Spanish Guidelines for the use of targeted deep sequencing in myelodysplastic syndromes and chronic myelomonocytic leukaemia

Laura Palomo,[1] (iD) Mariam Ibáñez,[2,3,4] María Abáigar,[5] Iria Vázquez,[6,7] Sara Álvarez,[8] Marta Cabezón,[9] Bárbara Tazón-Vega,[10,11] Inmaculada Rapado,[12,13,14] Francisco Fuster-Tormo,[1] José Cervera,[2,3,15] Rocío Benito,[5] María J. Larrayoz,[6,7] Juan C. Cigudosa,[8] Lurdes Zamora,[9] David Valcárcel,[10,11] María T. Cedena,[12,13,14] (iD) Pamela Acha,[1] Jesús M. Hernández-Sánchez,[5,16] Marta Fernández-Mercado,[6,17,18] Guillermo Sanz,[2,3] Jesús M. Hernández-Rivas,[5,16,19] María J. Calasanz,[6,7] Francesc Solé,[1] Esperanza Such,[2,3,4] (iD) On behalf of the Spanish Group of MDS (GESMD)

[1]Josep Carreras Leukaemia Research Institute, ICO Badalona-Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Sadalona, [2]Department of Haematology, Hospital Universitari i Politècnic La Fe, València, [3]Centro de Investigacion Biomédica en Red de Cáncer (CIBERONC), Instituto Carlos III, Madrid, [4]Departamento de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad CEU Cardenal Herrera, València, [5]Institute of Biomedical Research of Salamanca (IBSAL), Cancer Research Centre (IBMCC-CIC; Univ. of Salamanca-CSIC), Salamanca, [6]Haematological Diseases Laboratory, CIMA LAB Diagnostics, University of Navarra, [7]Navarra Institute for Health Research (IdiSNA), Pamplona, [8]NIMGenetics, Genómica y Medicina, S.L., Madrid, [9]Haematology Service, ICO Badalona-Hospital Germans Trias i Pujol, Josep Carreras Leukaemia Research Institute, Badalona, [10]Department of Haematology, Vall d'Hebron Institute of Oncology, Vall d'Hebron University Hospital, [11]Department of Medicine, Universitat Autònoma de Barcelona, Barcelona, [12]Haematology Department, Hospital Universitario 12 de Octubre, [13]Haematological Malignancies Clinical Research Unit, CNIO, [14]Centro de investigación Biomédica en Red Cáncer (CIBERONC), Madrid, [15]Genetics Unit, Hospital Universitario y Politécnico La Fe, Valencia, [16]University of Salamanca (USAL), Salamanca, [17]Advanced Genomics Laboratory, Centre for Applied Medical Research (CIMA), University of Navarra, Haemato-Oncology, Pamplona, [18]Biomedical Engineering Department, School of Engineering, University of Navarra, San Sebastian and [19]Hospital Universitario de Salamanca, Salamanca, Spain

Summary

The landscape of medical sequencing has rapidly changed with the evolution of next generation sequencing (NGS). These technologies have contributed to the molecular characterization of the myelodysplastic syndromes (MDS) and chronic myelomonocytic leukaemia (CMML), through the identification of recurrent gene mutations, which are present in >80% of patients. These mutations contribute to a better classification and risk stratification of the patients. Currently, clinical laboratories include NGS genomic analyses in their routine clinical practice, in an effort to personalize the diagnosis, prognosis and treatment of MDS and CMML. NGS technologies have reduced the cost of large-scale sequencing, but there are additional challenges involving the clinical validation of these technologies, as continuous advances are constantly being made. In this context, it is of major importance to standardize the generation, analysis, clinical interpretation and reporting of NGS data. To that end, the Spanish MDS Group (GESMD) has expanded the present set of guidelines, aiming to establish common quality standards for the adequate implementation of NGS and clinical interpretation of the results, hoping that this effort will ultimately contribute to the benefit of patients with myeloid malignancies.

The development of next generation sequencing (NGS) techniques during the last decade has contributed to the molecular characterization of haematological malignancies, including myeloid neoplasms, such as the myelodysplastic syndromes (MDS) and chronic myelomonocytic leukaemia (CMML) (Arber *et al*, 2016). According to the World Health Organization (WHO) classification (Arber *et al*, 2016), MDS comprise a group of clonal haematopoietic stem cell disorders characterized by ineffective haematopoiesis in one or more myeloid cell lines, abnormal dysplastic cell morphology and potential for clonal evolution. On the other hand, CMML is included in the WHO group of myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and is characterized by

Correspondence: Francesc Solé, Institut de Recerca Contra la Leucèmia Josep Carreras (IJC), Crta. de Can Ruti, Cami de les Escoles, Edifici IJC, Badalona 08916, Barcelona, Spain.
E-mail: fsole@carrerasresearch.org

First published online 16 October 2019
doi: 10.1111/bjh.16175

absolute peripheral monocytosis in conjunction with both effective (myeloproliferative variant, MP-CMML $\geq 13 \times 10^9$/l leucocytes) and ineffective (myelodysplastic variant, MD-CMML $< 13 \times 10^9$/l leucocytes) haematopoiesis. MDS and CMML are very heterogeneous at the clinical, morphological and genetic level and both and have the risk of progressing to acute myeloid leukaemia (AML). The natural history of these diseases is highly variable and a risk-adapted treatment strategy is mandatory.

Myeloid neoplasms arise through the serial acquisition of chromosomal alterations and somatic mutations that affect genes involved in several mechanisms. These mutations drive disease evolution from asymptomatic clonal haematopoiesis to overt disease and, eventually, progression to AML (Kennedy & Ebert, 2017). Recurrent mutations in more than 40 different genes have been identified in MDS and CMML and at least one mutation is detected at diagnosis in >80% of patients (Papaemmanuil et al, 2013; Haferlach et al, 2014; Elena et al, 2016). These mutations are related to the pathophysiological features of these diseases and play a role in their clinical heterogeneity. These molecular markers can complement current diagnostic criteria in MDS and CMML, as well as contribute to the risk assessment of these patients (Bejar et al, 2011; Haferlach et al, 2014; Elena et al, 2016). Therefore, diagnostic laboratories are including NGS genomic analyses in their routine clinical practice, in an effort to personalize the diagnosis, prognosis and treatment of MDS and CMML. Thorough evaluation of technical aspects, data analysis and variant interpretation is required for the correct implementation of NGS in routine diagnosis. Thus, the aim of this collaborative project within the Spanish MDS Group (GESMD) was to develop a set of guidelines, agreed by expert consensus panels, to standardize the use of targeted deep sequencing in the routine genetic testing of MDS and CMML patients, for the detection of single nucleotide variants (SNVs) and small insertions/deletions (indels). Our main objective is to harmonize analyses and variant interpretation, ensuring high standards in clinical reporting of genetic data. These consensually proposed NGS guidelines involve the following major sections, which will be further discussed in detail: (i) mutational landscape in MDS and CMML, (ii) sequencing workflow and quality control, (iii) data analysis and variant filtering, (iv) variant categorization/interpretation and (v) clinical reports.

Mutational landscape of MDS and CMML

Over 40 different genes are found recurrently mutated in MDS and CMML (Papaemmanuil et al, 2013; Haferlach et al, 2014; Elena et al, 2016). However, in contrast to other diseases, such as myeloproliferative neoplasms (MPN), none of them are specific to these disorders. Still, a few of these genes have been proved to be useful for diagnosis or to predict response to specific treatments (Papaemmanuil et al, 2011; Itzykson et al, 2011; Bejar et al, 2014; Traina et al,

2014). Moreover, some mutations are also associated with shorter survival and a higher risk of progression to AML and therefore can be used for prognostic stratification (Table I) (Bejar et al, 2011, 2012; Papaemmanuil et al, 2013; Haferlach et al, 2014; Elena et al, 2016; Makishima et al, 2017). Thus, an MDS/CMML targeted sequencing panel should at least include all those genes that have been proven relevant for the disease (Malcovati et al, 2013; Arber et al, 2016; Greenberg et al, 2017) (Table II). As NGS is a high throughput technique, it can be complemented with genes that are altered in other myeloid neoplasms to generate a pan-myeloid sequencing panel, in view of a wider utility of the tool (e.g. for MDS, MPN and AML) (Table III). In this regard, the Association for Molecular Pathology recently reviewed the clinical relevance of small DNA variants in chronic myeloid neoplasms, summarizing key findings that support clinical utility, and defining the need for gene inclusion in high-throughput sequencing testing panels (McClure et al, 2018).

Driver genes in MDS and CMML, which are common among the spectrum of myeloid malignancies, affect specific cellular processes and can be categorized according to their function (Greenberg et al, 2017; Kennedy & Ebert, 2017; McClure et al, 2018) (Fig 1).

Mutations in RNA-splicing machinery are the most frequent type of somatic mutations detected in MDS. Recurrent mutations have been reported in the spliceosome components of the SF3B1, SRSF2, U2AF1 and ZRSR2 genes, while mutations in PRPF40B, SF3A1, SF1 and U2AF2 (U2AF65) genes have been described in only 1–2% of MDS patients (Yoshida et al, 2011; Makishima et al, 2012; Papaemmanuil et al, 2013). SF3B1 mutations are associated with a higher overall survival rate and an inferior risk of AML progression, as they are the only ones that are clearly associated with a better prognosis, especially as a single mutation (Papaemmanuil et al, 2011; Malcovati et al, 2014). SRSF2 mutations are associated with monocytosis and marked thrombocythaemia and are especially frequent in CMML (Yoshida et al, 2011; Meggendorfer et al, 2012). Mutations in SRSF2 and U2AF1 have been associated with less favorable outcomes in some studies (Graubert et al, 2011; Thol et al, 2012; Meggendorfer et al, 2012; Haferlach et al, 2014; Makishima et al, 2017).

Epigenetic regulators are the second most common class of mutation in MDS, and can be divided into DNA methylation enzymes (TET2, DNMT3A and IDH1/2 genes) and chromatin modifiers (ASXL1 and EZH2 genes) (Delhommeau et al, 2009; Walter et al, 2011; Gelsi-Boyer et al, 2012; Shih et al, 2012; Ganguly & Kadam, 2016). TET2 mutations are very common in CMML and the combination of TET2 and SRSF2 is highly associated with this disorder (Malcovati et al, 2014). TET2 mutations do not have a prognostic impact but they are associated with an increased response rate to hypomethylating agents (Itzykson et al, 2011; Bejar et al, 2014) (Table III). DNMT3A mutations are associated with a more aggressive clinical course

Table I. Clinical relevance of mutated genes in MDS and CMML (Malcovati *et al*, 2013; Arber *et al*, 2016; Greenberg *et al*, 2017).

| Gene | MDS | | CMML | |
	Incidence	Clinical impact	Incidence	Clinical impact
ASXL1	5–25%	Unfavourable	40–50%	Unfavourable
CSNK1A1	<1%	Uncertain	<1%	Unknown
	5–15% MDS with del(5q)	Associated with del(5q)		
DNMT3A	12–18%	Unfavourable in patients without SF3B1 mutations	2–10%	Uncertain
EZH2	5–10%	Unfavourable	5–12%	Unfavourable
IDH1	<5%	Uncertain	<1%	Uncertain
IDH2	<5%	Uncertain	5–10%	Unfavourable
JAK2	<5%	MDS with del(5q), 5–7%	2–10%	Associated with MP-CMML
KRAS	5–10%	Uncertain	10–20%	Unfavourable
				Associated with MP-CMML
NRAS	5–10%	Unfavourable	10–20%	Unfavourable
				Associated with MP-CMML
RUNX1	10–15%	Unfavourable	10–30%	Unfavourable
		Can be of germline origin		
SETBP1	<5%	Unfavourable	5–10%	Unfavourable
SF3B1	20–30%	Associated with RS	5–10%	Unknown
	80% MDS-RS	Favourable		
SRSF2	10–15%	Unfavourable	30–50%	Uncertain
STAG2	5–10%	Unfavourable	5–10%	Unfavourable
TET2	20–25%	Uncertain	45–60%	Uncertain
TP53	8–12%	Unfavourable	<5%	Unfavourable
		Associated with CK (50%), del(5q) (15–20%)		
		Lower response rate to lenalidomide		
		Can be of germline		
U2AF1	8–12%	Unfavourable	5–10%	Unfavourable
ZRSR2	5–10%	Unfavourable	5–10%	Uncertain

CK, complex karyotype; CMML, chronic myelomonocytic leukaemia; HMA, hypomethylating agents; MDS, myelodysplastic syndrome; MDS-RS, MDS with ring sideroblasts; MP-CMML; myeloproliferative CMML; NK, normal karyotype; RS, ring sideroblasts.

and rapid progression to AML in both MDS and CMML patients (Walter *et al*, 2011; Bejar *et al*, 2014; Patnaik *et al*, 2017). *ASXL1* mutations are independently associated with a poor outcome in the spectrum of myeloid diseases, including a shorter overall survival and a higher risk of progression to AML (Boultwood *et al*, 2010; Gelsi-Boyer *et al*, 2012; Itzykson *et al*, 2013a). *EZH2* mutations are associated with worsened overall survival in both low and high risk MDS and CMML (Bejar *et al*, 2011; Grossmann *et al*, 2011; Bejar *et al*, 2012).

The multiprotein cohesin complex is involved in the cohesion of sister chromatids and the post-replicative DNA repair system, and it is encoded by the genes *STAG1/2, SMC1A, SMC3* and *RAD21*, which are mutated in up to 11% of low risk and 17% of high risk MDS patients (Kon *et al*, 2013; Thota *et al*, 2014). Mutations in *STAG2* are more frequent in patients with multilineage dysplasia and excess of blasts and seem to be associated with shorter overall survival (Thota *et al*, 2014).

Loss-of-function mutations in a transcription factor gene, such as *RUNX1, GATA2* or *ETV6*, are detected in approximately 20% of MDS (Chen *et al*, 2007; Bejar *et al*, 2011; Wall *et al*, 2012). *RUNX1* mutations are associated with a high risk phenotype, thus being more frequent in high risk MDS and CMML and in secondary AML (Bejar *et al*, 2011; Cazzola *et al*, 2013; Elena *et al*, 2016).

MDS and CMML patients frequently harbour mutations in signal transduction genes, which constitutively activate a signalling pathway involved in cell proliferation, apoptosis and cell differentiation (*CBL, CSNK1A1, JAK2, N/KRAS, MPL, NF1, PTPN11, KIT,* and *FLT3* genes). These mutations are more frequent in myeloproliferative CMML cases and usually correspond to secondary events that may lead to AML progression (Pich *et al*, 2009; Kohlmann *et al*, 2010; Ricci *et al*, 2010; Schwaab *et al*, 2012; Itzykson *et al*, 2013b; Smith *et al*, 2015; Elena *et al*, 2016).

Other genes recurrently mutated in MDS and CMML that are involved in other cellular processes, include *TP53, SETBP1, BCOR* and *BCORL1* (Bejar *et al*, 2011; Jädersten *et al*, 2011; Damm *et al*, 2013a,2013b; Bejar *et al*, 2014; Elena *et al*, 2016). *TP53* mutations are more frequent in high risk patients and are associated with adverse prognosis features, including advanced disease, complex karyotypes, monosomal karyotypes, excess of blasts, severe thrombocytopenia and therapy-related MDS (Kita-Sasai *et al*, 2001; Bejar *et al*, 2011; Kulasekararaj *et al*, 2013; Ok *et al*, 2015).

Table II. List of GESMD-recommended genes for studying the clinical management of MDS and CMML.

Gene	Region	Type of mutation	Frequency	
			MDS	CMML
ASXL1	Exons 10–13 Codons: all	Nonsense, frameshift	5–25%	40–50%
CSNK1A1	Exons 2–4 Codons: all	Missense	5–10%	<1%
DNMT3A	Complete coding region Hotspot codon: R882	All	12–18%	2–10%
EZH2	Complete coding region Codons: all	Nonsense, frameshift	5–10%	5–12%
IDH1	Exon 4 Hotspot codon: R132	Missense	<5%	<1%
IDH2	Exon 4 Hotspot codons: R140 and R172	Missense	<5%	5–10%
JAK2	Complete coding region Hotspot codon: V617F	Missense	<5%	2–10%
KRAS	Exons 2 and 3 Hotspot codons: G12, G13, Q61 and G146	Missense	5–10%	10–20%
NRAS	Exons 2 and 3 Hotspot codons: G12, G13 and Q61	Missense	5–10%	10–20%
RUNX1	Complete coding region Codons: all	Nonsense, frameshift	10–15%	10–30%
SETBP1	Exon 4 Codons: 858–870	Missense	<5%	5–10%
SF3B1	Exons 10–16 Codons: 622–781	Missense	20–30% 80% RS	5–10%
SRSF2	Complete coding region Hotspot codon: P95	Missense	10–15%	30–50%
STAG2	Complete coding region Codons: all	Nonsense, frameshift, splicing	5–10%	5–10%
TET2	Complete coding region Codons: 1134–1444 or 1842–1921	All	20–25%	45–60%
TP53	Complete coding region Codons: all	All	8–12%	<5%
U2AF1	Exons 2–6 Hotspot codons: S34 and Q157	Missense	8–12%	5–10%
ZRSR2	Complete coding region Codons: all	Nonsense, frameshift	5–10%	5–10%

GESMD, Spanish Group of MDS; CMML, Chronic Myelomonocytic Leukaemia; MDS, Myelodysplastic Syndrome; RS, ring sideroblasts.

TP53 mutations are associated with a very poor prognosis in all MDS subtypes, including patients who have undergone an haematopoietic stem cell transplantation (Bejar *et al*, 2011; Kulasekararaj *et al*, 2013; Bejar *et al*, 2014). *TP53* mutations are also associated with the presence of del (5q) and with a lower response rate to lenalidomide treatment in these patients (Jädersten *et al*, 2011; Meggendorfer *et al*, 2017).

## Myeloid neoplasms with germline predisposition

Although most cases of MDS or AML are sporadic diseases, a number of cases arise as the result of genetic predisposition due to the presence of inherited germline mutations, which has led the WHO classification to include a new section on myeloid neoplasms with germline predisposition. This section comprises cases of MDS, MDS/MPN and AML that arise on the background of a predisposing germline mutation (Arber *et al*, 2016). Several genes have been associated with a genetic predisposition to myeloid malignancies, including *ANKRD26, CBL, CEBPA, DDX41, ELANE, ETV6, GATA2, GSKIP, MPL, NF1, SAMD9, SAMD9L, PTPN11, RUNX1, SRP72* and *TP53*, among others (Kennedy & Shimamura, 2019). The majority of these genes are included in myeloid-related targeted panels focused on somatic mutations. In addition, recent studies suggest that the frequency of germline variants in some of these genes, like *DDX41*, may be underappreciated, suggesting that they should also be

Table III. Other myeloid-related genes more frequent in AML, MPN and other MDS/MPN.

Gene	Region Frequent mutations	Type of mutation	Frequency MDS (%)	Frequency CMML (%)
Frequent in myeloproliferative neoplasms				
CALR	Exon 9	Frameshift	<1	<1
	Codons: all			
CBL	Exons 8 and 9	Missense	<5	8–18
	Codons: 366–420			
CSF3R	Complete coding region	Missense	<1	3–4
	Hotspot codons: 618			
GATA2	Exons 2 and 6	Missense, frameshift	<5	<1
	Codons: all			
MPL	Complete coding region	Missense	<1	<1
	Codons 505 and 515			
NF1	Complete coding region	Nonsense, frameshift, splicing	<5	<5
	Codons: all			
PTPN11	Exons 3 and 7	Missense	<1	4
	Codons: all			
Frequent in acute myeloid leukaemia				
BCOR	Complete coding region	All	<5	<5
	Codon: all			
BCORL1	Complete coding region	Nonsense, frameshift	<1	<1
	Codons: all			
CEBPA	Complete coding region	Missense, frameshift	<5	<5
	Codons: all			
ETV6	Complete coding region	Nonsense, frameshift	<5	<1
	Codons: all			
FLT3	Exons 13–15 and 20	Missense, frameshift	<5	<5
	Hotspot codons: *FLT3*-ITD and D835			
KIT	Exons 2, 8–11, 13 and 17	Missense, frameshift	<3	<1
	Codons: all			
NPM1	Exon 11	Frameshift	<5	<5
	Hotspot codons: W288			
WT1	Exons 7 and 9	Missense, frameshift	<3	<3
	Codons: all			

CMML, chronic myelomonocytic leukaemia; ITD, internal tandem duplication; MDS, myelodysplastic syndrome.
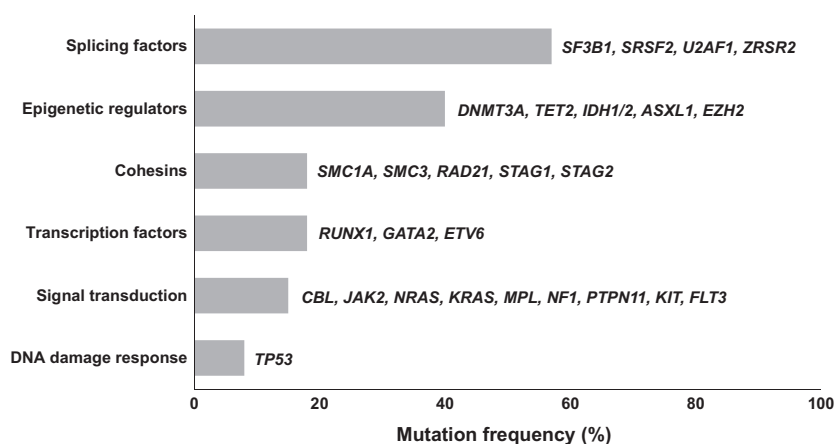
Fig 1. Classification of frequently mutated genes in MDS and CMML according to their functional category (modified from Kennedy & Ebert, 2017; Kennedy & Ebert, 2017).

considered for inclusion in a panel of recommended genes (Berger *et al*, 2017; Tawana *et al*, 2018). This may be notably relevant in the context of the search for a related donor. Considering all the above, when performing targeted sequencing in MDS patients, the possibility of detecting a germline mutation must be considered. Although a variant allele frequency (VAF) close to 50% or 100% may be suggestive of a germline mutation, its nature should be confirmed using a control germline sample before acting on it, and should always be evaluated in the clinical and familial context of each patient. Even though germline genetic predisposition is frequently associated with MDS with early onset (children and young adults), it is increasingly recognized also in MDS cases diagnosed in advanced ages. The prevalence of hereditary myeloid malignancies has not been clearly established but is suspected to concern at least 5% of adult patients with MDS/AML (Tawana *et al*, 2018). In addition, currently known MDS/AML predisposition genes account for only 25% of familial cases, so other risk *loci* remain to be discovered (Tawana *et al*, 2018). When a germline mutation is confirmed, referral of patients to professionals with expertise in cancer predisposing syndromes and in genetic counselling is recommended, due to the need for highly specialized and rapidly advancing evaluations and tailored treatments for these patients (Godley & Shimamura, 2017).

Clonal haematopoiesis of uncertain significance

Recurrent somatic mutations in MDS-associated genes, such as *DNMT3A*, *TET2*, *ASLX1*, *TP53* and *JAK2* genes, have been identified in the peripheral blood of healthy individuals with advanced age and normal blood counts (Genovese *et al*, 2014; Jaiswal *et al*, 2014; Xie *et al*, 2014). These mutations, usually detected at very low VAFs (<10%), provide a selective advantage to the haematopoietic stem cells in which they occur, but cells still maintain the ability to differentiate into circulating leucocytes (Steensma *et al*, 2015). This phenomenon has been defined as clonal haematopoiesis of indeterminate potential (CHIP) (Table IV) (Steensma *et al*, 2015). The incidence of CHIP increases with age, with more than 10% of healthy people older than 70 years of age carrying a mutation in one of those genes (Genovese *et al*, 2014; Jaiswal *et al*, 2014). These individuals have an increased risk

of cardiovascular disease and a higher risk of developing an haematological malignancy, although thankfully the rate of malignant transformation is very low (0·5–1% per year) (Jaiswal *et al*, 2014, 2017). This risk increases when the VAFs are higher (>10%) or more than one mutation is detected. In addition, recent studies have shown that patients with solid tumours who have CHIP before being exposed to treatment, are at increased risk of developing a therapy-related myeloid neoplasm (TRMN) (Takahashi *et al*, 2013; Gillis *et al*, 2017). Factors that influence the natural history of CHIP are currently under investigation, but it is known that the acquisition of further mutations drives the progression of CHIP to overt malignancy.

Apart from healthy individuals, mutations in these MDS-associated genes can also be detected in 35% of patients with persistent blood cytopenias for which no explanation is apparent, usually called idiopathic cytopenias of undetermined significance (ICUS) (Table IV). These cases, that present with both clonal haematopoiesis and cytopenias, are designated clonal cytopenias of uncertain significance (CCUS). Patients with CCUS are at a higher risk of developing a myeloid malignancy compared to those individuals with CHIP not presenting any cytopenia, especially in the presence of two or more mutations or if a single mutation in a splicing gene is detected (Arber *et al*, 2016; Bejar, 2017; Jaiswal *et al*, 2017) (Table IV). In addition, average VAFs in patients with CCUS (30–40%) are higher compared to those found in CHIP.

Taking all these observations into account, when performing NGS routine tests in a patient with an indication of MDS, diagnosis should always be performed in the context of other laboratory and clinical data.

Sequencing workflow and quality criteria

Targeted NGS panels commonly include a selected number of specific genes, or coding regions within genes that are clinically-actionable in the disease of interest, or that are known to harbour mutations that contribute to the pathogenesis of the disease. Focusing on a limited set of genes allows greater depth of coverage for increased analytical sensitivity and specificity. Thus, targeted sequencing is often used in clinical care because, while keeping the cost relatively low, it provides greater confidence and facilitates the

Table IV. Characteristics of CHIP, ICUS, CCUS and MDS.

	CHIP	ICUS	CCUS	MDS
Cytopenia	No	Yes (≥1)	Yes (≥1)	Yes (≥1)
Dysplasia	No	No, or minimum	No, or minimum	Yes
Mutations	Yes ($n = 1$)	No	Yes ($n \sim 1, 6$)	Yes >85% ($n \sim 2, 6$)
VAF	2–12%	–	30–40%	30–50%
Progression Risk	0.5–1% per year	>10% in 5 years	>85% in 5 years	

CCUS, clonal cytopenia of uncertain significance; CHIP, clonal haematopoiesis of uncertain significance; ICUS, idiopathic cytopenia of uncertain significance; MDS, myelodysplastic syndrome; VAF, variant allele frequency.

interpretation of the findings in their clinical context. The choice of sequencing strategy will depend on several factors, including size of the panel, sequencing platform, turnaround time (TAT) for reporting the results and cost per sample.

Panel design strategy

When designing a custom panel, it is recommended to select targets and transcripts only from genes with potential clinical relevance. There are several available online tools that can be used to design the specific primers/probes to enrich the targets of interest. Although custom panels are usually flexible and can be modified over time, they require a long process of optimization and validation, which is skipped when using off-the-shelf panels. On the other hand, commercial panels offer a closed design, but do not require a previous design phase and are already optimized. Of note, these panels are commercially available as ready-to-use, but quite often they still require validation in each laboratory.

Library preparation

In MDS and CMML, as in most haematological neoplasms, it is recommended to perform molecular analyses on bone marrow-derived cells. Some studies suggest that, due to the high sensitivity of NGS, there is little difference between bone marrow and peripheral blood samples (Duncavage *et al*, 2017; Martin *et al*, 2018). Therefore, if bone marrow is not available, peripheral blood can be useful, especially for molecular monitoring or in myeloproliferative disorders like CMML. Although DNA from fresh samples is the optimal input material, targeted NGS can be performed on any DNA sample as long as the quality and quantity are sufficient. DNA purity can be measured by spectrophotometry (i.e. Nanodrop device, Thermo Fisher Scientific, Waltham, MA, USA), while DNA integrity can be assessed by capillary electrophoresis (i.e. Tape Station or Bioanalyzer devices, Agilent, Santa Clara, CA, USA). Input DNA and DNA libraries should be quantified using a fluorometric assay (i.e. Qubit or Quant-iT PicoGreen, Thermo Fisher Scientific) that can distinguish and measure the concentration of double-stranded DNA. The manufacturer's recommendations should be strictly followed during library preparation.

Two main strategies for library preparation are widely used in targeted NGS: amplicon-based and hybrid-capture-based. Amplicon panels are based on the use of specific primers and the amplification of the regions of interest by polymerase chain reaction (PCR)-based methods (single or multiplex PCR, or emulsion PCR). On the other hand, target enrichment is performed in capture panels using probe hybridization-based methods. In both cases, DNA fragments generated during library preparation contain adapter sequences on both ends, which are complementary to platform-specific sequencing primers. Samples can be tagged with a unique oligonucleotide barcode that enables pooling of different patient samples in the same sequencing run (Table S1). In addition, some strategies use unique molecular barcodes that tag each original DNA fragment and allow identification and removal of PCR duplicates during bioinformatic processing of sequencing data.

Sequencing platforms

Sequencers for NGS, based on the ability to perform an elevated number of chemical reactions in parallel, are increasingly being used in the clinical setting. Sequencing instruments can be classified according to the mean read length (short or long), the type of sequencing (single-end and paired-end sequencing) and the sequencing chemistry (mainly by synthesis, ligation or hybridization). Multiple factors should be considered when choosing a sequencing platform, such as run time, size of sequenced region, required depth of coverage, read length, TAT requirements and cost per sample. According to the GESMD, Illumina (San Diego, CA, USA) and Thermo-Fisher Scientific, short-read sequencing platforms are ideal for the implementation of targeted deep sequencing in diagnostic laboratories (Table S2).

There is a number of sequencing parameters that define the scope and quality of data output of a given NGS technology: sequencing capacity, type and length of the reads, sequencing output, depth of coverage, percentage of reads mapped correctly and uniformity of the reads (Table S3). Some of these parameters can be influenced by other variables, such as input DNA quality, adequate library preparation, correct design of the primers/probes, choice of sequencing platform, and the initial estimation of the number of required reads. Regarding the depth of coverage, we recommend that ≥95% of the bases targeted in the panel design are covered by at least 100 reads (≥100×), and that the mean coverage for each sample is ≥1000×. This will enable the identification of variants with a sensitivity of at least 5% in most regions, as long as variant quality criteria are fulfilled (we recommend the presence of at least 25 reads for the alternative allele). Of note, even though this is our recommendation, less conservative approaches are also likely to be highly valid, especially for certain hotspot locations, and should be validated in each laboratory. The minimum output required per library can be estimated by taking into account the intended depth of coverage together with the size of the panel (including on- and off-target regions). It is recommended that the percentage of on-target mapped reads is higher than 70%.

Test validation

During test development, iterative cycles of performance optimization should be executed until all the different components of the assay are optimized, including panel design, library preparation conditions, sequencing parameters, number of samples that should be pooled in order to achieve the

desired coverage level, as well as analysis settings and pipeline. TAT for data reports need to be estimated and should be clinically appropriate. Once the entire panel conditions have been established, the assay needs to be validated, and performance characteristics (such as sensitivity, specificity and reproducibility) should be determined.

Two rounds of validation should be consecutively performed. Firstly, a technical validation round aimed at evaluating the quality of the design and identifying the regions with poor coverage. For technical validation, input DNA requirements, library generation and sequencing should be performed, strictly following manufacturer's recommendations. During this process, the percentage of bases covered at different depths of coverage should be determined, as well as the regions with low or no coverage, which should be then redesigned or removed from the design. Biological replicates should also be assessed, including sequencing two libraries generated from the same input DNA and sequencing two libraries from two different DNA extractions of the same sample. Recurrent artefacts should also be identified. For example, variants that appear at greater frequency than expected in a large cohort or that have reads supporting them in a large number of samples, especially if they all cluster at similar VAFs, are likely to be technical artefacts. Generation of internal databases with known technical artefacts can be helpful in order to filter these artefacts out during the analysis-filtering steps. Secondly, a clinical validation round should also be performed with the aim of determining if the designed test is able to detect known alterations associated with the disease of interest. In order to do so, previously characterized samples with known SNVs and/or indels, should be analysed with the new test. Whenever possible, previously undescribed variants and complex indels should be validated by other technologies, such as Sanger sequencing, amplification refractory mutation system (ARMS)-PCR, allele-specific oligonucleotide (ASO)-PCR, digital PCR or other NGS platforms. Finally, sequencing of serial dilutions from the same sample will help to determine the sensitivity of the test.

In NGS, standards for TAT should be established by each laboratory based on the clinically proper indication for testing. Multiplexing samples from different diseases and using small-scale sequencing systems can help reduce TAT. In real world scenario of diagnosis of MDS and CMML, these TAT should be consistent with other cytogenetic tests (karyotype and fluorescence *in situ* hybridisation), and should not exceed, for most cases, 15 working days (Rack *et al*, 2019). Urgent referrals should be prioritized and, for these cases, results should try to be reported within 10 days.

Data analysis and variant filtering

Data analysis

The goal of data analysis is to use bioinformatic tools within an analysis pipeline in order to transform the raw data coming from the sequencer into a list of variants that can be visualized, filtered and interpreted. The challenge of data analysis includes the huge number of available tools, the constantly evolving analysis pipelines and the lack of consensus regarding which tools to use. Typical NGS data analysis workflows include base calling, read alignment variant calling and variant annotation. Data analysis steps and tools that are recommended, and most commonly used, by the GESMD are summarized in (Data S1, Tables S4, S5). It should be noted that several open-source and commercial tools have been developed to facilitate NGS data analysis in the clinical setting, in the form of user-friendly interfaces. Some of these tools, used by the GESMD, include MiSeq Reporter (Illumina), Variant Studio (Illumina), IonReporter (Thermo-Fisher Scientific), DNAnexus (DNAnexus, Santa Clara, CA, USA) and Sophia DDM (Sophia Genetics, Lausanne, Vaud, Switzerland).

Variant filtering

After data analysis, variant filtering should then be performed in order to obtain a list of candidate variants that will later be categorized, interpreted and reported. Of note, variant filtering criteria should always be updated according to the advances made in NGS technologies and in the disease of interest. The variant filtering workflow proposed by the GESMD is described in Table V and Fig 2.

Variant interpretation and categorization

Interpretation systems for classifying variants are useful to standardize the way in which variants are reported to clinicians. In response to the classification discrepancies that often exist between laboratories, the use of unified guidelines is highly recommended. On one hand, germline variants classification systems aim to classify variants according to their pathogenicity in a given disease (Richards *et al*, 2015; Matthijs *et al*, 2016). A joint consensus between the American College of Medical Genetics and Genomics and the Association for Molecular Pathology, for example, proposes updated standards for variant assessment and promotes the systematic evidence-based classification of variants found in Mendelian disorders (Richards *et al*, 2015). On the other hand, in cancer, somatic variants are assessed for diagnostic, prognostic, predictive and/or therapeutic impact in the context of tumour site and histology (Sukhai *et al*, 2016). Given their relevance, a system to prioritize variants detected by whole exome sequencing (WES) as well as two somatic tier-based variant classification systems have been reported in the literature (Van Allen *et al*, 2014; Sukhai *et al*, 2016; Li *et al*, 2017). Sukhai *et al* (2016) propose a classification scheme in which variants are categorized from Class 1 (highest priority) to Class 5 depending on the influence that the variant shows on diagnosis, prognosis or treatment of the specific tumour being evaluated. Similarly, Li *et al* (2017) classify variants

Table V. Variant filtering workflow detailed information proposed by the GESMD.

Step	Description
Variant pre-filtering	

Filter according to variant region	• Preserve variants located in:
	
	   ○ Coding regions (exonic)
	   ○ Splicing sites (±12 bp)
	
	• Remove variants located at: intergenic regions, downstream, upstream, non-coding RNAs, intronic regions far from splicing sites.

Remove sequencing errors	• Remove sequencing errors non-detected previously by the software of analysis. Some commonly detected errors include:
	
	   ○ Non-uniform coverage in the region flanking the variant
	   ○ Strand bias: variants only covered by forward or reverse reads
	   ○ Small indels located at repetitive and homopolymeric regions
	   ○ Edge effect: variants located at the end of the amplicon
	
	• A genomic viewer that can open alignment files (BAM or SAM), such as IGV (Broad Institute), should be used to visualize the data and identify the errors (Robinson *et al.*, 2011).

Variant filtering	
Polymorphisms	• MAF refers to the frequency at which a variant occurs in a given population. Variants with a MAF ≥ 1% are considered as polymorphisms in somatic mutation analysis. The clinical implication of polymorphisms in MDS and CMML is currently not known; therefore, we recommend not reporting these variants for now. • Population databases provide comprehensive information about frequencies of alternative (minor) alleles at a given locus in a large cohort of individuals. The following population databases can be used to identify and filter out polymorphisms: 1000 Genomes Project, Exome Variant Server, ExAC, dbSNP, dbVar and gnomAD.
Synonymous variants	• Synonymous (or silent) variants are SNVs that do not alter the encoded amino acid sequence. Their clinical relevance in MDS and CMML is unknown. Based on this current knowledge, we recommend that these variants should be filtered out for now, except well annotated pathogenic variants (i.e. *TP53* synonymous variants that are known to affect splicing).
Variants in UTR regions	• The clinical relevance of variants located at 3'UTR or 5'UTR regions is currently unknown. Based on this we recommend that these variants should be filtered out for now.
Quality criteria	
Coverage	• Coverage of the locus: ≥100×. We recommend that the genomic position in which the variant is located is covered by at least 100 reads. • Coverage of the variant: ≥25×. We recommend the presence of at least 25 reads for the alternative allele. • If the variant does not fulfil the quality criteria, sequencing should be repeated or the variant should be validated by a different technique.

BAM, binary alignment map; bp, base pairs; CMML, chronic myelomonocytic leukaemia; GESMD, Spanish MDS Group; IGV, Integrative Genomics Viewer; MAF, minor allele frequency; MDS, myelodysplastic syndrome; SAM, sequence alignment map; SNV, single nucleotide variant; UTR, untranslated region.

from Tier I (Variants with Strong Clinical Significance) to Tier IV (Benign or Likely Benign Variants) based on the clinical impact of a given variant, which is determined according to currently available evidence. It is of high importance to include a separate category for benign variants, to inform clinicians and patients and to reduce the burden on laboratories (Hoskinson *et al*, 2017). Based on our experience, the use of specific standard terminology (pathogenic, likely pathogenic, uncertain significance, likely benign and benign) facilitates the use of classification systems, especially for clinicians.

In light of this, and based on the same tier-based classification system of the American Association for Molecular Pathology, American Society of Clinical Oncology and College of American Pathologists (Li *et al*, 2017), we propose a system to classify somatic variants detected in myeloid neoplasms (Fig 2).

Tools for variant interpretation

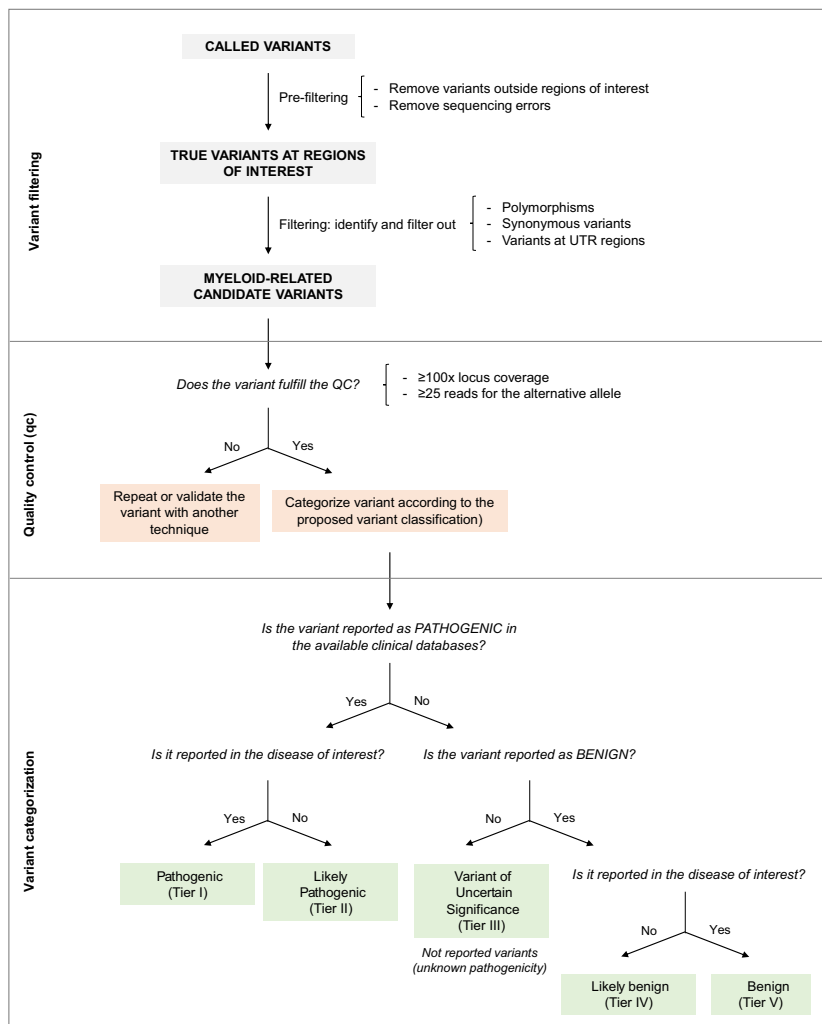In order to properly classify and annotate the detected somatic variants, several currently available repositories and

Fig 2. Proposed workflow for variant filtering and categorization. UTR, untranslated region.

genomic databases can be used. Before using these databases, clinical laboratories should make an effort to understand their content and note their limitations (Li *et al*, 2017). Likewise, establishing and consulting an in-house database of variants identified in the laboratory is recommended. The tools recommended by the GESMD are listed in Table VI.

Reference sequence databases. Reference sequence databases encompass the human reference genome and related information for the unequivocal localization of a variant in the genome. These databases annotate variant location (coding, non-coding, splicing site or untranslated region) and the strand representation (positive or negative) within the corresponding gene. Frequently consulted resources include Ensembl [European Molecular Biology Laboratory- European Bioinformatics Institute (EMBL-EBI) and the Wellcome Trust Sanger Institute; http://www.ensembl.org/index.htm], RefSeq [National Center for Biotechnology Information (NCBI) Reference Sequence Database; https://www.ncbi.nlm.

nih.gov/refseq] and Locus Reference Genomic (LRG, NCBI and EMBL-EBI; https://www.lrg-sequence.org/). This information is essential for the correct nomenclature of the variants according to the Human Genome Variation Society (HGVS) (Li *et al*, 2017).

Population databases. Population databases are useful for obtaining the frequency of variants at a given locus in a variety of geographically distinct populations (Cottrell *et al*, 2014; Richards *et al*, 2015). They are commonly used to filter out polymorphic variants based on an established cut-off of minor allele frequency (MAF). Although a standardized MAF to discriminate between polymorphic or benign variants has not been established yet, current practice within GESMD sets 1% as the cut-off point in studies of somatic variants. Additionally, an ethnic specificity of the variant should be considered based on the ethnic background of the patient. Finally, we would emphasise a cautionary note when consulting these databases, as they were built under the assumption that the population included in them is healthy, but some somatic

Table VI. List of web resources useful for variant interpretation.

Database	Website URL
Reference sequence	
NCBI reference sequence database	https://www.ncbi.nlm.nih.gov/genome
Ensembl genome browser	http://www.ensembl.org/index.htm
Locus reference genomic	https://www.lrg-sequence.org/
RefSeqGene	https://www.ncbi.nlm.nih.gov/refseq
MitoMap	https://www.mitomap.org/MITOMAP/HumanMitoSeq
UCSC genome browser	https://genome.ucsc.edu/cgi-bin/hgTables
Population databases	
dbSNP	https://www.ncbi.nlm.nih.gov/snp/
Exome variant server	http://evs.gs.washington.edu/EVS/
1000 genomes project	http://browser.1000genomes.org
dbVar	https://www.ncbi.nlm.nih.gov/dbvar
Exome Aggregation Consortium (ExAC)	http://exac.broadinstitute.org/
Genome Aggregation Database (gnomAD)	http://gnomad.broadinstitute.org/
Short genetic variation	https://www.ncbi.nlm.nih.gov/snp
Somatic and constitutional variants databases	
National Cancer Institute's Genome Data Commons	https://gdc.cancer.gov
Catalogue of Somatic Mutations in Cancer (COSMIC)	http://cancer.sanger.ac.uk/cosmic
IARC *TP53* mutation database	http://p53.iarc.fr/TP53GeneVariations.aspx
TP53 website	http://p53.fr/
Personalized cancer therapy	https://pct.mdanderson.org
cBioPortal	http://www.cbioportal.org
Intogen	https://www.intogen.org/search
ClinicalTrials.gov	https://clinicaltrials.gov
Pediatric Cancer Genome Project	http://explorepcgp.org
My Cancer Genome	http://www.mycancergenome.org
International Cancer Genome Consortium (ICGC)	https://dcc.icgc.org
The Cancer Genome Atlas (TCGA)	https://cancergenome.nih.gov/
VarSome The Human Genomic Variant Search Engine	https://varsome.com
DECIPHER	https://decipher.sanger.ac.uk/
ClinVar	http://www.ncbi.nlm.nih.gov/clinvar
Online Mendelian Inheritance in Man (OMIM)	https://www.omim.org/
Human Gene Mutation Database (HGMD)	http://www.hgmd.cf.ac.uk/ac/index.php
Leiden Open Variation Database (LOVD)	http://www.lovd.nl
In-house laboratory databases	
RESMDmol	https://www.gesmd.es/carrerasresearch/index.php
Algorithms for *in silico* prediction	
PolyPhen2	http://genetics.bwh.harvard.edu/pph2
SIFT	http://sift.jcvi.org
Mutation Assessor	http://mutationassessor.org
Mutation Taster	http://www.mutationtaster.org
PROVEAN	http://provean.jcvi.org/index.php
CoVEC	https://sourceforge.net/projects/covec/files
CADD	http://cadd.gs.washington.edu
GERP++	http://mendel.stanford.edu/sidowlab/downloads/gerp/index.html
PhyloP and PhastCons	http://compgen.bscb.cornell.edu/phast
ConSurf	http://consurftest.tau.ac.il
FATHMM	http://fathmm.biocompute.org.uk
PANTHER	http://www.pantherdb.org/tools/csnpScoreForm.jsp
PhD-SNP	http://snps.biofold.org/phd-snp/phd-snp.html
SNPs & GO	http://snps-and-go.biocomp.unibo.it/snps-and-go
Align GVGD	http://agvgd.iarc.fr/agvgd_input.php
MAPP	http://mendel.stanford.edu/SidowLab/downloads/MAPP/index.html
MutPred	http://mutpred.mutdb.org
nsSNPAnalyzer	http://snpanalyzer.uthsc.edu

Table VI. (*Continued*)

Database	Website URL
Condel	http://bg.upf.edu/condel/home
LRT	http://www.genetics.wustl.edu/jflab/lrt_query.html
DANN	https://omictools.com/dann-tool
Splice site prediction	
Human splicing finder	http://www.umd.be/HSF3
MaxEntScan	http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html
NetGene2	http://www.cbs.dtu.dk/services/NetGene2
NN plice	http://www.fruitfly.org/seq_tools/splice.html
GeneSplicer	http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml
NNSplice	http://www.fruitfly.org/seq_tools/splice.html
FSPLICE	http://www.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind

variants may accidentally have been included as well. For example, common hotspot mutations, such as *DNMT3A* R882 or *JAK2* V617F, can be found in some of these databases and therefore a whitelist of known hotspot mutations could be useful to rescue these variants.

Recommended population databases include The Database of Short Genetic Variation (dbSNP, the NCBI database of genetic variation; https://www.ncbi.nlm.nih.gov/snp/), Exome Variant Server (EVS, NHLBI GO Exome Sequencing Project; http://evs.gs.washington.edu/EVS/), 1000 Genomes Project (International Genome Sample Resource, IGSR; http://browser.1000genomes.org), Exome Aggregation Consortium (ExAC, Broad Institute; http://exac.broadinstitute.org/) and Genome Aggregation Database (gnomAD, Broad Institute; http://gnomad.broadinstitute.org/). Databases such as ExAC or gnomAD are more accurate than dbSNP when it comes to filtering polymorphisms and neutral variants.

Somatic and germline variants in clinical databases. Both somatic and germline variants are included in clinical databases. The incidence and prevalence of a variant is often detailed in different subtypes of cancers and functional prediction algorithms are provided to categorize the variants according to their pathogenicity. Frequently, clinical databases also include information for accurate annotation and prioritization of somatic variants; bibliographic references with or without systematic review; additional information about the tissue in which the variant has been described; outcome disease data or targeted therapies, among others. An example is the National Cancer Institute's Genome Data Commons (https://gdc.cancer.gov), which contains all the genomic data generated at the National Cancer Institute, and which includes the well-known databases of The Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov/), the Therapeutically Applicable Research to Generate Effective Therapies (TARGET; https://ocg.cancer.gov/programs/target), and the Cancer Genome Characterization Initiative (CGCI; https://ocg.cancer.gov/programs/cgci). Another public database is the Catalog of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic) from the Wellcome

Sanger Institute, which contains millions of somatic variations described in numerous types of tumours and has also recently included intron variants. However, the information provided in these databases is not always up-to-date. In addition, a poor representation of pathological diagnostic standards is detected as well as a lack of an exhaustive validation of the findings and/or the sources from which the included variants were extracted.

Germline variants are also usually detected upon tumour sequencing. Such variants may be associated with cancer predisposition syndromes. To evaluate them, there are several germline mutations databases, such as Human Gene Mutation Database (Institute of Medical Genetics; http://www.hgmd.cf.ac.uk/ac/index.php) or ClinVar (NCBI; http://www.ncbi.nlm.nih.gov/clinvar).

Finally, for *TP53* gene variant interpretation, the International Agency for Research on Cancer (IARC) *TP53* Database (WHO; http://p53.iarc.fr/TP53GeneVariations.aspx) and *TP53* Website (Centre de Recherche des Cordeliers and Karolinska Institute; http://p53.fr/) are useful resources for evaluating their impact in haematological malignancies, because they include the frequencies of the variants observed in both the somatic and germinal contexts.

In-house laboratory databases. We recommend that each clinical laboratory should establish an in-house database to provide consistent variant annotation and categorization, to determine the frequency of mutations and to identify platform-specific artefacts or potential false-positive variants. A continuous review of new publications, clinical trials and databases used in variant interpretation, which are regularly updated, should be carried out to ensure that variant annotation is up-to-date. To increase the quality of interpretation, we recommend that in-house databases are complemented with further relevant annotation, including results from other techniques such as morphology, cytogenetic and immunophenotyping, because NGS results should always be combined and interpreted in the context of other laboratory and clinical diagnostic data. Moreover, it would be extremely useful to share those internal databases with other centres/

laboratories that perform similar determinations. Indeed, the GESMD is making that effort by building the RESMDmol database (https://www.gesmd.es/carrerasresearch/index.php), in view of facilitating the homogenization of results informed in the clinical reports.

Functional consequence of the variant. In silico predictive algorithms estimate the possible deleterious consequences of a variant on the encoded protein of a given gene. The criteria used to determine this effect varies across different tools. Algorithms designed to predict the functional consequence of a missense variant are commonly based on the evolutionary conservation of an amino acid or nucleotide, the location and context within the protein sequence and the biochemical consequence of the amino acid substitution (Brunak *et al*, 1991; Vreeswijk *et al*, 2009; Thusberg *et al*, 2011). In silico tools have also been developed to evaluate the consequence of splicing variants related to the creation or loss of splice sites at the exonic or intronic regions (Thusberg *et al*, 2011; Houdayer *et al*, 2012). However, in the context of cancer, the interpretation of these predictions is usually not easy, especially for gain-of-function mutations. Therefore, it is recommended that least four different prediction algorithms should be used to analyse each variant, although the obtained output should never be used as sole evidence for clinical decision making, as, to date, there is no consensus about the relevance of each predictor, nor about how to combine outputs from different prediction algorithms (Li *et al*, 2017).

In the case of variants with available information about their functional effect, two scenarios can be considered. On one hand, when the pathogenicity of the affected gene is associated with a loss of function, frameshift variants must be interpreted as probably pathogenic, as long as the variant does not affect the last exon of the gene, unless the pathogenicity of those variants affecting the last exon has been validated (i.e. variants in *ASXL1* and *CALR*). Loss of function variants located at the last exon of a gene will need evaluation according to other parameters, such as the type of protein domain that is altered and the effect of other proximity variants. Accordingly, these will be classified as variants of uncertain significance or probably pathogenic. On the other hand, when the pathogenicity of the affected gene is associated with a gain-of-function, and a frameshift variant is detected, this will be interpreted as a variant of uncertain significance unless there is strong evidence of pathogenicity based on functional studies in the literature.

Criteria for variant classification

We recommend to interpret and report variants that fulfil all three following criteria: (i) MAF < 1%, (ii) VAF ≥ 5% and (iii) minor allele coverage ≥25 reads. Although we recommend that cut-off of VAF ≥ 5% should be used, the analyst should consider informing variants with lower allele frequencies in particular cases, such as hotspot regions in genes with

clear clinical relevance, such as *TP53* or *JAK2*. However, in order to report variants with a VAF below 5%, the depth of coverage must be increased to maintain, at least, the minor allele coverage over 25 reads.

The GESMD proposes that variants should be categorised according to the following criteria: (i) relevance of the altered gene based on its actionability, defined as being relevant for the diagnosis/classification, prognosis and/or treatment (therapeutic target or related to sensitivity, toxicity or resistance to therapy) of the disease; (ii) presence of the variant in clinical databases and published literature that indicates the recurrence or pathogenicity of the identified variant; (iii) tissue and/or tumour histology in which the variant has been described; and (iv) in silico predictive algorithms and functional studies.

Therefore, the categorization of the variant must be based on its clinical impact on the specific disease and tissue under study. Due to the importance of the definition of the actionability of a gene, we strongly recommend following the evidence defined by Sukhai *et al* (2016). Of note, determining the actionability of a gene will be particularly relevant when using pan-haematological or oncological targeted NGS panels that include genes which have not been previously described in the disease of interest, as well as for WES and whole genome sequencing.

Variant classification system

According to our categorization criteria, variants will be classified into one of the following classes (Fig 2, Table VII):

Pathogenic (Tier I). Described in the literature as relevant for the clinical management of MDS/CMML or other myeloid neoplasms. The variant affects an actionable gene and has been established as a pathogenic variant in myeloid neoplasms.

Likely pathogenic (Tier II). Described in the literature but with no established relevance in the clinical management of MDS or other myeloid neoplasms. The variant affects an actionable gene but has been established as a pathogenic variant in solid tumours or in non-myeloid haematological neoplasms. This category also includes variants previously not described for which predictive algorithms strongly classify them as pathogenic.

Variant of uncertain significance (VUS) (Tier II). Previously described but with no sufficient evidence of its pathogenicity on actionable MDS genes, other haematological neoplasms or solid tumours. This category also includes variants not previously described for which predictive algorithms cannot strongly classify them into pathogenic or benign.

Likely benign (Tier IV) or benign (Tier V). Described in the literature as clinically insignificant because it has no effect on

Table VII. Classification variant system.

Category	Criterion				
	Gene actionability	Variant clinical significance	Variant recurrence in databases	Tissue and/or tumour histology	Predictive algorithms and functional studies
Pathogenic (Tier I)	Actionable	Diagnostic, prognostic and/or treatment significance in in the disease of interest (Biomarker)	Described and confirmed as pathogenic	Disease or tissue of interest	NA
Likely pathogenic (Tier II)	Actionable	Clinical significance in other haematological neoplasms or solid tumors	Described as pathogenic	Other haematological neoplasms or solid tumors	NA
Variant of uncertain significance (VUS) (Tier III)	Actionable/not actionable	Of uncertain significance	Unknown pathogenicity	NA	Likely pathogenic
Likely benign (Tier IV)	Actionable/not actionable	Clinically irrelevant	Described as benign	Other haematological neoplasms or solid tumors	Probably benign
Benign (Tier V)	Actionable/not actionable	Clinically irrelevant	Described and confirmed as benign	Disease or tissue of interest	Probably benign

NA, not applicable.

the protein. Frequently, these variants present MAF > 1% and, sometimes, VAF of approximately 50% or 100%. These levels of VAF may be indicative of the germline nature of the variant and, therefore, are neither acquired nor tumour-specific. However, the VAF criterion should be considered with caution, as there are somatic variants present in major clones with high VAF. This category also includes variants previously not described for which predictive algorithms strongly classify them as benign. We recommend that, to avoid confusion, benign and likely benign variants should not be included in the clinical report.

Clinical report

Next generation sequencing clinical reports should follow the general professional organizations' recommendations and guidelines (Richardson, 2002; Rack *et al*, 2019), such as avoiding long reports for the benefit of clarity, inclusion of pagination, and reviewing and signing by, preferably, two geneticists with the relevant expertise on haematological malignancies. We recommend that the report includes (at least) the information detailed below.

Patient identification

As in current standard tests, proper patient identification should be incorporated in the clinical report, including two unique patient identifiers, demographic data and referral information (reason for referral, suspected or confirmed diagnosis and clinical time point). Sample details should also be provided (type, source and received date) as well as hospital and referring physician. A statement of signed informed consent should also be made.

Methodology

Details on the methodology used should be specified, including library preparation chemistry and sequencing instrument. Regions included in the panel (genes according to the Human Genome Organisation nomenclature, exons and hotspots) and low covered regions should be detailed. Analysis settings, filters and cut-off values (such as minimum coverage or VAF) should be specified. The version of the human reference sequence to which sequence reads are aligned should also be included, as well as the clinical databases used for annotation. Key quality control metrics and limitations need also be listed.

Results and interpretation

A list of identified variants should be included in the results section, annotated in accordance to the HGVS mutation nomenclature. For each variant, we recommend including the following information: gene, exon, nucleotide change, protein change, type of variant (missense, frameshift,

nonsense, splicing), transcript RefSeq ID, variant ID from clinical databases, VAF and position depth of coverage. When using a variant classification system, the categorization of each variant should be included and the used classification system should be described. Regarding variant interpretation, we recommend including a critical summary of the clinical relevance of the variant in the disease of interest, including treatment or referenced clinical trials information, if applicable. For the categorization of the disease, the WHO 2017 nomenclature should be used (Arber *et al*, 2016). Further studies to validate the significance of the results obtained should be conducted when appropriate.

Conclusion

We have long awaited the introduction of NGS into the routine diagnostic armamentarium of myeloid malignancies. Currently, an increasing number of laboratories are implementing NGS procedures into the diagnostic algorithms of patients with haematological malignancies, opening new horizons for individualized clinical management of these patients. It is therefore of major importance to standardize the generation, analysis and clinical interpretation of NGS data. To that end, the GESMD has expanded the present set of guidelines, aiming at stablishing common quality standards for the adequate clinical interpretation of NGS results, hoping that this effort will ultimately contribute to the benefit of patients with myeloid malignancies.

Acknowledgements

Author contribution

All authors contributed in the development of these guidelines and in the revision of the manuscript.

Supporting Information

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

Table S1. Main characteristics and differences between amplicon- and capture-based panels.

Table S2. Comparison of Illumina and Thermo Fisher NGS platforms.

Table S3. Main sequencing parameters.

Table S4. List of most commonly used alignment tools.

Table S5. List of most commonly used somatic variant callers.

Data S1. Supplementary methods.

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