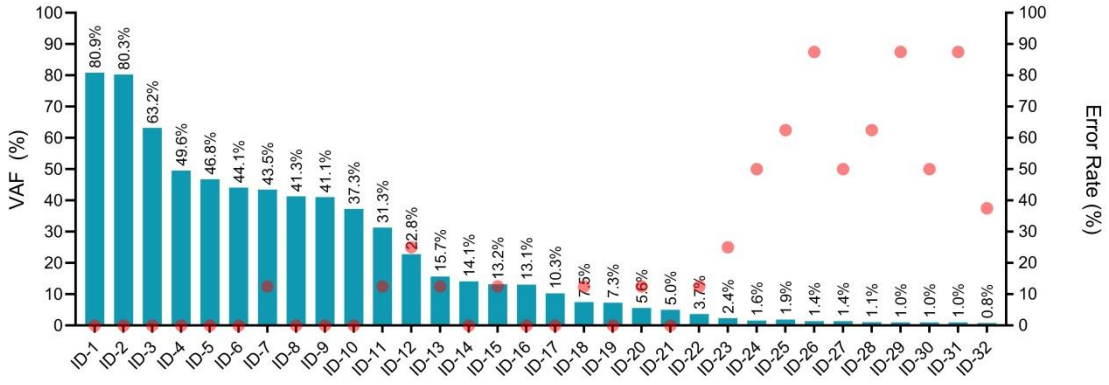


Figure S1. Third Cross-validation round results. Blue bars represent the allele frequency for the detected variants in the third cross-validation round. The red dots show the error rate in variant detection. The X-axis indicates the variant ID of table S2 for variant identification.

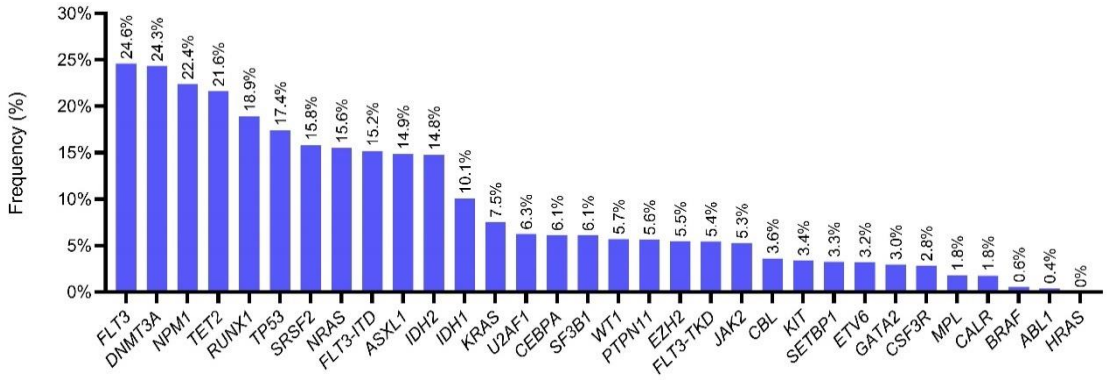


Figure S2. Mutational frequency of PETHEMA consensus genes in the global cohort (N=2856 samples).

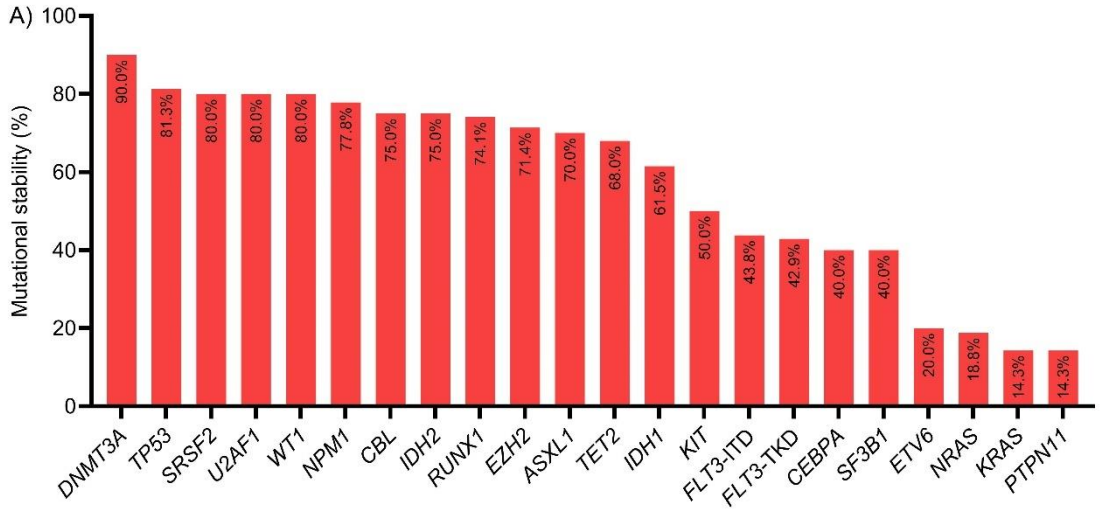


Figure S3. Stability rates in diagnosis-relapse samples (n=97 patients)

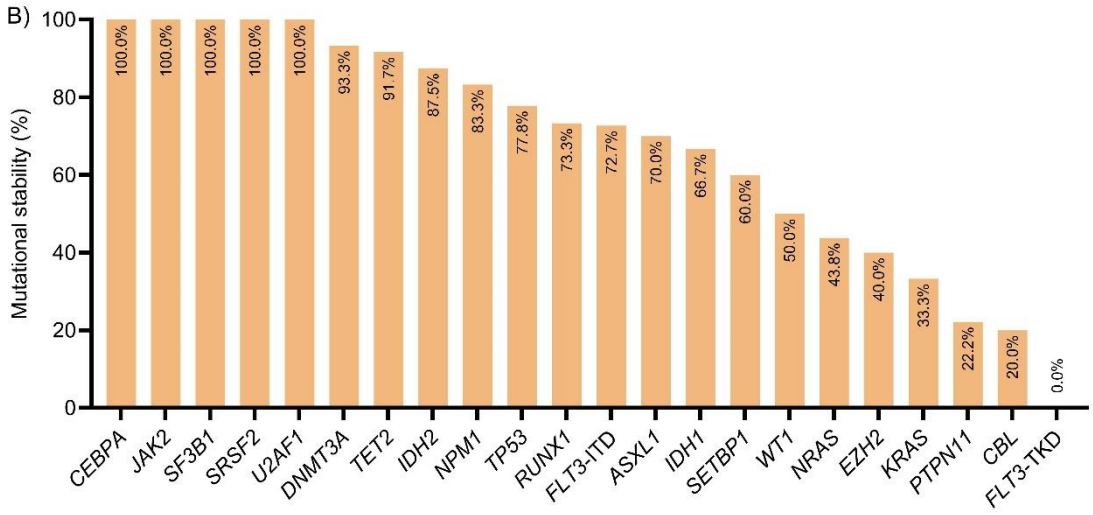
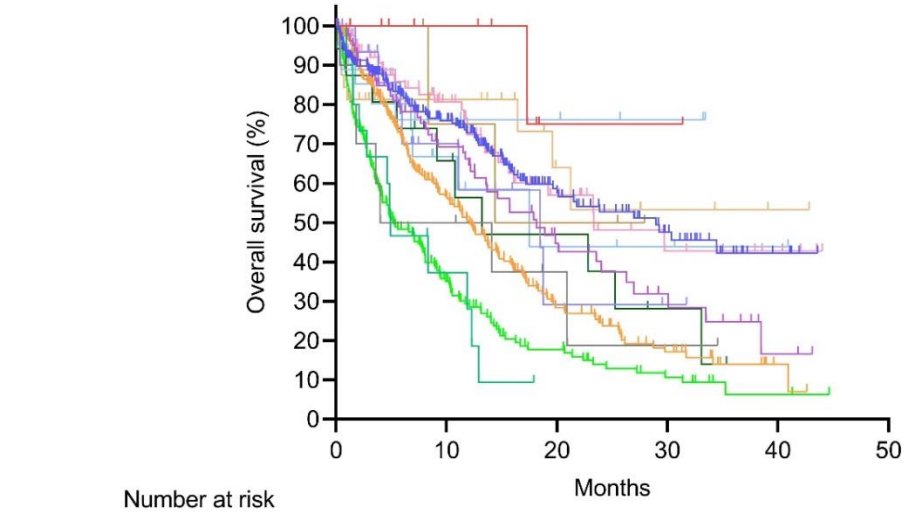


Figure S4. Stability rates in diagnosis-refractoriness samples (n=59 patients)



Number at risk					
NPM1	228	132	54	24	4
CEBPA bZIP	11	6	1	1	-
inv(3)	15	4	-	-	-
sAML1	83	46	21	9	2
sAML2	244	108	38	17	2
WT1	10	5	2	1	-
Trisomies	5	3	2	-	-
DNMT3A/IDH1-2	17	6	2	1	-
mNOS	79	41	17	8	4
No events	9	5	4	2	-
t(X;11)	17	8	5	2	-
inv(16)	32	14	6	3	1
TP53/CK	183	50	20	9	2
t(8;21)	21	8	3	2	1

Figure S5. Overall survival curves according to AML molecular classes.

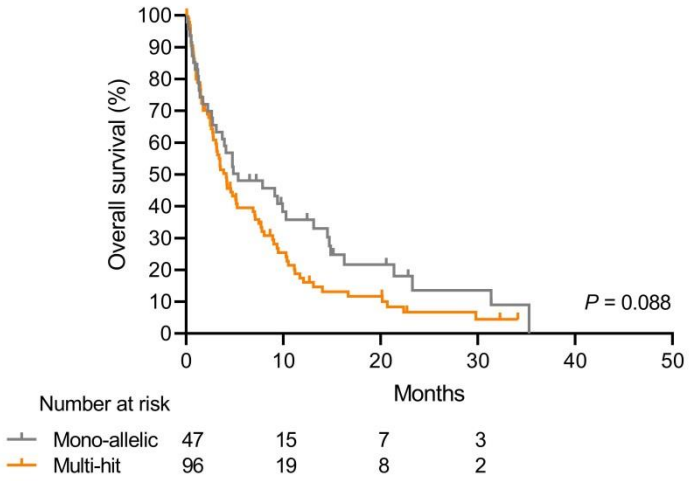


Figure S6: Overall survival curves of *TP53* mutated patients according to mono-allelic vs. multi-hit configurations.

Genetic analyses

NGS studies were performed in central laboratories according to specific protocols and sequencing platforms. Cytogenetic and molecular data were anonymously recruited by the diagnostic platform.

Title: Validation of the 2022 European LeukemiaNet risk classification in a real-life cohort of the PETHEMA group

Running title: 2022 ELN validation in the PETHEMA real-life cohort

Claudia Sargas [1], Rosa Ayala [2], María J. Larráyoz [3], María C. Chillón [4], Eduardo Rodriguez-Arboli [5], Cristina Bilbao [6], Esther Prados de la Torre [7], David Martínez-Cuadrón [8,10], Rebeca Rodríguez-Veiga [8], Blanca Boluda Pascual [8], Cristina Gil [11], Teresa Bernal [12], Juan Bergua [13], Lorenzo Algarra [14], Mar Tormo [15], Pilar Martínez-Sánchez [2], Elena Soria [5], Josefina Serrano [7], Juan M. Alonso-Dominguez [16], Raimundo García [17], María Luz Amigo [18], Pilar Herrera-Puente [19], María J. Sayas [20], Esperanza Lavilla-Rubira [21], Joaquín Martínez-López [2], María J. Calasanz [3], Ramón García-Sanz [4], José A. Pérez-Simón [5], María T. Gómez Casares [6], Joaquín Sánchez-García [7], *Eva Barragán [9,10], and *Pau Montesinos [8,10], (on behalf of PETHEMA group).

1 Grupo Acreditado de Investigación en Hematología, Instituto de Investigación Sanitaria La Fe (IIS La Fe), Valencia, Spain.

2 Hospital Universitario 12 de Octubre, CNIO, Complutense University, Madrid, Spain.

3 CIMA LAB Diagnostics-Universidad de Navarra, Pamplona, Spain.

4 Servicio de Hematología, Hospital Universitario de Salamanca (HUS/IBSAL), CIBERONC, Centro de Investigación del Cáncer-IBMCC (USAL-CSIC), Salamanca, Spain.

5 Hospital Universitario Virgen del Rocío, Instituto de Biomedicina (IBIS / CSIC), Universidad de Sevilla, Sevilla, Spain.

6 Hospital Universitario de Gran Canaria Dr. Negrín, Las Palmas de Gran Canaria, Spain.

7 IMIBIC, Hematology, Hospital Universitario Reina Sofía, UCO, Córdoba, Spain.

8 Servicio de Hematología, Grupo Acreditado de Investigación en Hematología, Hospital Universitario y Politécnico La Fe, Instituto de Investigación Sanitaria La Fe (IIS La Fe), Valencia, Spain.

9 Servicio Análisis Clínicos, Grupo Acreditado de Investigación en Hematología, Hospital Universitario y Politécnico La Fe, Instituto de Investigación Sanitaria La Fe (IIS La Fe), Valencia, Spain.

10 Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Instituto de Investigación Sanitaria La Fe (IIS La Fe), Valencia, Spain.
11 Hospital General Universitario de Alicante, Alicante, Spain.
12 Hospital Universitario Central de Asturias, Instituto Universitario (IUOPA), Instituto de investigación del Principado de Asturias (ISPA), Oviedo, Spain.
13 Hospital Universitario San Pedro de Alcántara, Cáceres, Spain.
14 Hospital Universitario General de Albacete, Albacete, Spain.
15 Hospital Clínico Universitario-INCLIVA, Valencia, Spain.
16 Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain.
17 Hospital Universitari General de Castelló, Castellón, Spain.
18 Hospital Universitario Morales Messeguer, Murcia, Spain.
19 Hospital Universitario Ramón y Cajal, Madrid, Spain.
20 Hospital Universitari Dr. Peset, Valencia, Spain.
21 Hospital Universitario Lucus Augusti, Lugo, Spain.
* Co-senior authors. These authors contributed equally to this work
Corresponding author: Pau Montesinos (montesinos_pau@gva.es)

Abstract

Next-Generation Sequencing is needed for the accurate genetic risk stratification of acute myeloid leukemia according to European LeukemiaNet (ELN) guidelines. We validated and compared the 2022 ELN risk classification in a real-life cohort of 546 intensively and 379 non-intensively treated patients. Among fit patients, those aged ≥65 years old showed worse OS than younger regardless risk classification. Compared with the 2017 classification, 14.5% of fit patients changed the risk with the 2022 classification, increasing the high-risk group from 44.3% to 51.8%. 3.7% and 0.9% *FLT3*-ITD mutated patients were removed from the favorable and adverse 2017 categories respectively to 2022 intermediate risk group. We suggest that midostaurin therapy could be a predictor for 3 years OS (85.2% with vs. 54.8% without midostaurin, *P*=0.04). Forty-seven (8.6%) patients from the 2017 intermediate group were assigned to the 2022 adverse-risk group as they harbored myelodysplasia (MDS)-related mutations. Patients with one MDS-related mutation did not reach median OS, while patients with ≥2 mutations had 13.6 months median OS (*P*=0.002). Patients with complex karyotype/*TP53* or inv(3) had a dismal prognosis (9.5 months median OS). We validate the prognostic utility of the

2022 ELN classification in a real-life setting providing supportive evidences to improve risk stratification guidelines.

Keywords

2022 ELN guidelines, Risk stratification, Next-Generation Sequencing, Prognostic utility validation, Real-life cohort.

Introduction

The application of Next-Generation Sequencing (NGS) has revealed successive novel molecular alterations in acute myeloid leukemia (AML) (1). This progress has substantially modified the diagnostic and prognostic classifications of AML, becoming molecular and cytogenetic alterations essential to properly diagnose and classify patients according to international guidelines (2–4).

In 2022, an updated version of the European LeukemiaNet (ELN) recommendations for diagnosis and management of AML was published (4). The ELN genetic risk classification was revised to include additional cytogenetic and molecular markers besides measurable residual disease assessment to refine individual risk assignment (5). One of the most important changes was the elimination of *FLT3*-ITD allelic ratio in the risk stratification; therefore, all patients with *FLT3*-ITD are now categorized as intermediate-risk irrespective of allelic ratio and concurrent *NPM1* mutation. Other major modification was the categorization of AML with myelodysplasia-related gene (MDS) mutations (*ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2*) as adverse genetic risk. In addition, the 2017 ELN risk classification only considered biallelic mutated *CEBPA* as favorable genetic abnormality; however recent studies (6–8) have shown that only in-frame mutations affecting the basic leucine zipper (bZIP) domain of *CEBPA* confer favorable outcome. Consequently, bZIP in-frame *CEBPA* mutations (*CEBPA* bZIP) are now categorized within the favorable-risk category irrespective of their occurrence as biallelic or monoallelic mutations. Regarding cytogenetics, additional abnormalities have been included as adverse-risk factors including t(3q26.2;v) involving the *MECOM* gene or t(8;16)(p11.2;p13.3) associated with *KAT6A::CREBBP* gene fusion (9,10). Furthermore, hyperdiploid karyotypes with multiple trisomies (or polysomies) without structural abnormalities are not considered complex karyotypes. Finally, adverse chromosomal abnormalities

define poor outcome irrespective of *NPM1* mutations (11). Although the new 2022 ELN risk stratification could refine and improve the former 2017 ELN classification, this should be confirmed in large AML series with complete NGS and cytogenetic datasets. Furthermore, validation of the 2022 ELN prognostic impact in a real-life cohort could be helpful to support its use in the routine clinical practice.

This study aims to compare and validate the 2022 ELN and 2017 ELN risk classifications in a large real-life series of newly diagnosed AML patients included in the *Programa Español de Tratamientos en Hematología* (PETHEMA) registry.

Methods

Patients and inclusion criteria

Since October 2017, bone marrow samples of 2434 patients with newly diagnosed AML (as per WHO 2016 criteria) were analyzed in the PETHEMA central laboratories (PLATAFO-LMA project). Pediatric patients (<18 years) and acute promyelocytic leukemias were excluded, and all eligible patients were registered regardless of the treatment received. The Institutional Ethics Committee for Clinical Research of each institution approved this study. Written informed consent in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki, and institutional regulations were obtained from all patients.

Genetic analysis

Molecular analyses were performed by NGS following harmonized criteria previously established by the PETHEMA group across 7 central laboratories (12). NGS panel included 32 genes: *ASXL1, BCOR, BRAF, CALR, CBL, CEBPA, CSF3R, DNMT3A, ETV6, EZH2, FLT3, GATA2, HRAS, IDH1, IDH2, JAK2, KIT, KRAS, MPL, NPM1, NRAS, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, STAG2, TET2, TP53, U2AF1, WT1* and *ZRSR2*. Quality parameter criteria: uniformity (>85%) and mean read depth of 1000X. Consensus criteria for variant report: All pathogenic or probably damaging variants with VAF≥5% in AML key genes were reported. For variants with 1-5% VAF, only those described in hotspot regions of clinically relevant genes were considered. Cytogenetic analyses were performed locally.

Statistics

All statistics were performed using SPSS version 22 (IBM, Armonk, NY, USA) and GraphPad Prism 4 (GraphPad, La Jolla, CA, USA) software programs. Chi square test was used to assess associations between categorical variables. Survival analyses were performed using the Kaplan–Meier method and the log-rank test. Cox proportional-hazards model was used to evaluate the risk of death among groups. *P*-value (*P*) <0.05 was considered as statistically significant test.

Results

Based on the full availability of clinical, cytogenetic and mutational data of the real-life PETHEMA cohort, 546 intensively treated patients were considered to ELN risk assessment classification (table S1). A separate analysis was conducted in 379 non-intensively treated patients according to the ELN guidelines.

2017 and 2022 ELN risk groups in intensively treated patients

According to 2017 ELN, 31.0% of patients were assigned to the favorable, 24.7% to the intermediate and 44.3% to the adverse-risk category. Although no significant differences were observed in risk distribution according to age, elderly patients (≥65 years) were mostly classified in the adverse risk group: <65 years: Favorable 33.7%, Intermediate 24.4% and adverse 42.0%; ≥65 years: Favorable 23.6%, Intermediate 25.7% and adverse 50.7% (*P*=0.07). We did not find statistically significant differences in the risk group stratification according sex (Figure 1A).

Regarding 2022 ELN risk stratification, fewer patients were classified in the favorable (27.8%) and intermediate (20.3%) risk groups, while there was an increase of adverse-risk patients (51.8%). Patients from adverse-risk category had lower leukocyte count (*P*<0.001) and lower percentage of BM blast (*P*=0.011) than patients in the favorable and intermediate categories (Table S2).

2022 ELN risk stratification was significantly different between younger and elderly AML patients. In younger patients, favorable and intermediate risk groups were overrepresented while adverse risk group was predominantly in elderly AML: <65 vs. ≥65: Favorable: 30.7% - 20.3%, intermediate: 21.6% - 16.9% and adverse: 47.7% - 62.8% (*P*=0.006). When comparing risk stratification according sex: 56.2% of male patients were classified in the

adverse-risk category compared to 46.2% of women (*P*=0.02). In contrast, 25.2% of women were classified in the intermediate category versus 16.6% of male patients. Similar distribution was observed in the favorable-risk category (27.3% - 28.6%) (Figure 1B).

Five patients (0.9%) classified according to the 2017 ELN risk stratification showed molecular alterations that allowed them to be classified in more than one risk group. Two patients had t(8;21) and *FLT3*-ITD (low ratio) mutations with wild-type (WT) *NPM1* and were classified to the favorable risk group. One patient had mutated *NPM1* and *FLT3*-ITD (high ratio) with mutated *RUNX1* and it was included in the intermediate risk group. Two patients had *FLT3*-ITD (high ratio) mutations with WT-*NPM1* and favorable risk alterations [t(8;21) and biallelic *CEBPA* mutations], both patients were assigned to the favorable risk group (Table S3).

We detected an increase in the percentage of patients with an ambiguous classification according to 2022 ELN (n=27, 4.9%) (Table S4). Nine patients with adverse-risk cytogenetic alterations and *FLT3*-ITD with WT-*NPM1* were assigned to the adverse risk group while three patients with t(8;21) and *FLT3*-ITD were assigned to the favorable risk group accordingly to the well-established prognostic value of cytogenetic alterations. Similarly, due to the recognition of AML with *CEBPA* bZIP mutations as a biological entity with favorable prognosis, one patient with a *CEBPA* bZIP mutation and *FLT3*-ITD was assigned to the favorable risk group. On the other hand, 14 patients harboring *FLT3*-ITD with mutated MDS-related genes were classified to the adverse risk group.

Outcomes according to 2017 and 2022 ELN risk in intensively treated patients

According to 2017 ELN risk stratification, median overall survival (OS) for the whole cohort was not reached in the favorable and intermediate risk groups; while in the adverse risk group median OS was 15.7 months (95%CI 11.3-20.1) (*P*<0.001) (Figure 2A). In <65 years old patients median OS was not reached in the favorable and intermediate risk groups and it was 19.9 months (95%CI 13.2-26.5) for adverse risk (*P*<0.001) (Figure 2B). Patients aged ≥65 years old had lower median OS for all risk groups: Favorable (30.4 months, 95%CI 15.3-45.4), intermediate (16.8 months, 95%CI 7.4-26.2) and adverse

(9.4 months, 95%CI 4.8-13.9) (*P*<0.01) (Figure 2C). Elderly patients classified in the favorable and intermediate risk groups had a worse prognosis than their respective risk groups of younger patients.

For the global cohort, the risk of death of intermediate and adverse-risk patients was 1.7 (95%CI 1.1-2.6; *P*<0.05) and 2.7 (95%CI 1.9-4.0; *P*<0.001) relative to favorable-risk group. According to age, elderly patients classified in the intermediate and adverse risk groups showed an increased risk of death of 2.3 (95%CI 1.2-4.3, *P*<0.01) and 1.6 (95%CI 1.1-2.4, *P*<0.05) regarding the same risk-group as young patients (Figure S1., Figure S3A).

According to 2022 ELN risk, median OS was not reached in the favorable and intermediate risk groups. Median OS in the adverse risk group was 15.2 months (95%CI 11.8-18.6) (*P*<0.001) (Figure 3A). Overall, intermediate risk group did not show a significant increased risk of death relative to favorable risk group [1.5 (95%CI 0.9-2.6; *P*=0.111)]. In contrast, adverse-risk patients had an increased risk of death of 3.3 (95%CI 2.2-5.0; *P*<0.001) (Figure S3B).

For younger patients, median OS was not reached in favorable and intermediate risk groups, while adverse risk patients showed a median OS of 19.9 months (95%CI 13.4-26.3) (*P*<0.001) (Figure 3B). In elderly patients, median OS in favorable and intermediate risk groups was 30.4 months (95%CI Not reached) and 18.8 months (95%CI 12.5-25.1) while adverse risk patients showed a median OS of 9.4 months (95%CI 6.3-12.5) (*P*<0.001) (Figure 3C). In both younger and elderly patients, only adverse-risk patients had a statistically significant increased risk of death compared to the favorable-risk group (Figure S3B).

We assessed the impact of age on the prognosis of each risk group. We detected and increased risk of death in elderly patients classified in the adverse risk group [HR 1.7 (95%CI 1.2-2.4; *P*<0.05)]. An increased risk of death was also detected regarding to young patients in the favorable and intermediate risk groups, although no significant differences were reached [Favorable: HR 2.1 (95%CI 0.9-4.5; *P*=0.069); Intermediate: HR 1.8 (95%CI 0.8-4.0; *P*=0.116)]. (Figure S2).

2017 and 2022 ELN in non-intensively treated patients

Among 379 non-intensively treated patients, 2017 ELN risk distribution was 18.2% favorable, 19.8% intermediate and 62% adverse. The median OS was

9.4 (95%CI 5.5-13.5), 11.5 (95%CI 5.6-17.4) and 6.5 (95%CI 4.8-8.2) months for favorable, intermediate and adverse-risk groups respectively (*P*=0.015) (Figure S4A).

According to 2022 ELN risk distribution for non-intensively treated patients was 16.1% favorable, 11.9% intermediate and 72% adverse. Poor OS was observed for all risk categories: Median OS favorable: 10.9 (95%CI 4.9-16.9), intermediate: 8.3 (95%CI 2.3-14.3) and adverse: 7.1 (95%CI 5.2-8.9) (*P*=0.219) (Figure S4B).

Comparison of risk category assignment between 2017 and 2022 ELN criteria

Among patients treated intensively, 79 patients (14.5%) were classified into different risk groups in each classification (Figure 4). Most transitions (12.5%) involved assignment to a worse prognosis group: 20 patients (3.7%) transitioned from the 2017 ELN favorable group to the 2022 ELN intermediate group since *FLT3*-ITD allelic ratio was not considered for risk assessment. Forty-seven (8.6%) intermediate risk patients according to 2017 ELN, were assigned to an adverse risk group in the 2022 ELN classification as they harbored mutations in MDS-related genes. Only one patient (0.2%) with double *CEBPA* mutations transitioned from favorable 2017 ELN risk group to an adverse 2022 ELN as no mutation affected the bZIP domain and MDS related gene mutations were detected. On the other hand, transitions from adverse to intermediate risk group were mostly due to the consideration of *FLT3*-ITD mutations as intermediate-risk despite of high allelic ratio (N=5, 0.9%). Finally, four patients (0.7%) harbored *CEBPA* bZIP mutations as the only clinically relevant alteration and transitioned from 2017 ELN intermediate category to the favorable group according to 2022 ELN classification (Table 1).

Outcomes of reclassified patients supported 2022 ELN modifications: new favorable patients harboring *CEBPA* bZIP mutations, showed good prognosis with no reported deaths at 31 months (Figure S5A). Furthermore, outcome of new intermediate risk patients (21.8 months; 95%CI not reached) was similar to those already classified as intermediate risk patients (*P*=0.679) (Figure S5B). Finally, new adverse risk patients showed a similar prognosis (13.7

months; 95%CI 7.0-20.5) to those previously classified in the adverse risk category (*P*=0.794) (Figure S5C).

Survival in genetic subgroups of the 2022 ELN risk categories

No differences in OS were detected between favorable risk genetic subgroups [*NPM1*mut without *FLT3*-ITD, *CEBPA* bZIP, inv(16) and t(8;21); *P*=0.741](Table 2; Figure S6A). Patients with these mutations showed 2-years OS rates between 61% and 75%.

Analysis for the genetic subsets within the intermediate risk group (*NPM1*mut with *FLT3*-ITD, *NPM1*-WT with *FLT3*-ITD, t(9;11) and other cytogenetic and molecular abnormalities), did not show significant differences in OS among subgroups (*P*=0.201) (Table 2; Figure S6B). Twenty-two out of 111 patients with *FLT3*-ITD mutations were treated with midostaurin-based regimens, resulting in a significant (*P*=0.042) better outcome than those treated with standard therapy with 3-years OS rates of 85.2% and 54.8%, respectively (Figure S7).

We found significant distinct outcomes (*P*=0.014) among adverse genetic abnormalities [inv(3), (-5, -7, -17), complex karyotype (CK)/*TP53*, MDS mutated genes, t(X;11), t(6;9) and t(9;22)]. Patients with inv(3) and CK/*TP53* showed the worst median OS: 8.3 months (95%CI 0-16.8) and 9.5 (95%CI 5.0-14.0), respectively (Table 2; Figure S6C). Patients harboring t(6;9) (N=5) or t(9;22) (N=3) were excluded from the analysis because of the small sample size.

When grouped together, patients harboring inv(3) or CK/*TP53* genetic abnormalities showed a worse median OS (9.5 months; 95%CI 5.4-13.6) as compared to other adverse risk genetic groups (*P*<0.001) (Figure S8). Together inv(3) and CK/*TP53* ("very adverse risk group") had a higher risk of death than "other adverse-risk group" in 2022 ELN [1.9 (95%CI 1.3-2.8; *P*=0.001)] (Figure 5) (Figure S9).

MDS-related genes as per 2022 ELN risk stratification had an adverse outcome with a median OS of 19.9 months (95%CI 13.1-26.6). However, patients with only one mutated MDS gene did not reach median OS while patients with two or more mutated genes showed a median OS of 13.6 months (95%CI 9.0-18.1) (*P*=0.002) (Figure 6A). Furthermore, OS in patients with one MDS mutated gene was similar to patients classified in the 2022 ELN

intermediate group (3-year OS rate of 57.6% vs. 59.6%; *P*=0.978) while patients with ≥2 MDS genes showed an OS similar to the adverse-risk group (3-year OS rate of 25.7% vs. 30.6%; *P*=0.391) (Figure 6B).

Presence of MDS-related mutations in the favorable risk group (N=28; 18.4%) did not impact OS (*P*=0.986) and although MDS mutations were weakly represented in the intermediate group (N=6; 5.4%) they conferred worse outcome (*P*=0.011).

DISCUSSION

In this study we have compared the 2017 and 2022 ELN classifications in order to validate new modifications in a real-life cohort of patients from the PETHEMA cooperative group in a centralized laboratory network using harmonized NGS studies. We have validated the prognostic impact of 2022 ELN classification, which is able to properly discriminate among the three risk-groups, like the 2017 ELN. The main prognostic difference between both classifications is that the 2022 ELN increases the burden of the adverse risk group, leading to slightly better survival rates among the favorable and intermediate groups as compared to the 2017 ELN.

For both editions the adverse risk group was the most represented, followed by the favorable and intermediate risk groups. Similarly to Lachowiez *et al*. (13), our analysis showed an increase of 7.5% of patients classified in the 2022 ELN adverse risk group mainly due the association of MDS-mutations with high-risk disease. Furthermore, Papaemmanuil and others have recently described stronger enrichment of MDS mutations in secondary AML and older patients (14,15). This finding could explain our results in elderly patients with more than 60% of patients allocated in the adverse risk category following 2022 ELN criteria. In contrast, younger patients were more likely to belong to favorable and intermediate risk groups due to higher incidence of *NPM1* and *FLT3* mutations, which also had a significant impact in terms of eligibility for targeted therapy treatment (16). It should be noted that our study validates 2022 ELN risk groups in a real-world setting, contrarily to the BEAT-AML clinical trial analyses (13), thus supporting broad applicability of these classifications in routine practice. Nevertheless, we should highlight that current ELN proposal for risk assessment increase the demand to perform an NGS panel at diagnosis, and this test is not yet widely affordable for many

patients. This would harm applicability of the 2022 ELN classification in the real-world setting.

Among intensively treated patients, older age was strongly associated with a worse prognosis regardless of the ELN risk group, suggesting less applicability for elderly patients (17,18). This has been widely described by several cooperative group trials and population-based studies which have demonstrated that advanced age at the time of AML diagnosis is clearly associated with poor outcome (19–21) On the other hand, the ELN risk classifications (both 2017 and 2022) might be used for clinical management of intensively treated patients. According to our results and previous studies (24) non-intensively treated patients have dismal prognosis regardless of ELN risk group.

The consideration of all *FLT3*-ITD mutations as intermediate risk markers into the 2022 ELN classification led to reallocate 3.7% of patients from the 2017-favorable to the 2022-intermediate risk and in 0.9% from the 2017-adverse to the 2022-intermediate categories. However, it is difficult to interpret the benefit of this risk-adjustment based on 1) several studies showing the impact of *FLT3*-ITD allelic ratio, and in particular a recently reported series of 2901 patients by PETHEMA supporting a cutoff of 0.5 for OS and 0.8 for relapse-free survival(22); and 2) the impact of targeted therapy with midostaurin in patients with *FLT3*-ITD mutations. In fact, we confirm in the real-life setting that patients receiving front-line midostaurin had significant improved OS than those receiving standard regimens (23,24). According to our results, we can even speculate that at least some *FLT3*-ITD mutated patients treated with midostaurin could be reallocated to a more favorable risk category.

Our results showed that patients with CK/*TP53* or inv(3) could be grouped in an independent risk category with a very poor prognosis (25). AML with mutated *TP53* has been widely recognized as a molecular subgroup with a very poor prognosis (26,27). Some authors refer to it as "the worst of any" with a particularly dismal prognosis especially when a complex karyotype is also present (28). Our finding is consistent with the refinement of the 2017 ELN classification suggested by Herold *et al*., who already proposed a very adverse risk subgroup which encompasses patients with *TP53* mutations and complex katyotype (29). Furthermore, the updated genomic AML classification of Tazi

et al., considers CK/*TP53* and inv(3) as molecular markers of highly chemoresistant disease and relapse-related mortality (30). This result contrasted with our results in the genetic subgroup of *CEBPA*-bZIP mutations which were the genetic abnormalities with best OS, in line with previous analyses (13).

MDS-related gene mutations have become significant in recent studies of the molecular basis of AML although its prognostic value lacks unanimous agreement. The last AML genomic classification (30), established a specific association with adverse outcomes for patients with two or more MDS gene mutations. However, ELN 2022 recommendations have not supported any differences in this respect. We show that up to 26% of patients will fall into the adverse risk 2022 ELN category based in the presence of MDS-gene mutations, becoming the biggest genetic subgroup now. Furthermore, the main risk group change between 2017 and 2022 ELN classifications was driven by the implementation of this new adverse risk subgroup. However, we show that patients with only one mutated MDS gene, representing roughly 40% of this category, had similar outcomes than intermediate risk group, while patients with two or more mutations had an OS similar to the remaining adverse risk group patients. Our results support the observations of Tazi *et al*., of lower response rate to induction chemotherapy and higher benefit after hematopoietic stem cell transplantation in patients harboring two or more MDS mutations (30). Nonetheless, we recommend reassessing the appropriateness of classifying as adverse risk AML patients with a single mutation in one of the so called MDS-related genes (*ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2)* and no other adverse genetic factor(31). Furthermore, although in the intermediate risk group our results are not consistent due to the small sample size, it would be interesting to assess the impact of MDS mutations in this risk group.

Our study has some limitations: 1) to be comparable with other studies validating ELN classifications we pick-up OS as the main predicted outcome. However, we believe that genetic risk classifications should anticipate chemoresistance and/or relapse occurrence, as patients can die by treatment toxicity or other causes unrelated to leukemic biology itself; 2) although we analyze a modern series of patients, the therapeutic landscape in AML is

rapidly evolving and we cannot properly analyze the impact of novel approaches in genetic risk assessment; and 3) our registry departed from 2434 patients with NGS results, but only 546 intensively and 379 non-intensively treated subjects had full data-set available and were used to validate the 2022 ELN classification. We are working to increase the evaluable sample size and provide further insights in future analyses.

In summary, our study provides first validation of 2022-ELN risk stratification in the real-world setting. When compared with the 2017 ELN, 14.5% of patients were reclassified according to novel 2022 ELN criteria increasing the burden of the adverse risk group. Additional studies are needed to better define risk stratification among *FLT3*-ITD patients in the era of targeted inhibitors. The allocation of AML patients into the adverse risk group based on the presence of a single MDS-related gene mutation remains as another critical issue to be solved.

Acknowledgements

The authors would like to thank María D. García, Carlos Pastorini, Rafael Vianney, Yolanda Mendizabal and Alvaro Fernández for data collection and management.

Author Contributions

CS was responsible for writing the manuscript and analyzing data. EB and PM were responsible for writing the manuscript, conceiving the work, and conducting the search. All authors: CS, RA, MJL, MCC, ERA, CB, EPT, DMC, RRV, BBP, CG, TB, JB, LA, MT, PMS, ES, JS, JMA, RG, MLA, PHP, MJS, ELR, JML, MJC, RGS, JAPS, MGC, JSG, EB and PM were involved in data interpretation, writing, reviewing, and editing the manuscript and approved the final version for submission.

Funding

This research was partially funded by Ministry of Economy and Competitiveness | Instituto de Salud Carlos III, Spain: PI18/01340, PI19/00730, FI19/00059.

Competing Interests:

The authors declare no competing financial interest in relation to the work described.

Data availability statement

The datasets generated during the current study are available from the corresponding author on reasonable request.

References

1. Llop M, Sargas C, Barragán E. The role of next-generation sequencing in acute myeloid leukemia. Curr Opin Oncol. 2022 Nov 1;34(6):723–8.

2. Khoury JD, Solary E, Abla O, Akkari Y, Alaggio R, Apperley JF, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. Leukemia. 2022 Jul 22;36(7).

3. Arber DA, Orazi A, Hasserjian RP, Borowitz MJ, Calvo KR, Kvasnicka HM, et al. International Consensus Classification of Myeloid Neoplasms and Acute Leukemia: Integrating Morphological, Clinical, and Genomic Data. Blood [Internet]. 2022 Jun 29 [cited 2022 Sep 13]; Available from: https://pubmed.ncbi.nlm.nih.gov/35767897/

4. Döhner H, Wei AH, Appelbaum FR, Craddock C, DiNardo CD, Dombret H, et al. Diagnosis and Management of AML in Adults: 2022 ELN Recommendations from an International Expert Panel. Blood [Internet]. 2022 Jul 7 [cited 2022 Aug 2]; Available from: https://pubmed.ncbi.nlm.nih.gov/35797463/

5. Ravandi F, Walter RB, Freeman SD. Evaluating measurable residual disease in acute myeloid leukemia. Blood Adv [Internet]. 2018 Jun 12 [cited 2022 Dec 5];2(11):1356–66. Available from: https://ashpublications.org/bloodadvances/article/2/11/1356/16057/Evaluating-measurable-residual-disease-in-acute

6. Wakita S, Sakaguchi M, Oh I, Kako S, Toya T, Najima Y, et al. Prognostic impact of CEBPA bZIP domain mutation in acute myeloid leukemia. Blood Adv. 2022 Jan 11;6(1):238–47.

7. Tarlock K, Lamble AJ, Wang YC, Gerbing RB, Ries RE, Loken MR, et al. CEBPA-bZip mutations are associated with favorable prognosis in de novo AML: a report from the Children's Oncology Group. Blood. 2021 Sep 30;138(13):1137–47.

8. Taube F, Georgi JA, Kramer M, Stasik S, Middeke JM, Röllig C, et al. CEBPA mutations in 4708 patients with acute myeloid leukemia: differential impact of bZIP and TAD mutations on outcome. Blood. 2022 Jan 6;139(1):87–103.

9. Sun J, Konoplev SN, Wang X, Cui W, Chen SS, Medeiros LJ, et al. De novo acute myeloid leukemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2): a clinicopathologic and cytogenetic study of an entity recently added to the WHO classification. Modern Pathology 2011 24:3 [Internet]. 2010 Nov 26 [cited 2022 Dec 4];24(3):384–9. Available from: https://www.nature.com/articles/modpathol2010210

10. Lamble AJ, Gerbing RB, Smith JL, Ries RE, Kolb EA, Alonzo TA, et al. Crebbp Alterations Are Associated with a Poor Prognosis in De Novo AML. Blood [Internet]. 2021 Nov 23 [cited 2022 Dec 4];138(Supplement 1):3451–3451. Available from: https://ashpublications.org/blood/article/138/Supplement 1/3451/479594/Crebbp-Alterations-Are-Associated-with-a-Poor

11. Angenendt L, Röllig C, Montesinos P, Martínez-Cuadrón D, Barragan E, García R, et al. Chromosomal abnormalities and prognosis in NPM1-mutated acute myeloid leukemia: A pooled analysis of

individual patient data from nine international cohorts. Journal of Clinical Oncology. 2019 Oct 10;37(29):2632–42.

12. Sargas C, Ayala R, Chillón MC, Larráyoz MJ, Carrillo-Cruz E, Bilbao C, et al. Networking for advanced molecular diagnosis in acute myeloid leukemia patients is possible: the PETHEMA NGS-AML project. Haematologica [Internet]. 2021 Dec 1 [cited 2022 Aug 2];106(12):3079–89. Available from: https://pubmed.ncbi.nlm.nih.gov/33179471/

13. Lachowiez CA, Long N, Saultz JN, Gandhi A, Newell LF, Hayes-Lattin B, et al. Comparison and validation of the 2022 European LeukemiaNet guidelines in acute myeloid leukemia. Blood Adv [Internet]. 2022 Nov 28 [cited 2022 Dec 21]; Available from: https://ashpublications.org/bloodadvances/article/doi/10.1182/bloodadvances.2022009010/493353/Comparison-and-validation-of-the-2022-European

14. Wang SY, Cheng WY, Mao YF, Zhu YM, Liu FJ, Ma TT, et al. Genetic alteration patterns and clinical outcomes of elderly and secondary acute myeloid leukemia. Hematol Oncol [Internet]. 2019 Oct 1 [cited 2022 Dec 5];37(4):456–63. Available from: https://pubmed.ncbi.nlm.nih.gov/31348835/

15. Prassek V v., Rothenberg-Thurley M, Sauerland MC, Herold T, Janke H, Ksienzyk B, et al. Genetics of acute myeloid leukemia in the elderly: mutation spectrum and clinical impact in intensively treated patients aged 75 years or older. Haematologica [Internet]. 2018 Nov 1 [cited 2022 Dec 5];103(11):1853–61. Available from: https://haematologica.org/article/view/8663

16. Hellesøy M, Engen C, Grob T, Löwenberg B, Valk PJM, Gjertsen BT. Sex disparity in acute myeloid leukaemia with FLT3 internal tandem duplication mutations: implications for prognosis. Mol Oncol [Internet]. 2021 Sep 1 [cited 2022 Dec 4];15(9):2285–99. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/1878-0261.13035

17. Appelbaum FR, Gundacker H, Head DR, Slovak ML, Willman CL, Godwin JE, et al. Age and acute myeloid leukemia. Blood [Internet]. 2006 May 1 [cited 2022 Dec 4];107(9):3481–5. Available from: https://ashpublications.org/blood/article/107/9/3481/133476/Age-and-acute-myeloid-leukemia

18. Sekeres MA, Guyatt G, Abel G, Alibhai S, Altman JK, Buckstein R, et al. American Society of Hematology 2020 guidelines for treating newly diagnosed acute myeloid leukemia in older adults. Blood Adv [Internet]. 2020 Aug 11 [cited 2022 Dec 4];4(15):3528–49. Available from: https://ashpublications.org/bloodadvances/article/4/15/3528/461693/American-Society-of-Hematology-2020-guidelines-for

19. Pulte D, Gondos A, Brenner H. Improvements in survival of adults diagnosed with acute myeloblastic leukemia in the early 21st century. Haematologica [Internet]. 2008 Apr 1 [cited 2022 Dec 4];93(4):594–600. Available from: https://haematologica.org/article/view/4820

20. Juliusson G, Antunovic P, Derolf Å, Lehmann S, Möllgård L, Stockelberg D, et al. Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. Blood [Internet]. 2009 Apr 30 [cited 2022 Dec 4];113(18):4179–87. Available from: https://ashpublications.org/blood/article/113/18/4179/25710/Age-and-acute-myeloid-leukemia-real-world-data-on

21. de Moor JS, Mariotto AB, Parry C, Alfano CM, Padgett L, Kent EE, et al. Cancer Survivors in the United States: Prevalence across the Survivorship Trajectory and Implications for Care. Cancer Epidemiol Biomarkers Prev [Internet]. 2013 Apr [cited 2022 Dec 4];22(4):561. Available from: /pmc/articles/PMC3654837/

22. Ayala R, Carreño-Tarragona G, Barragán E, Boluda B, Larráyoz MJ, Chillón MC, et al. Impact of FLT3-ITD Mutation Status and Its Ratio in a Cohort of 2901 Patients Undergoing Upfront Intensive Chemotherapy: A PETHEMA Registry Study. Cancers (Basel) [Internet]. 2022 Dec 1 [cited 2022 Dec 24];14(23). Available from: https://pubmed.ncbi.nlm.nih.gov/36497281/

23. Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, Bloomfield CD, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. N Engl J Med [Internet]. 2017 Aug 3 [cited 2022 Dec 21];377(5):454–64. Available from: https://pubmed.ncbi.nlm.nih.gov/28644114/

24. Abbas HA, Alfayez M, Kadia T, Ravandi-Kashani F, Daver N. Midostaurin In Acute Myeloid Leukemia: An Evidence-Based Review And Patient Selection. Cancer Manag Res [Internet]. 2019 [cited 2022 Dec 21];11:8817–28. Available from: https://pubmed.ncbi.nlm.nih.gov/31632141/

25. Silva P, Badiola J, Martín-Rojas RM, Gómez-Centurión I, Rodríguez-Macias G, Gómez-Llobell M, et al. Patients with Acute Myeloid Leukemia on Non-Intensive Therapy: Applicability of the European Leukemia Net Risk Classification. Blood. 2021 Nov 5;138(Supplement 1):3382–3382.

26. Granowicz EM, Jonas BA. Targeting TP53-Mutated Acute Myeloid Leukemia: Research and Clinical Developments. Onco Targets Ther [Internet]. 2022 [cited 2022 Dec 4];15:423. Available from: /pmc/articles/PMC9037178/

27. Grob T, al Hinai ASA, Sanders MA, Kavelaars FG, Rijken M, Gradowska PL, et al. Molecular characterization of mutant TP53 acute myeloid leukemia and high-risk myelodysplastic syndrome. Blood [Internet]. 2022 Apr 14 [cited 2022 Dec 4];139(15):2347–54. Available from: https://ashpublications.org/blood/article/139/15/2347/483799/Molecular-characterization-of-mutant-TP53-acute

28. Granowicz EM, Jonas BA. Targeting TP53-Mutated Acute Myeloid Leukemia: Research and Clinical Developments. Onco Targets Ther [Internet]. 2022 [cited 2022 Dec 4];15:423. Available from: /pmc/articles/PMC9037178/

29. Herold T, Rothenberg-Thurley M, Grunwald V v., Janke H, Goerlich D, Sauerland MC, et al. Validation and refinement of the revised 2017 European LeukemiaNet genetic risk stratification of acute myeloid leukemia. Leukemia [Internet]. 2020 Dec 1 [cited 2022 Dec 4];34(12):3161–72. Available from: https://pubmed.ncbi.nlm.nih.gov/32231256/

30. Tazi Y, Arango-Ossa JE, Zhou Y, Bernard E, Thomas I, Gilkes A, et al. Unified classification and risk-stratification in Acute Myeloid Leukemia. medRxiv [Internet]. 2022 Mar 12 [cited 2022 Jul 14];2022.03.09.22271087. Available from: https://www.medrxiv.org/content/10.1101/2022.03.09.22271087v1

31. Jentzsch M, Bischof L, Ussmann J, Backhaus D, Brauer D, Metzeler KH, et al. Prognostic Impact of the 2022 European Leukemia Net Risk Classification in Patients with Acute Myeloid Leukemia Undergoing

Allogeneic Stem Cell Transplantation. Blood [Internet]. 2022 Nov 15 [cited 2023 Jan 2];140(Supplement 1):10601–2. Available from: https://ashpublications.org/blood/article/140/Supplement 1/10601/490513/Prognostic-Impact-of-the-2022-European-Leukemia

Figure and table legends:

Figure 1. Risk categories distribution in the global cohort and according age and sex for A) 2017 ELN and B) 2022 ELN. Green section: Favorable risk category; Yellow section: Intermediate risk category and Red section: Adverse risk category.

Figure 2. Overall survival according to 2017 ELN risk: A) Global cohort, B) young patients (<65 years) and C) elderly patients (≥65 years)

Figure 3. Overall survival according to 2022 ELN risk: A) Global cohort, B) young patients (<65 years), C) elderly patients (≥65 years)

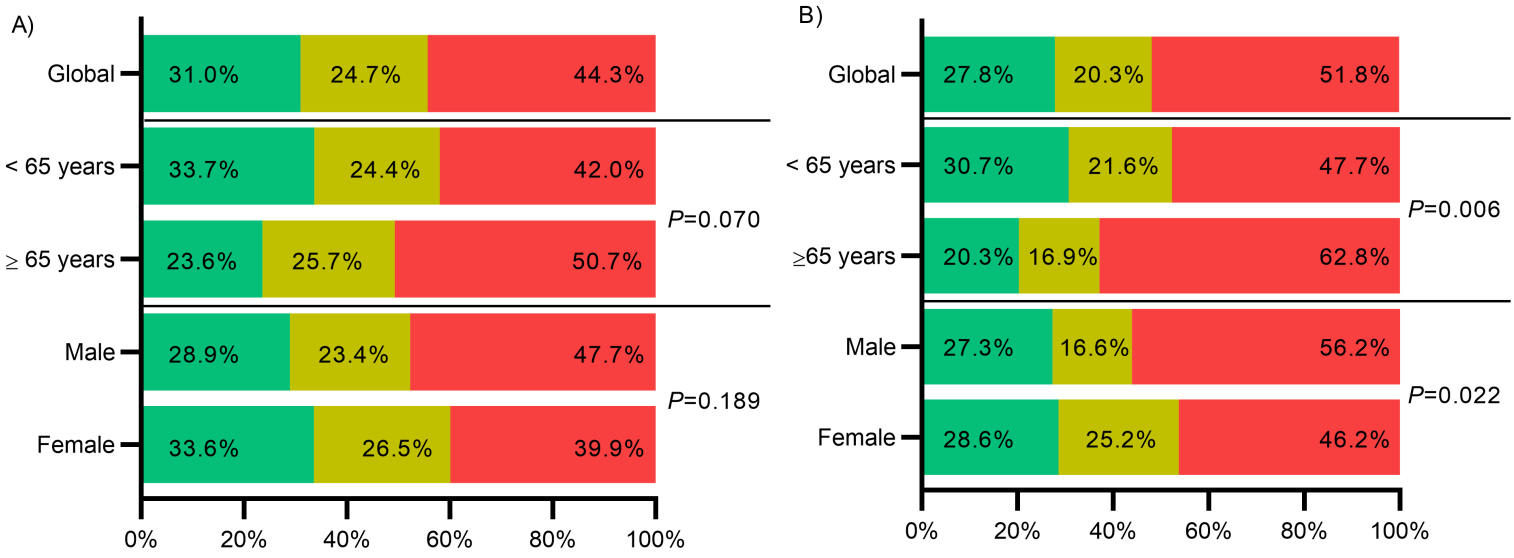
Figure 4. Sankey diagram comparing 2017 ELN and 2022 ELN risk categories.

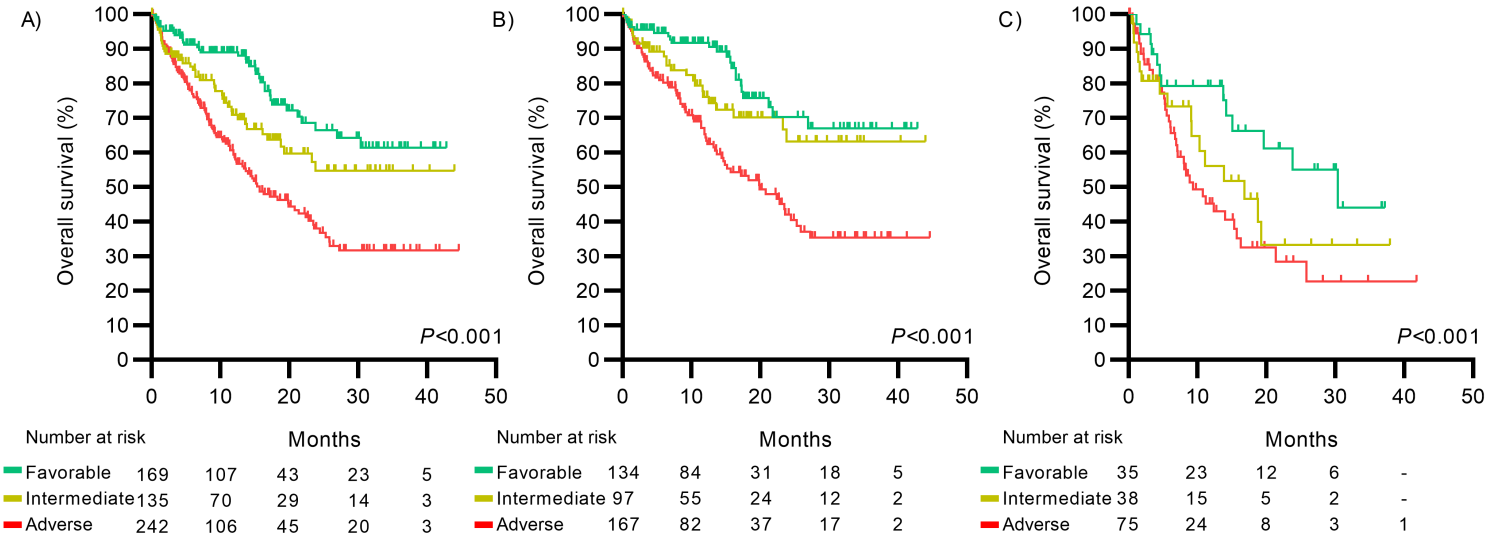
Figure 5. Outcomes of patients according to the proposed refinement of the 2022 ELN risk categories.

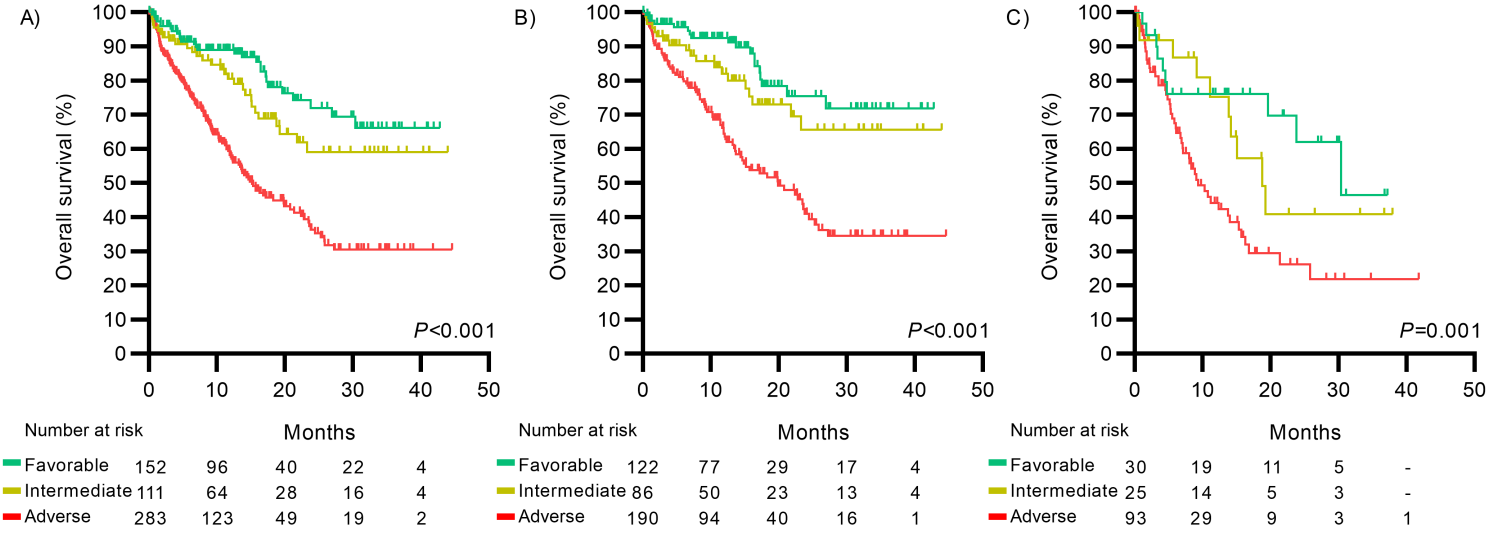
Figure 6. Outcomes of MDS mutated patients. A) According to the number of mutated genes and B) regarding intermediate and adverse 2022 ELN risk groups. MDS: myelodysplasia-related genes.

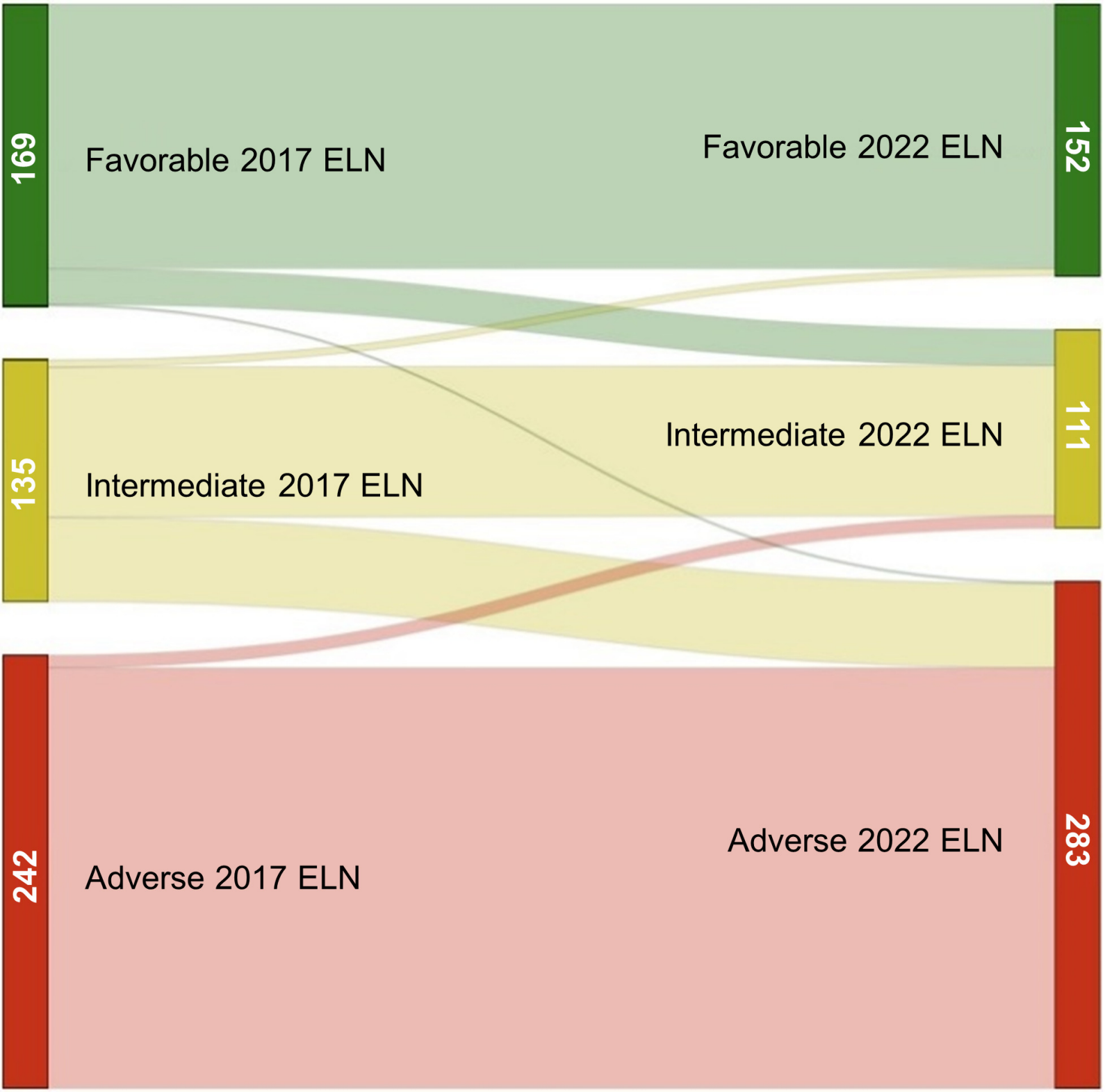
Table 1. Patients with different risk classification according to 2017 ELN and 2022 ELN.

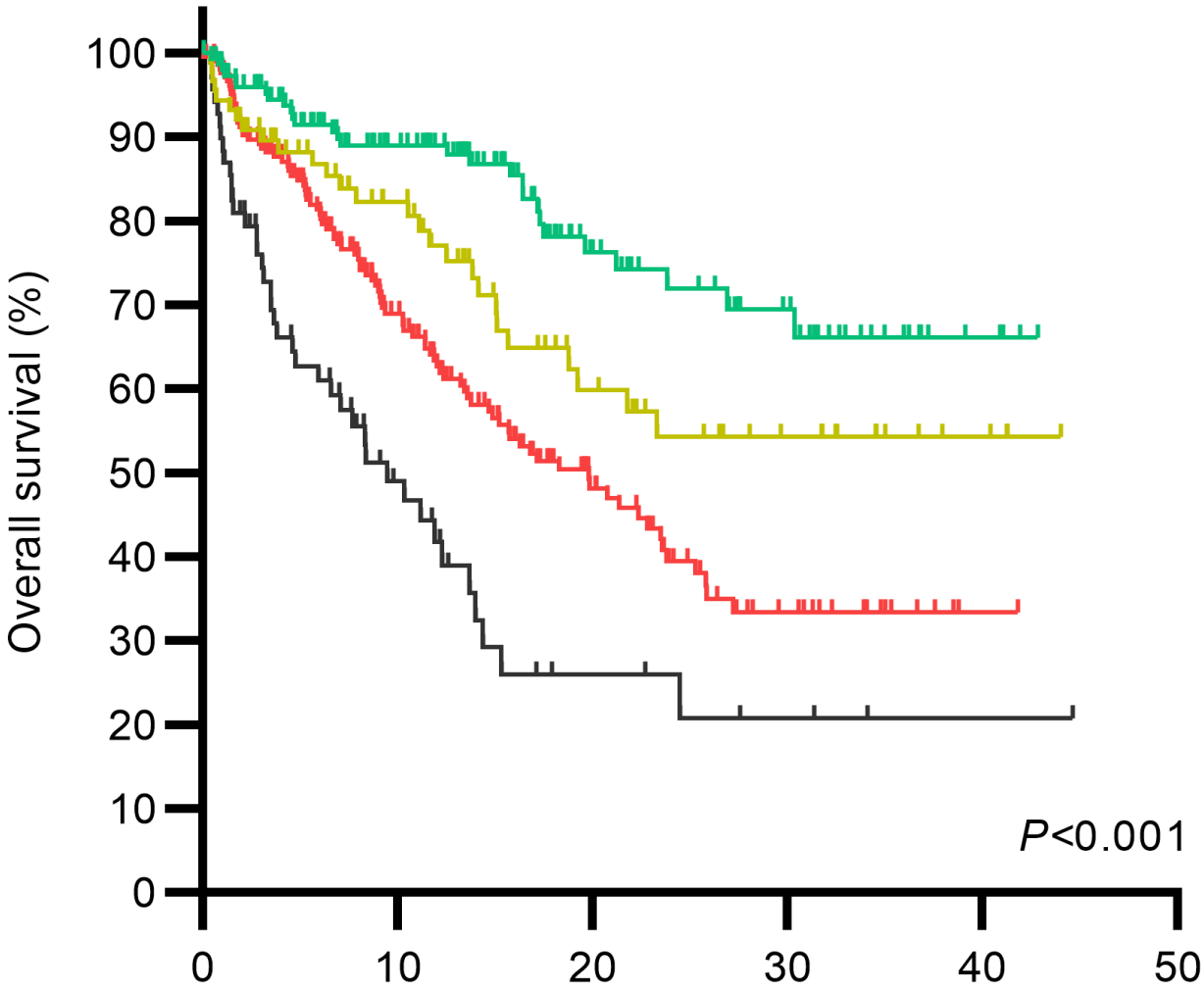
Table 2. Outcomes according to genetic subsets within the 2022 ELN.


A)
Global
31.0%
24.7%
44.3%
< 65 years
33.7%
24.4%
42.0%
≥ 65 years
23.6%
25.7%
50.7%
P=0.070
Male
28.9%
23.4%
47.7%
Female
33.6%
26.5%
39.9%
P=0.189
0%
20%
40%
60%
80%
100%
B)
Global
27.8%
20.3%
51.8%
< 65 years
30.7%
21.6%
47.7%
≥65 years
20.3%
16.9%
62.8%
P=0.006
Male
27.3%
16.6%
56.2%
Female
28.6%
25.2%
46.2%
P=0.022
0%
20%
40%
60%
80%
100%


A)
B)
C)
Overall survival (%)
Months
P<0.001
Number at risk
Favorable 169 107 43 23 5
Intermediate 135 70 29 14 3
Adverse 242 106 45 20 3
Favorable 134 84 31 18 5
Intermediate 97 55 24 12 2
Adverse 167 82 37 17 2
Favorable 35 23 12 6 -
Intermediate 38 15 5 2 -
Adverse 75 24 8 3 1


A)
Overall survival (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
Months
P<0.001
Number at risk
Favorable 152 96 40 22 4
Intermediate 111 64 28 16 4
Adverse 283 123 49 19 2
B)
Overall survival (%)
Months
P<0.001
Number at risk
Favorable 122 77 29 17 4
Intermediate 86 50 23 13 4
Adverse 190 94 40 16 1
C)
Overall survival (%)
Months
P=0.001
Number at risk
Favorable 30 19 11 5 -
Intermediate 25 14 5 3 -
Adverse 93 29 9 3 1


169
Favorable 2017 ELN
135
Intermediate 2017 ELN
242
Adverse 2017 ELN
Favorable 2022 ELN
Intermediate 2022 ELN
Adverse 2022 ELN
152
111
283



Number at risk					
Favorable	152	96	40	22	4
Intermediate	89	49	24	13	4
Adverse	213	102	43	16	1
Very Adverse CK/*TP53*, inv(3)	70	21	6	3	1

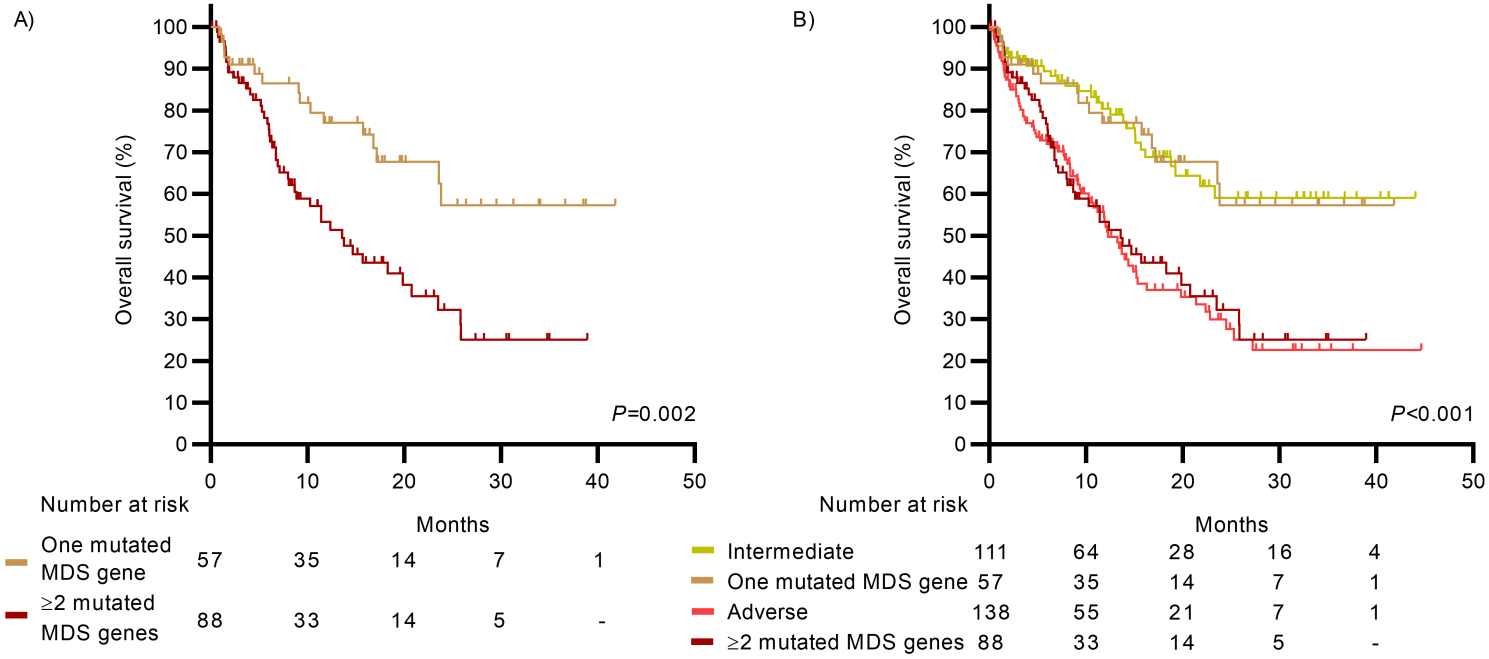

A)
Overall survival (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
Months
P=0.002
Number at risk
One mutated MDS gene 57 35 14 7 1
≥2 mutated MDS genes 88 33 14 5 -
B)
Overall survival (%)
P<0.001
Number at risk
Months
Intermediate 111 64 28 16 4
One mutated MDS gene 57 35 14 7 1
Adverse 138 55 21 7 1
≥2 mutated MDS genes 88 33 14 5 -

Table 1. Patients with different risk classification according to 2017 ELN and 2022 ELN.

N	(%)	Molecular features	2017 ELN risk classification	2022 ELN risk classification
20	3.7%	Mutated *NPM1* with low allelic ratio *FLT3*-ITD	Favorable	Intermediate
1	0.2%	Biallellic mutated *CEBPA* (not bZIP domain) + Mutated MDS genes	Favorable	Adverse
4	0.7%	bZIP in frame mutated *CEBPA* (only one *CEBPA* mutation)	Intermediate	Favorable
47	8.6%	Mutated MDS genes (not *RUNX1* and *ASXL1)*	Intermediate	Adverse
4	0.7%	High allelic ratio *FLT3*-ITD	Adverse	Intermediate
1	0.2%	Hyperdiploid karyotype + high allelic ratio *FLT3*-ITD	Adverse	Intermediate
2	0.4%	Hyperdiploid karyotype	Adverse	Intermediate

Table 2. Outcomes according to genetic subsets within the 2022 ELN.

Category	N	1-year OS	2-year OS	3-year OS	*P*
Favorable					
NPM1 mut, *FLT3*-ITD WT	99	86.9%	75.1%	66.4%	0.741
CEBPA-bZIP	9	100.0%	75.0%	-	
inv(16)	25	91.8%	68.5%	68.5%	
t(8;21)	17	81.4%	61.0%	61.0%	
Intermediate					
NPM1 mut, *FLT3*-ITD mut	45	74.7%	47.9%	47.9%	0.201
NPM1 WT, *FLT3*-ITD mut	11	74.1%	74.1%	74.1%	
Other abnormalities	44	88.6%	72.7%	72.7%	
t(9;11)	1	-	-	-	
Adverse					
inv(3)	10	15.6%	-	-	0.014
(-5, -7, -17)	35	56.8%	34.4%	23.0%	
Complex karyotype/*TP53*	60	40.3%	22.6%	22.6%	
MDS-mutated genes	145	59.5%	38.2%	38.2%	
t(X;11)	12	54.1%	27.0%	-	
t(6;9)	5	100.0%	-	-	
t(9;22)	3	-	-	-	

SUPPLEMENTARY MATERIAL:

Figures

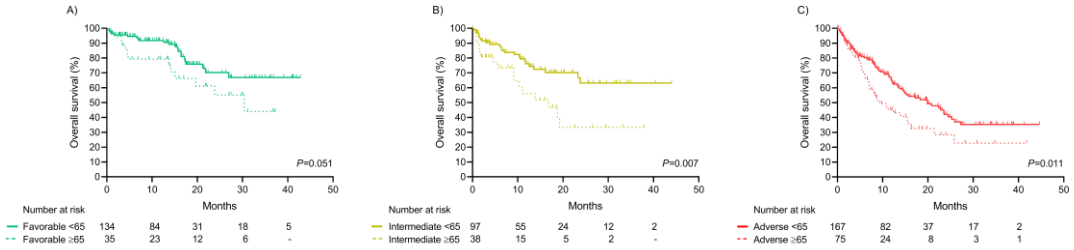


Figure S1. Overall survival assessment between young and elderly AML patients for the 2017 ELN risk categories: A) Favorable, B) Intermediate and C) Adverse.

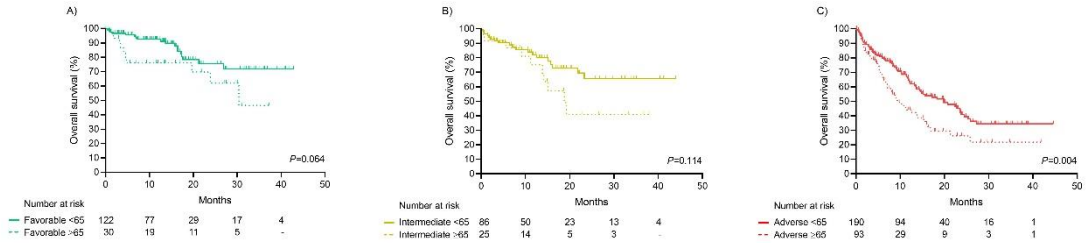


Figure S2. Overall survival assessment between young and elderly AML patients for the 2022 ELN risk categories: A) Favorable, B) Intermediate and C) Adverse.

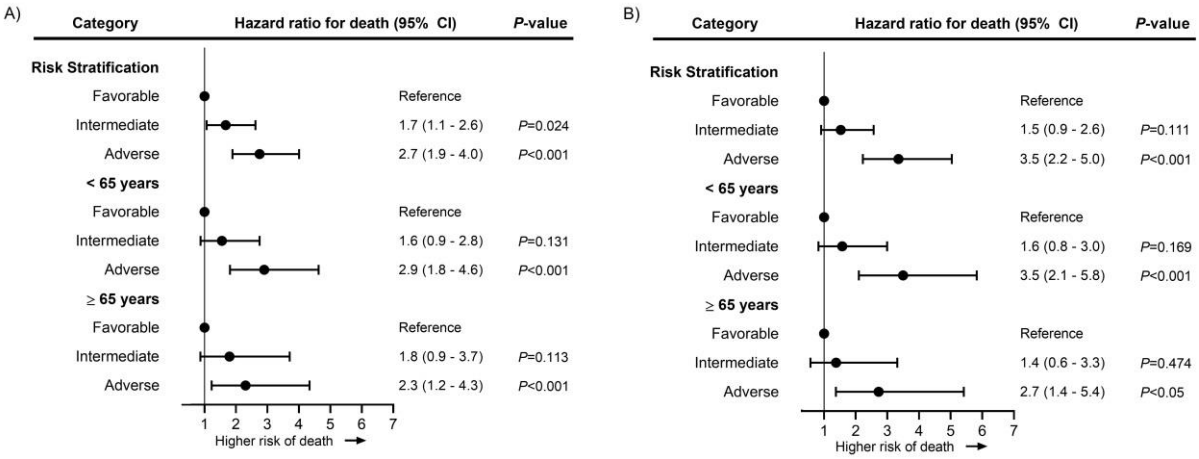


Figure S3. Hazard ratio for A) 2017 ELN and B) 2022 ELN risk categories.

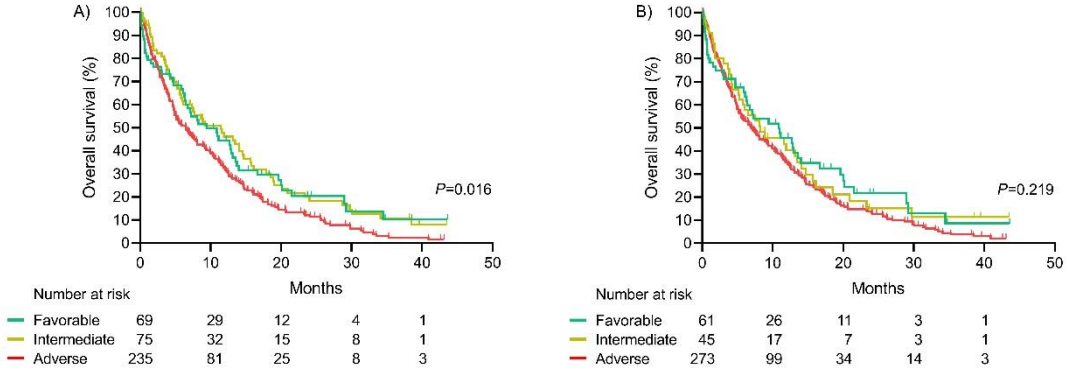


Figure S4. Overall survival analysis in non-intensive treated patients according to: A) 2017 ELN and B) 2022 ELN risk categories.

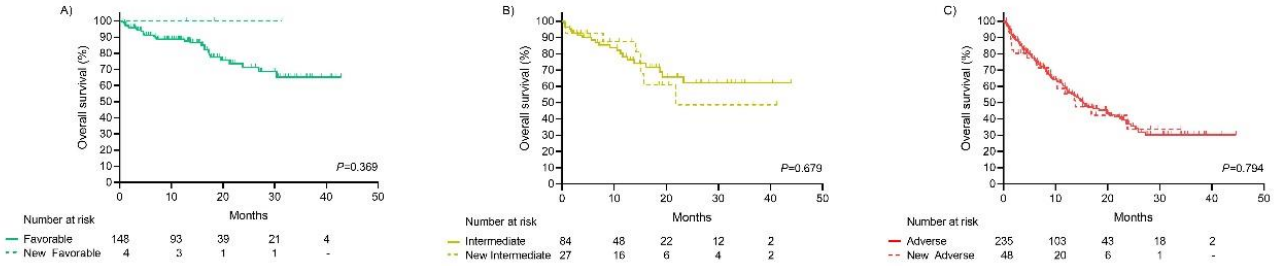


Figure S5. Overall survival analysis of reclassified A) new favorable, B) new intermediate and c) new adverse risk patients according to 2022 ELN and patients who remained in the risk category.

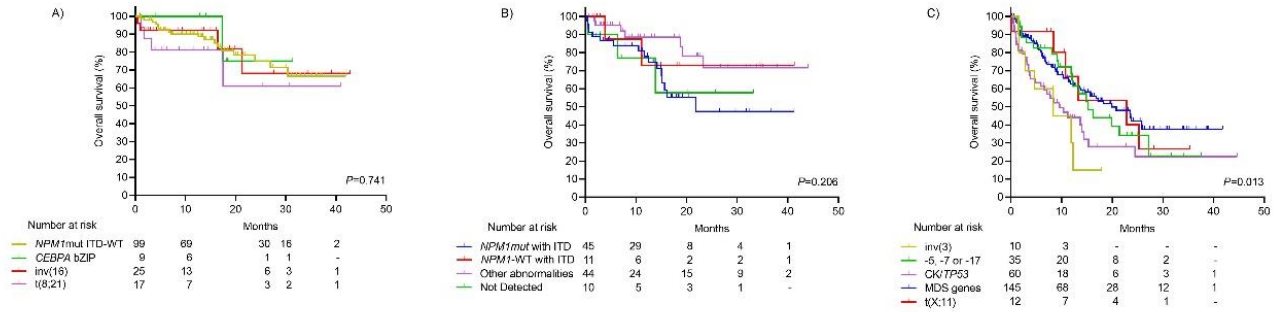


Figure S6. Outcomes of specific genetic subsets with in the 2022 ELN risk categories. WT: wild-type; CK: Complex karyotype; MDS: myelodysplasia-related genes.

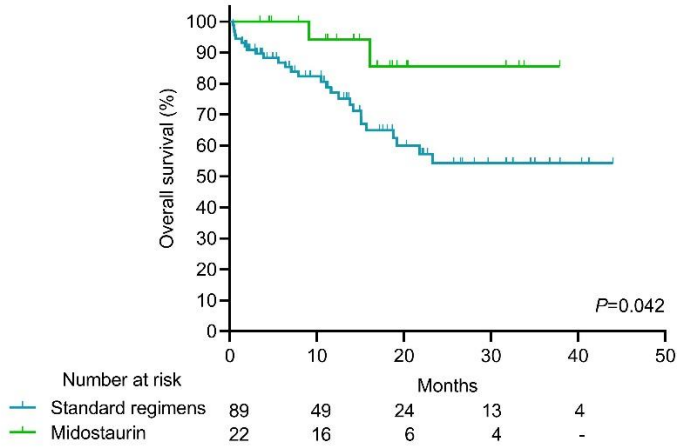


Figure S7. Outcomes from intermediate risk patients with *FLT3*-ITD AML treated with midostaurin inhibitor.

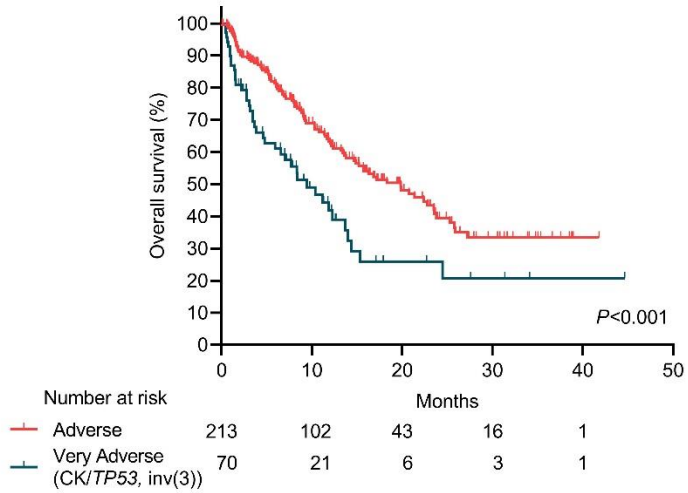


Figure S8. Outcomes from 2022 ELN adverse risk patients and those classified in the proposed as very high-risk.

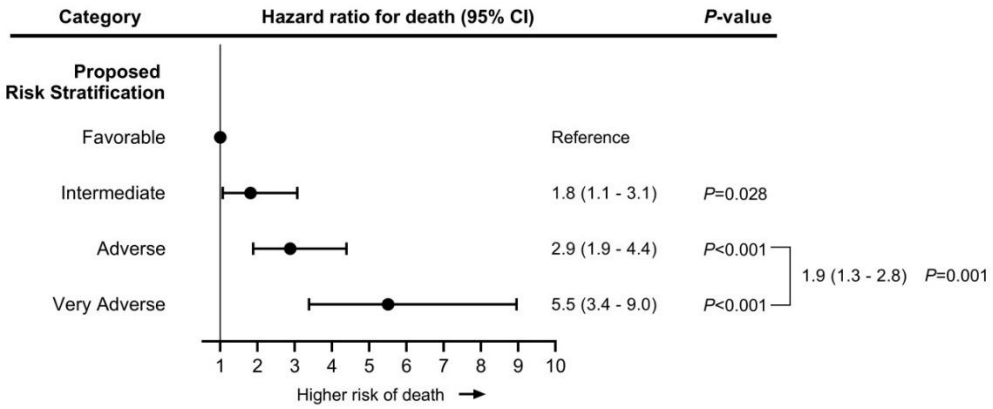


Figure S9. Hazard ratio for the new risk stratification proposal according to the PETHEMA cohort.

Tables

Table S1. Demographic and baseline characteristics of the study population (N=546).

Characteristic	Mean	Median	Range	N	(%)
Age, years	56.1	58.4	18-88	546	100
<65				398	72.9
≥65				148	27.1
Sex				546	100
Male				308	56.4
Female				238	43.6
ECOG				465	100
0				235	50.5
1				187	40.2
2				31	6.7
3				9	1.9
4				3	0.6
Not available				81	
WBC (×10^9/L)	33.0	8.7	0.2-374	406	
BM blast cells, %	54.1	53.0	0-100	463	
Creatinine, mg/dL	1.0	0.8	0.4-10.3	470	
MRC cytogenetic risk				546	100
Favorable				44	8.1
Intermediate				95	17.4
Adverse				134	24.5
Normal karyotype				273	50.0
AML FAB subtype				318	100
M0				31	9,7
M1				64	20,1
M2				50	15,7
M4				81	25,5
M5				76	23,9
M6				11	3,5
M7				5	1,6
Not available				228	
Type of AML				546	100
De novo				392	71.8
Secondary				154	28.2

Table S2. Patients' characteristics according to 2022 ELN risk group (N=546).

Characteristic	2022 ELN risk categories			P
	Favorable N=152	Intermediate N=120	Adverse N=274	
Age, years	53.7 (17.4-75.8)	58.2 (22.9-88.1)	60.9 (18.0-83.9)	<0.001
Age, group				
<65	122 (30.7)	86 (21.6)	190 (47.7)	0.006
≥65	30 (20.3)	25 (16.9)	93 (62.8)	
Sex				
Male	84 (27.3)	51 (16.6)	173 (56.2)	0,022
Female	68 (28.6)	60 (25.2)	110 (46.2)	
ECOG				
0	66 (28.1)	55 (23.4)	114 (48.5)	0,09
1	52 (27.8)	28 (15)	107 (57.2)	
2	10 (32.3)	9 (29)	12 (38.7)	
3	0 (0)	3 (33.3)	6 (66.7)	
4	2 (66.7)	0 (0)	1 (33.3)	
Not available				81
WBC (×10^9/L)	20.9 (0.6-339)	16.7 (0.3-374)	5.2 (0.2-314.3)	<0.001
BM blast cells, %	58 (20-100)	56.5 (8-100)	48 (4-100)	0.011
Creatinine, mg/dL	0.9 (0.4-3.9)	0.8 (0.4-10.3)	0.9 (0.4-7.4)	0.098
MRC cytogenetic risk				
Favorable	44 (100)	0 (0)	0 (0)	<0.001
Intermediate	15 (15.8)	24 (25.3)	56 (58.9)	
Unfavorable	0 (0)	3 (2.2)	131 (97.8)	
Normal karyotype	93 (34.1)	84 (30.8)	96 (35.2)	
AML FAB subtype				
M0	1 (3.2)	8 (25.8)	22 (71)	<0.001
M1	14 (21.9)	20 (31.3)	30 (46.9)	
M2	21 (42.0)	9 (18.0)	20 (40.0)	
M4	38 (46.9)	18 (22.2)	25 (30.9)	
M5	22 (28.9)	19 (25)	35 (46.1)	
M6	1 (9.1)	1 (9.1)	9 (81.8)	
M7	0 (0)	0 (0)	5 (100)	
Not available				228
Type of AML				
De novo	105 (26.8)	82 (20.9)	205 (52.3)	0.654
Secondary	47 (30.5)	29 (18.8)	78 (50.6)	

Table S3. Ambiguous patients according to the 2017 ELN risk categories.

N	Karyotype	Genetic abnormalities for ELN-2017 risk categories			Consensus
		Favorable genetic abnormalities	Intermediate genetic abnormalities	Adverse genetic abnormalities	
1	46,XY	Biallelic mutated *CEBPA*	-	Wild-type *NPM1* with *FLT3*-ITD^{high}	Favorable
2	46,XY	-	Mutated *NPM1* with *FLT3*-ITD^{high}	Mutated *RUNX1*	Intermediate
3	45,XY,t(8;21)(q22;q22.1)-Y	t(8;21)(q22;q22.1)	Wild-type *NPM1* with *FLT3*-ITD^{low}	-	Favorable
4	46,XY,t(8;21)(q22;q22.1)	t(8;21)(q22;q22.1)	Wild-type *NPM1* with *FLT3*-ITD^{low}	-	Favorable
5	46,XY,t(8;21)(q22;q22.1)	t(8;21)(q22;q22.1)	-	Wild-type *NPM1* with *FLT3*-ITD^{high}	Favorable

Table S4. Ambiguous patients according to the 2022 ELN risk categories.

N	Karyotype	Genetic abnormalities for 2022 ELN risk categories			Consensus
		Favorable risk alteration	Intermediate risk alteration	Adverse risk alteration	
1	46,XY	*CEBPA* bZIP in-frame	Wild-type *NPM1* with *FLT3*-ITD	-	Favorable
2	47XY, +8	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
3	46,XY	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
4	46,XX	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
5	46,XX,t(6,9)(p23,q34)	-	Wild-type *NPM1* with *FLT3*-ITD	t(6;9)(p23;q34)	Adverse
6	54,XX,+4,+6,+8,+9,+10,+13,+5,add(16)(q24),+21	-	Wild-type *NPM1* with *FLT3*-ITD	Complex/Monosomal karyotype	Adverse
7	46,XX,t(6,9)(p23,q34)	-	Wild-type *NPM1* with *FLT3*-ITD	t(6;9)(p23;q34)	Adverse
8	46,XY,t(11,19)(q23,p13)	-	Wild-type *NPM1* with *FLT3*-ITD	t(v;11q23.3)	Adverse
9	46,XY,t(3,3)(q21,q26.2)	-	Wild-type *NPM1* with *FLT3*-ITD	t(3;3)(q21;q26.2)	Adverse
10	46,XX,del(7)(q21),del(5)(q31)	-	Wild-type *NPM1* with *FLT3*-ITD	-7, -5 + Mutated MDS-related genes	Adverse
11	46,XX	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
12	45,XY,t(8;21)(q22;q22.1)-Y	t(8;21)(q22;q22.1)	Wild-type *NPM1* with *FLT3*-ITD	-	Favorable
13	46,XY,t(8;21)(q22;q22.1)	t(8;21)(q22;q22.1)	Wild-type *NPM1* with *FLT3*-ITD	-	Favorable
14	46,XY	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
15	46, XY	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
16	47, XY, +19	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
17	47,XX,+8	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
18	45,X,-Y	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
19	46,XX	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
20	47,XY,+8	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
21	46XY	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
22	47 XY,+8, t(11,19)(q23,p13)	-	Wild-type *NPM1* with *FLT3*-ITD	t(v;11q23.3)	Adverse
23	46,XY	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse
24	46,XY,t(6,9)(p22,q34)	-	Wild-type *NPM1* with *FLT3*-ITD	t(6;9)(p23;q34)	Adverse
25	46,XY,t(6,9)(p23,q34)	-	Wild-type *NPM1* with *FLT3*-ITD	t(6;9)(p23;q34)	Adverse
26	46,XY,t(8;21)(q22;q22.1)	t(8;21)(q22;q22.1)	Wild-type *NPM1* with *FLT3*-ITD	-	Favorable
27	46,XX,del(11)	-	Wild-type *NPM1* with *FLT3*-ITD	Mutated MDS-related genes	Adverse

Appendix A

Institutions and clinicians participating in the PETHEMA epidemiologic registry of acute myeloid leukemia and acute promyelocytic leukemia.

Argentina (Grupo Argentino para el Tratamiento de la Leucemia Aguda - GATLA)— Hospital de Clínicas, Buenos Aires: F. Rojas; H. Longoni; Fundaleu, Buenos Aires: G. Milone, I. Fernández, Clínica Conciencia, Neuquén: R. Ramirez; Hospital Rossi ,La Plata : C. Canepa, S. Saba, G. Balladares, Hospital General San Martin, Parana : G. Milone, C. Ventiurini, R. Mariano, P. Negri; Hospital Italiano de La Plata, La Plata: M. V. Prates, J. Milone; Hospital General San Martín, La Plata:P. Fazio, M. Gelemur; Hospital Clemente Alvarez, Rosario: G. Milone, S. Ciarlo, F. Bezares; Hospital de Córdoba, Córdoba: L. López , Hospital Privado de Córdoba, Córdoba: J. J.García; Instituto Privado Hematología, Paraná: P. Negri, M. Giunta, G. Milone; Hospital Teodoro Alvarez, Buenos Aires: M. Kruss; Hospital Tornú, Buenos Aires: D. Lafalse, G. Milone; Hospital Gobernador Centeno, La Pampa: E. Marquesoni, M. F. Casale; Hospital Italiano de Buenos Aires, Buenos Aires: A. Gimenez , E. B. Brulc, M. A. Perusini; Complejo Médico Policía Federal, La Plata: G. Milone, L. Palmer; Colombia (Asociación Colombian de Hematología y Oncología - ACHO)— Clínica La Estancia, Popayán: M. E. Correa; Fundación Valle del Lili , Cauca: F.J. Jaramillo, J. Rosales; Instituto FOSCAL, Bucaramanga: C. Sossa, J. C. Herrera ; Hospital Pablo Tobón Uribe, Antioquia: M. Arango; Poland (Polish Adult Leukemia Group - PALG)—City Hospital Legnica, Baja Silesia: J. Holojda; IHIT Hematology and transfusiology institute, Warszawa: A. Golos, A. Ejduk; Wojewódzki Szpital Specjalistyczny w Olsztynie, Olsztyn: B. Ochrem; WIM (Military Institute of Medicine in Warsaw), Warszawa: G. Małgorzata; Poland Medical University of Warsaw Banacha, Warszawa:A. Waszczuk-Gajda, J. Drozd-Sokolowska, M. Czemerska, M. Paluszewska; Medical University School Gdansk, Gdansk: E. Zarzycka; Wojewódzki Szpital Specjalistyczny im. Św.. Jadwigi Śląskiej, Opole: A. Masternak; Hospital Brzozow, Brzozow: Dr. Hawrylecka; Medical University Lublin, Lublin: M. Podhoreka, K. Giannopoulos, T. Gromek; Medical University Bialystok, Bialystok: J. Oleksiuk; Silesian Medical University Katowice, Katowice:bA. Armatys, G. Helbig; Universitary Hospital Wroclaw ,Wroclaw: M. Sobas; Poznan University of Medical Sciences, Pozna: A. Szczepaniak; Rydigier City Hospital Krakow, Krakow: E. Rzenno, M. Rodzaj; Collegium Medicum Jagiellonian University Krakow, Krakow: B. Piatkowska-Jakubas; City Hospital Rzeszów,Rzeszów: A. Skret; Medical University Lodz, Lodz: A. Pluta, M. Czemerska; Center of Oncology Kielce, Kielce: E. Barańska; Medical University of Warsaw , Warsaw : M. Paluszewska; Portugal—Hospital de Santa Maria-Lisboa, Lisboa:G. Vasconcelos, J. Brioso; IPOFG Lisboa, Lisboa: A. Nunes, I. Bogalho; Centro Hospitalar e Universitário de Coimbra, Coimbra: A. Espadana, M. Coucelo, S. Marini, J. Azevedo, A. I. Crisostomo, L. Ribeiro, V. Pereira; Centro Hospitalar de Lisboa Central E. P. E, Lisboa: A. Botelho; Instituto Português Oncologia do Porto Francisco Gentil, Porto: J. M. Mariz; Centro Hospitalar São João, Porto: J. E. Guimaraes, E. Aguiar; Centro Hospitalar do

Porto E.P.E. Porto: J. Coutinho; Spain (Programa Español de Tratamiento de las Hemopatías Malignas, PETHEMA)—Complejo Hospitalario Universitario A Coruña, A Coruña: V. Noriega, L. García, C. Varela, G. Debén, M. R. González; Hospital Clínico Universitario de Santiago, A Coruña: M. Encinas, A. Bendaña, S. González, J.L. Bello, M. Albors; Hospital General de Albacete, Albacete: L. Algarra, J.R.Romero, J.S.Bermon, M.J. Varo; Hospital Vinalopó, Alicante: V. López, E. López; Hospital Virgen de los Lirios, Alcoy: C. Mora, C. Amorós; Hospital General Elche, Alicante: E. López, A. Romero; Hospital Torrevieja Salud, Alicante: A. Jaramillo, N. Valdez, I. Molina, A. Fernández, B. Sánchez; Hospital de la Marina Baixa Villajoyosa, Alicante: A. García; Hospital General de Elda, Alicante: V. Castaño, T. López, J. Bernabeu; Hospital de Denia-Marina Salud, Alicante: M.J. Sánchez; Hospital de la Vega Baja de Orihuela, Alicante: C. Fernández; Hospital General de Alicante, Alicante: C. Gil, C. Botella, P. Fernández, M. Pacheco, F. Tarín; J.J. Verdú; Complejo Hospitalario Torrecardenas, Almeria: M.J. García, A. Mellado, M.C. García, J. González; Hospital Central de Asturias, Asturias: T. Castillo, E. Colado, S. Alonso; Complejo Asistencial Ávila, Ávila: I. Recio, M. Cabezudo, J. Davila, M. J. Rodríguez, A. Barez, B. Díaz; Hospital Don Benito-Villanueva, Badajoz: J. Prieto; Institut Catala d'Oncologia L´Hospitalet, Barcelona: M. Arnan, C. Marín, M. Mansilla; Hospital de Cruces, Bizkaia: A. Balaberdi, M. E. Amutio, R. A. del Orbe, I. Ancin, J. C. Ruíz; Hospital Galdakao-Usansolo, Bizkaia: M. Olivalres,C. Gómez, I. gonzález, M. Celis, K. Atutxa, T. Carrascosa, T. Artola, M. Lizuain; Basurtuko Ospitalea, Bizkaia: J .I. Rodriguez, O. Arce, J. A. Márquez, J. Atuch, F. Marco de Lucas, Z. Díez, B. Dávila; Hospital Santos Reyes, Burgos: R. Cantalejo, M. Díaz; Hospital Universitario de Burgos, Burgos: J. Labrador, F. Serra, G. Hermida, F. J. Díaz, P. de Vicente, R. Álvarez: Hospital Santiago Apóstol, Burgos: C. Alonso, Hospital San Pedro de Alcántara, Cáceres: J. M. Bergua; Hospital Campo Arañuelo, Cáceres: N. Ugalde; Hospital Virgen del Puerto, Cáceres: E. Pardal; Hospital General Jerez de la Frontera, Cádiz: R. Saldaña, F. Rodríguez, E. Martín, L. Hermosín; Hospital Universitario Puerta del Mar, Cádiz: M. P. Garrastazul, I. Marchante, J. A. Raposo, F. J. Capote; Hospital U. Marqués de Valdecilla, Cantabria: M. Colorado, A. Batlle, L. Yañez, S. García, P. González, E. M. Ocio, M. Briz, A. Bermúdez, S. García; Consorcio Hospitalario Provincial de Castellón, Castellón: C. Jiménez, S. Beltrán; Hospital de Vinaroz: M. Montagud; Hospital Universitario de La Plana, Castellón: I. Castillo; Hospital General de Castellón, Castellon: R. García, A. Gascón, J. Clavel, A. Lancharro, L. Lnares; Hospital Santa Bárbara , Ciudad Real: M. M. Herráez, A. Milena; Hospital Virgen de Altagracia, Ciudad Real: M. J. Romero, Hospital General de Ciudad Real, Ciudad Real: B. Hernández, C. Calle, R. Benegas; Hospital Gutierrez Ortega de Valdepeñas, Ciudad Real: Dr. Bolívar; Hospital General La Mancha Centro, Ciudad Real: M. A. Pozas; Hospital Reina Sofia, Córdoba: J. Serrano, F. J. Dorado, J. Sánchez, M. C. Martínez; Hospital Virgen de la Luz, Cuenca: C. J. Cerveró, M. J. Busto; Hospitales HUVN-HC San Cecilio de Ganada, Granada: M. Bernal, E. López, L. Moratalla, Z. Mesa, M. Jurado, A. Romero, P. González; Complejo Hospitalario Universitario Granada, Granada: L. Moratalla, A. Romero, L. López; Hospital

Universitario de Guadalajara, Guadalajara: M. Díaz, D. De Miguel, A. B. Santos, J. Arbeteta; Hospital Donostia, Donosti: E. Pérez, N. Caminos, N. Uresandi, N. Argoitiaituart, T. Artola, J. Swen, A. Uranga, I. Olazaba, M. Lizuain, E. Gainza, P. Romero; Hospital Juan Ramón Jimenez Huelva, Huelva: E. Gil, A. J. Palma, K. G. Gómez, M. Solé, J. N. Rodríguez; Hospital San Jorge, Huesca: I. M. Murillo, J. Marco, J. Serena, V. Marco; Hospital de Barbastro, Huesca: M. Perella, L. Costilla; Hospital General Ciudad de Jaen, Jaén: J. A. López, A. Baena, P. Almagro; Hospital San Pedro de Logroño, La Rioja: M. Hermosilla, A. Esteban, B. A. Campeny, M. J. Nájera, P. Herrra; Hospital Insular de Las Palmas, Las Palmas: R. Fernández, J. D. González, L. Torres; Hospital Dr. Negrín, Las Palmas: S. Jiménez; M. T. Gómez, C. Bilbao, C. Rodríguez; Hospital Doctor José Molina Orosa, Las Palmas: A. Hong, Y. Ramos de Laón, V. Afonso; Hospital Universitario de León, León: F. Ramos, M. Fuertes; Hospital Comarcal del Bierzo, León: E. de Cabo, C. Aguilera, M. Megido; Hospital Universitari Arnau de Vilanova de Lleida, Leida: T. García; Hospital Lucus Augusti, Lugo: E. Lavilla, M. Varela, S. Ferrero, M. J. Sánchez, L. López, J. Arias, L. Vizcaya; Hospital Infanta Sofía, Madrid: A. Roldán, A. Vilches, M. J. Penalva, J. Vázquez; Hospital Central de la Defensa Gómez Ulla, Madrid: M. T. Calderón, A. Matilla, C. Serí, M. J. Otero, N. García, E. Sandoval; Hospital de Fuenlabrada, Madrid: C. Franco, R. Flores, P. Bravo, A. López; Hospital Fundación Jiménez Díaz, Madrid: J. L. López, C. Blas, A. Díez, J. M. Alonso, C. Soto, A. Arenas; Hospital U. Príncipe de Asturias, Madrid: J. García, Y. Martín, P. S. Villafuerte, E. Magro; Hospital Puerta de Hierro, Madrid: G. Bautista; A. De Laiglesia; Hospital Gregorio Marañón, Madrid: G. Rodríguez, L. Solán, M. Chicano, P. Balsalobre, S. Monsalvo, P. Font, D. Carbonell, C. Martínez; Hospital U. La Paz, Madrid: K. Humala, A. E. Kerguelen, D. Hernández, M. Gasior, P. Gómez, I. Sánchez; Hospital Madrid Norte Sanchinarro, Madrid: S. Redondo, L. Llorente, M. Bengochea, J. Pérez; Hospital Sanitas Torrejón, Madrid: A. Sebrango, M. santero, A. Morales; Hospital La Princesa, Madrid: A. Figuera, P. Villafuerte, A. Alegre, E. Fernández; Hospital Ruber Internacional, Madrid: A. Alonso; Hospital 12 de Octubre, Madrid: M. P. Martínez, J. Martínez, M. T. Cedena, L. Moreno; MD Anderson Cancer Center, Madrid: A. De la Fuente; Hospital Sanitas La Zarzuela, Madrid: D. García; Hospital Universitario Quiron, Madrid: C. Chamorro, V. Pradillo, E. Martí, J. M. Sánchez, I. Delgado, A. Alonso; Hospital Rey Juan Carlos, Madrid: B. Rosado, A. Velasco, C. Miranda, G. Salvatierra, J. M. Alonso,, J. L. López; Hospital Infanta Leonor, Madrid: M. Foncillas, J. A. Hernández; Hospital Universitario de Getafe, Madrid: C. Escolano, L. García, I. Delgado; Hospital Clínico San Carlos, Madrid: C. Benabente, R. Martínez, M. Polo, E. Anguita; Hospital Universitario Severo Ochoa, Madrid: R. Riaza, G. Amores, M. J. Requena; Hospital Universitario Fundación Alcorcón, Madrid: F. Javier, L. Villaloón; Hospital Universitario Moncloa, Madrid: C. Aláez, V. Pradillo, S. Nistal, B. Navas; Hospital Universitario de Móstoles, Madrid: J. Sánchez, M. A. Andreu; Hospital Ramon y Cajal, Madrid: P. Herrera, J. López; Hospital U. Virgen de la Victoria, Málaga: M. García, M. J. Moreno, A. Fernández, M. P. Queipo; Hospital Quirónsalud Málaga, Málaga: A. Hernández; Hospital Regional de Málaga, Málaga: M. Barrios, A.

Heiniger, A. Jiménez, A. Contento, F. López, M. Alcalá; Hospital Vithas Xanit Internacional, Málaga: S. Lorente, M. González, E. M. Morales, J. Gutierrez; Hospital Virgen del Castillo, Murcia: M. J. Serna, V. Beltrán; Hospital Santa Lucía de Cartagena, Murcia: M. Romera, M. Berenguer, A. Martínez, A. Tejedor; Hospital Morales Meseguer, Murcia: M. L. Amigo, F. Ortuño, L. García, A. Jerez, O. López; Hospital U. Virgen de la Arrixaca, Murcia: J. M. Moraleda, P. Rosique, J. Gómez, M. C. Garay; Hospital Los Arcos Mar Menor, Murcia: P. Cerezuela, C. Martínez, A. B. MArtínez, A. González; Hospital STª Mª del Rosell, Murcia: J. Ibáñez; Clínica San Miguel, Navarra: M. J. Alfaro; Complejo Hospitalario de Navarra, Navarra: M. Mateos, M. A. Goñi, M. A. Araiz, A. Gorosquieta, M. Zudaire, M. Viguria, A. Zabala, M. Alvarellos, I. Quispe, M. P. Sánchez, G. Hurtado, M. Pérez, Y. Burguete, N. Areizaga, T. Galicia; Clínica Universitaria de Navarra, Navarra: J. Rifón, A. Alfonso, F. Prósper,M. Marcos, L. E. Tamariz, V. Riego. A. Manubens, M. J. Larrayoz, M. J. Calasanz, A. Mañú, B. Paiva, I. Vázquez, L. Burgos; Complejo Hospitalario de Ourense (CHOU), Ourense: M. Pereiro, M. Rodríguez, M. C. Pastoriza, J. A. Mendez, J. L. Sastre, M. Iglesias, C. Ulibarrena, F. Campoy; Hospital Valdeorras, Ourense: D. Jaimes; Hospital Rio Carrión, Palencia: J. M. Alonso, B. Albarrán, J. Solano, A. Silvestre; Complexo Hospitalario Universitario de Vigo, Vigo: C. Albo, S. Suarez, C. Loureiro, I. Figueroa, M. Rodríguez, M. A. Fernández, A. Martínez, C. Poderós, J. Vazquez, L. Iglesias, A. Nieto, T. Torrado, A. M. Martínez; Hospital Provincial de Pontevedra, Pontevedra: M.L. Amador, P. Oubiña, E. Feijó, A. Dios, I. Loyola, R. Roreno; Hospital POVISA, Pontevedra: A. Simiele, L. Álvarez, V. Turcu; Hospital U. Salamanca, Salamanca: B. Vidriales, M. González, R. García, A. Avendaño, C. Chillón, E. Pérez, V. González; Hospital General La Palma, Santa Cruz de Tenerife: J. V. Govantes, S. Rubio, M. Tapia; Hospital General de Segovia, Segovia: C. Olivier, J. A. Queizán; Hospital U. Virgen Macarena, Sevilla: : O. Pérez, J. A. Vera, C. Muñoz, A. rodriguez, N. González; Hospital U. Virgen del Rocio, Sevilla: J. A. Pérez, E. Soria, I.Espigado, J. Falantes, I. Montero, P. García, E. Rodríguez, E. Carrillo, T. Caballero, C. García; Hospital Virgen de Valme, Sevilla: C. Couto, I. Simón, M. Gómez; Hospital Virgen del Mirón de Soria, Soria: C. Aguilar; Hospital Universitario Canarias, Tenerife: B. J. González, S. Lakhwani, A. Bienert, B. González; Hospital Universitario Nuestra Señora de Candelaria, Tenrife: A. Cabello, A. Y. Oliva, H. González; Hospital Obispo Polanco, Teruel: N. González, Hospital de Alcañiz, Teruel: L. Sancho, M. Paricio, L. Perdiguer; Hospital General Nuestra Señora del Prado, Toledo: F. Solano, A. Lerma, M. D. Martínez; Hospital Virgen de la Salud de Toledo, Toledo: M. I. Gómez, A. Yeguas; Hospital U. La Fe, Valencia: P. Montesinos, E. Barragán, C. Sargas, R. Amigo, D. Martinez, B. Boluda, R. Rodríguez, E. Acuña, I. Cano; Hospital de Requena, Valencia: A. Escrivá, M. Pedreño; Hospital de Lluis Alcanyis de Xativa, Valencia: R. Renart; IVO (Instituto Valenciano de Oncología), Valencia: A. Navalón; Hospital de Sagunto, Valencia: I. Castillo, M. Orts; Hospital Dr. Peset, Valencia: M. J. Sayas, M. J. Fernández, M. L. Juan, E. Gómez, M. Gimeno, E. Donato, M. Cejalvo, J. Marco; Hospital Clínico Universitario, Valencia: M. Tormo, M. Calabuig, B. Navarro, I. Martin, E. Villamont,

A. Miralles; Hospital de La Ribera, Valencia: R. Lluch; Hospital Casa de la Salud, Valencia: J. García; Hospital de Gandía, Valencia: M. Moragues, M. A. Ruiz; Hospital Arnau de Vilanova, Valencia: A. López, C. Benet, M. Valero; Hospital General de Valencia, Valencia: M. Linares, R. Collado, M. Orero, P. Ibañez, M. J. Lis, P. L. Pérez, M. Roig, M. López, A. V. Mena; Hospital Manises, Valencia: I. Picón, V. Cánovas, A. Palacios, E. Martí; Hospital Clínico de Valladolid, Valladolid: R. Cuello, J. Borrego, M. burgois; Hospital Rio Hortega, Valladolid: A. Cantalapiedra, O. Norberto, E. Angomas, B. Cidoncha; Hospital Universitario Araba, Victoria: L. Cuevas, D. Robles, A. Mendiazabal, I. Oiartzabal, J. M. Guinea de Castro; Hospital Virgen de la Concha, Zamora: C. Montes, M. Pérez, L. García; Hospital Royo Villanova, Zaragoza: V. Carrasco, A. Pérez, L. López, J. J. Moneva; Hospital Clínico U. Lozano Blesa, Zaragoza: M. Olave, E. Bonafonte, L. Mayor, G. Azaceta, L. Palomera; Hospital Ernest Lluch Martin, Zaragoza: M. Malo, M. J. Escobar; Hospital Quiron Salud Zaragoza, Zaragoza: J. M. Grasa; Hospital Miguel Servet, Zaragoza: B. De Rueda, A. Aulés, C. Salvador, V. Ansó, A. Iborra, P. Delagado, A. Rubio; Uruguay —Hospital de Clínicas, Montevideo: M. Stevenazzi, I. Alpire, V. Irigoin, L. Díaz, C. Guillermo, R. Guadagna, S. Grille, C. Oliver, M. Boada, V. Vales; Hospital Maciel, Montevideo: A. I. Prado; COMERO (Cooperativa Medica de Rocha), Rocha: A. P. De los Santos.