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Standards of Genetic Testing in the Diagnosis and Prognostication of Systemic Mastocytosis in 2022: Recommendations of the EU-US Cooperative Group

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Mastocytosis comprises rare heterogeneous diseases characterized by an increased accumulation of abnormal mast cells in various organs/tissues. The pathogenesis of mastocytosis is strongly linked to the presence of *KIT*-activating mutations. In systemic mastocytosis (SM), the most frequent mutation encountered is *KIT* p.D816V, whose presence constitutes one of the minor diagnostic criteria. Different techniques are used to search and quantify the *KIT* p.D816V mutant; however, allele-specific quantitative PCR and droplet digital PCR are today the most sensitive. The analysis of the *KIT* p.D816V allele burden has undeniable interest for diagnostic, prognostic, and therapeutic monitoring. The analysis of non−mast cell hematological compartments in SM is similarly important because *KIT* p.D816V multilineage involvement is associated with a worse prognosis. In addition, in advanced forms of SM, mutations in genes other than *KIT* are frequently identified and affect negatively disease outcome and response to therapy. Thus, combined quantitative and sensitive analysis of *KIT* mutations and next-generation sequencing of other recurrently involved myeloid genes make it possible to better characterize the extent of the affected cellular compartments and additional molecular aberrations, providing a more detailed overview of the complex mutational landscape of SM, in relation with the clinical

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Abbreviations used
AdvSM- advanced SM
AHN- associated hematologic neoplasm
ASM- aggressive SM
ASO-qPCR- allele-specific quantitative PCR
ASXL1- additional sex combs-like 1
BM- bone marrow
BST- basal serum tryptase
DNMT3A- DNA cytosine 5 methyl transferase 3A
ddPCR- droplet digital PCR
EAB- expressed allele burden
ECD- extracellular domain
ECNM- European Competence Network on Mastocytosis
EU- European Union
ISM- indolent SM
MC- mast cell
MCL- mast cell leukemia
NGS- next-generation sequencing
OS- overall survival
PB- peripheral blood
RUNX1- Runt-related transcription factor 1
S/A/R- SRSF2/ASXL1/RUNX1
SM- systemic mastocytosis
SM-AHN- SM with an associated hematologic neoplasm
SRSF2- serine/arginine-rich splicing factor 2
SSM- smoldering SM
TK- tyrosine kinase
VAF- variant allele fraction
WoCo- working conference
WP- work package

heterogeneity of the disease. In this article, we report the latest recommendations of the EU-US Cooperative Group presented in September 2020 in Vienna during an international working conference, on the techniques we consider standard to detect and quantify the *KIT* p.D816V mutant in SM and additional myeloid mutations found in SM subtypes. **(J Allergy Clin Immunol Pract 2022;10:1953-63)**

Key words: *Mast cell; mastocytosis;* KIT *mutations; diagnosis; next-generation sequencing; allele burden; prognosis*

INTRODUCTION

The Year 2020 International Working Conference of the EU-US Cooperative Group

The year 2020 international working conference (WoCo) on mast cell (MC) disorders was held in Vienna between August 30 and September 1. It was undertaken within a European Union (EU)-US multicenter cooperative initiative, comprising members of the European Competence Network on Mastocytosis (ECNM)[1] and of the American Initiative in Mast cell diseases,[2] for more standardized diagnostics in daily practice within and outside clinical trials in MC disorders, including mastocytosis. Work Packages (WPs) were initiated several months before the WoCo venue to identify consensus opinions on the topics covered by each WP. WP4 was specifically devoted to molecular biomarkers in mastocytosis and the techniques to be considered standard to detect and quantify *KIT* p.D816V in bone marrow (BM) and peripheral blood (PB) samples from patients diagnosed with or suspected of systemic mastocytosis (SM). In addition, the use of next-generation sequencing (NGS) techniques for detection of additional somatic mutations in genes other than *KIT* was discussed. In this report, we present the consensus recommendations approved by the faculty. These consensus recommendations focus on SM in adults and include the algorithm and technical procedures to be followed for the detection of *KIT* mutations and the interpretation of the results for diagnostic, risk-stratification, and monitoring of therapy in SM. Overall, the proposed recommendations extend those forwarded by the ECNM in 2015.[3] In addition, we present new recommendations agreed upon during the WoCo regarding the investigations of additional somatic mutations in genes other than *KIT* that occur frequently in SM.

Mastocytosis

Mastocytosis is an orphan disease of heterogeneous clinical presentations, characterized by the expansion and/or accumulation of abnormal (neoplastic) MCs in 1 or more tissues and/or organs.[4,5] According to 2016 World Health Organization classification, mastocytosis can be classified into 3 major categories, namely cutaneous mastocytosis, various subtypes of SM, and the rare category of MC sarcoma.[6] Mastocytosis affects both children and adults. In children, the disease is usually confined to the skin (cutaneous mastocytosis) and has a good prognosis,[7,8] whereas in adults, the systemic involvement (eg, SM) of extracutaneous tissues is detected.[6] SM diagnosis is established when at least the major and 1 minor criterion, or 3 minor criteria in the absence of the major criterion, are found.[6] The major diagnostic criterion is the presence of dense multifocal infiltrates consisting of cohesively aggregated MCs (>15 MCs) in biopsy of BM and/or other extracutaneous organs. Minor criteria include (a) an altered MC morphology with more than 25% of all MCs being atypical on BM smears or spindle-shaped in infiltrates detected in sections of other extracutaneous (eg, visceral) organs; (b) expression of CD25 with or without CD2 on MCs in BM or another extracutaneous organ; (c) baseline serum tryptase (BST) level more than 20 ng/mL; and (d) presence of a *KIT* point mutation at codon 816 in the BM or another extracutaneous organ.[6,9] The diagnostic subtypes of SM are defined by the presence *versus* absence of (a) the so-called B- and C- findings, (b) an associated hematologic neoplasm (AHN), and (c) the percentage of neoplastic MCs in a BM-aspirated smear. B-findings are indicative of a greater MC burden and include (1) more than 30% MCs in the BM and BST levels more than 200 ng/mL, (2) hepatomegaly or splenomegaly without impairment of organ function, and (3) signs of myelodysplasia or myeloproliferation in the absence of diagnostic criteria of another hematological malignancy. C-findings reflect organ dysfunction and may include (1) cytopenia(s) due to massive BM infiltration, (2) impaired liver function with ascites and/or portal hypertension, (3) hypersplenism, (4) large (eg, >2 cm) osteolytic bone lesions with or without pathological fractures, and (5) malabsorption characterized by hypoalbuminemia and/or weight loss. Thus, SM can be categorized, according to the above criteria, into (1) indolent SM (ISM) with no or 1 B-finding and no C-findings, (2) smoldering SM (SSM) when 2 or more B-findings (but no C-findings) are found,[10] (3) aggressive SM (ASM) in case 1 or more C-findings are found in the absence of another AHN and if

criteria for MC leukemia (MCL) are not met,[9] (4) SM with an associated hematologic neoplasm (SM-AHN), when another coexisting non-MC hematologic neoplasm is diagnosed,[11] and (5) the very rare variant MCL, when more than 20% abnormal MCs are present on a BM smear.[12] ASM, SM-AHN, and MCL are collectively termed advanced SM (AdvSM).[13]

KIT mutations

Activating mutations of *KIT* are closely associated with mastocytosis.[14,15] KIT/CD117 is a type III transmembrane receptor with tyrosine kinase (TK) activity, which binds stem cell factor.[16] The *KIT* gene consists of 21 exons (Figure 1) located in chromosome 4q12. The resulting protein is a glycoprotein of 145 kDa and 976 amino acids.[17−19] The structure of KIT is similar to that of other transmembrane receptors with TK activity (Figure 1).[20] Thus, KIT consists of an N-terminal extracellular domain (ECD) that binds stem cell factor and an intracellular domain (C-terminal), linked by a transmembrane domain.[21] The ECD is made up by a series of 5 immunoglobulin-like domains, which are relevant for ligand binding and receptor dimerization. The transmembrane domain allows anchoring of the receptor in the plasma membrane.[21] The intracellular domain contains all sequences related to the TK activity and can be divided into (a) a juxtamembrane domain, an autoinhibitory domain of the TK activity,[22] and (b) a kinase domain. In children, *KIT* mutations frequently affect the ECD of the KIT receptor,[23,24] whereas in adult patients, the *KIT* p.D816V mutation, located in the phosphotransferase domain of the receptor encoded by exon 17, is predominantly found in neoplastic BM MCs because it is detected in virtually all patients with ISM and in more than 90% of patients with AdvSM, accounting for more than 90% of all patients with SM.[9,25,26] In addition to *KIT* p.D816V, other activating mutations located in the juxtamembrane domain (eg, *KIT* p.V560G) and ECD (eg, *KIT* p.D419del) of KIT can be found more rarely in patients with SM, particularly in patients with ASM, MCL, and MC sarcoma.[27-30] In contrast to adult patients with SM, in skin biopsies of children with mastocytosis, the *KIT* p.D816V mutant is found in only approximately 30% of cases, whereas other *KIT*-activating mutations, mainly located in the ECD of the receptor, are found in approximately 40% of the cases (Figure 2).[23] Interestingly, in a recent study, no significant association between evolution (spontaneous regression or persistence at adolescence) and the type of *KIT* mutation was found in a cohort of pediatric cases.[31] An updated list of *KIT*-activating mutations reported in mastocytosis is presented in Table I, and the distribution of *KIT* p.D816V and other *KIT* mutations within the different diagnostic categories of SM is presented in Table II.

KIT p.D816V mutant allele burden as a biomarker

In a proportion of ISM cases, the *KIT* mutation is found exclusively in the neoplastic MC compartment. However, in a significant fraction of patients with ISM, the mutation is detected in other mature BM and PB cells such as basophils, eosinophils, neutrophils, as well as B- and T-lymphocytes.[32,33] Interestingly, multilineage *KIT* p.D816V involvement has been found to be the most important prognostic criterion for progression of ISM to more advanced SM subtypes.[34] In SM-AHN, the *KIT* p.D816V mutation can be found not only in the MC compartment but often also in the malignant cells of the AHN.[35-38] The *KIT* p.D816V mutant allele burden is a surrogate for the extent of multilineage involvement in SM.[39,40]

Kristensen et al[41] described a correlation between BST levels, the *KIT* p.D816V variant allele fraction (VAF; DNA-based) and the percentage of neoplastic BM MCs in ISM. These data were further confirmed by Erben et al[42] in 2014, who demonstrated a correlation between BST levels and the *KIT* p.D816V−expressed allele burden (EAB; RNA-based). This later study also showed a strong link between the *KIT* p.D816V EAB and the subtype of SM (indolent *vs* advanced SM; $P < .001$) and thereby, overall survival (OS).[42] Broesby-Olsen et al[43] found no association between clinical manifestations (skin, gastrointestinal, etc) and *KIT* p.D816V VAF in a study restricted to patients with ISM, whereas Hoermann et al[44] showed significant differences in the *KIT* p.D816V VAF according to the different subtypes of mastocytosis and proposed a prognostic threshold of 2% *KIT* p.D816V VAF. More recently, Greiner et al[45] used droplet digital PCR (ddPCR) to investigate the *KIT* p.D816V mutation burden in formalin-fixed paraffin-embedded BM tissue sections of 116 patients with SM (91 with ISM and 25 with AdvSM). These authors found that the *KIT* p.D816V mutation burden in the tissue was significantly higher than in blood and BM-aspirated samples, and that it correlated better with the BM MC infiltration ($r = 0.68$ *vs* 0.48) and BST levels ($r = 0.68$ *vs* 0.58) compared with that derived from the analysis of PB and BM specimens. Furthermore, the *KIT* p.D816V tissue mutation burden (a) was significantly higher in AdvSM *versus* ISM and (b) was an independent predictor of patient survival in multivariate analysis.[45] In addition, Carter et al[46] demonstrated a correlation of serum tryptase with PB and BM *KIT* p.D816V VAF in pediatric patients with ISM. Altogether these data indicate that the *KIT* p.D816V VAF is a reliable and reproducible prognostic marker of SM,[44] which has been subsequently incorporated into prognostic scoring systems, such as the REMA (Spanish Network on Mastocytosis) score.[40]

In parallel, the *KIT* p.D816V mutant allele burden has also been used as a biomarker for response to treatment. In 2014 Hoermann et al[44] reported a significant decrease in the *KIT* p.D816V VAF in 11 patients with AdvSM in response to nonspecific cytoreductive drugs, such as cladribine, hydroxyurea, or IFN-α. The same year, Erben et al[42] confirmed that the *KIT* p.D816V EAB can also be useful to monitor therapeutic treatment (chemotherapy, allogeneic stem cell transplantation [Allo-SCT]) in patients with AdvSM. In the era of KIT-targeted TK inhibitors, such as midostaurin or avapritinib, monitoring of molecular responses to such drugs has become critical, to better assess their efficacy and to early detect resistance.[47] In this regard, Jawhar et al[48] have evaluated the impact of molecular markers at baseline and during follow-up in midostaurin-treated patients with AdvSM. *KIT* p.D816V EAB was measured by ASO-q-PCR in PB for all patients.[48] Depending on the relative reduction of the *KIT* p.D816V EAB in PB at month 6 of therapy, patients were classified as *KIT* responders (decrease in EAB $\geq$25%) or *KIT* nonresponders (decrease in EAB <25%). In univariate analyses, 25% or more reduction in *KIT* p.D816V EAB, 50% or more in tryptase, and 50% or more in alkaline phosphatase at month 6 of therapy were significantly associated with an

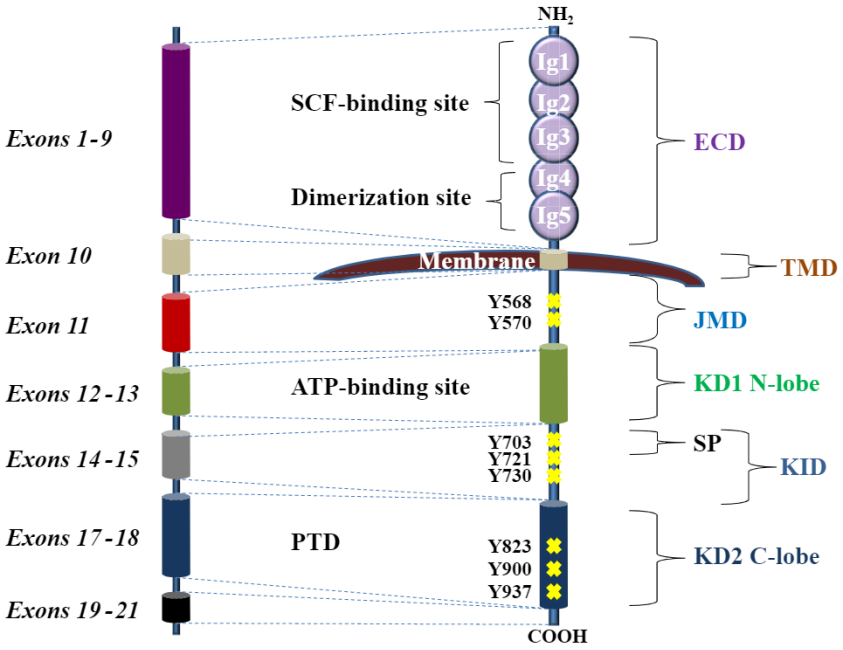

FIGURE 1. Structure of the *KIT* gene and of the corresponding receptor (KIT/CD117). The *KIT* gene (left), located on chromosome 4q12 in humans, contains 21 exons transcribed/translated into a transmembrane receptor TK (RTK) of 145 kDa and 976 amino acids (right). The figure shows the receptor under its monomeric form, comprising 5 immunoglobulin (Ig)-like subunits in the ECD, encoded by exons 1 to 9 with a ligand-binding site for SCF (SCF-binding site), aa dimerization site, and a cytoplasmic region with a transmembrane domain (TMD), encoded by exon 10, made by a single helix. The cytoplasmic region of KIT contains an autoinhibitory juxta-membrane domain (JMD), encoded by exon 11, and a kinase domain (KD) splitted by a large kinase insert domain (KID) of approximately 60 to 100 aa residues (encoded by exons 14 and 15) into 2 subdomains: KD1 (ATP-binding site in green), encoded by exons 12 and 13, and KD2 (phosphotransferase domain in blue; PTD), encoded by exons 17 and 18. The "Switch Pocket"(SP), located between the N and C lobes (and adjacent to the ATP-binding site), allows the kinase to adopt an active or inactive conformation (ON/OFF switch). The positions of major intracellular tyrosine residues that are phosphorylated on dimerization of the receptor are represented by yellow crosses. *SCF*, Stem cell factor. Adapted from Bibi et al.[17]

improved OS.[48] In multivariate analysis, only the 25% or more *KIT* p.D816V EAB reduction remained an independent on-treatment marker for improved OS.[48]

Mutations in genes other than *KIT*

In addition to *KIT* mutations, several non-*KIT* somatic mutations have been described in SM.[49-55] These additional molecular abnormalities can be found in and contribute to the pathophysiology of SM and are usually associated with more advanced and poorer-prognostic forms of the disease.[25] Indeed, these additional mutations are found mainly in AdvSM, and particularly in SM-AHN,[25] although SSM and a smaller fraction of patients with ISM may also harbor such additional defects, which have an adverse impact on patient outcome when present at a high VAF.[40] Of note, most of these additional mutations affect genes involved in epigenetic regulation or regulation of transcription or splicing, and are usually found mutated in other myeloid malignancies. Indeed, in patients with AdvSM, additional genetic defects are quite frequently found and affect *TET2* (ten-eleven-translocation 2) (although the impact of *TET2* mutations remains controversial),[48,50] *ASXL1* (additional sex combs-like 1),[53] *SRSF2* (serine/arginine-rich splicing factor 2),[54] *SF3B1* (splicing factor 3B subunit 1),[52] *RUNX1* (Runt-related transcription factor 1),[55] or *JAK2* (Janus kinase 2).[51,53] Other mutations more rarely encountered affect *NRAS* (neuroblastoma RAS viral oncogene homolog), *KRAS* (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), *DNMT3A* (DNA cytosine 5 methyl transferase 3A), *ETV6* (translocation-Ets-leukemia virus), *EZH2* (enhancer of zeste homolog 2), or *SETBP1* (SET-binding protein 1).[49-54] Of note, some patients may present with up to 5 or even 6 additional mutations besides that of *KIT*,[55] the prognosis becoming progressively less favorable as the number of additional mutations increases.[55] Despite the variety of additional mutations described in AdvSM, it appears that only *ASXL1*, *SRSF2*, and *RUNX1* (SRSF2/ASXL1/RUNX1 [S/A/R] panel), as well as *NRAS*, *DNMT3A*, or *EZH2* mutations, have a significant adverse impact on patient survival.[50,55-57] Interestingly, mutations in *SRSF2*, *ASXL1*, or *RUNX1* have been shown to impact the prognosis of the disease and are already detectable at diagnosis, whereas *RAS* or *TP53* mutations appear usually during the follow-up when the disease progresses. In addition, these non-*KIT* mutations affect response to treatment. In particular, Jawhar et al[48] have analyzed the impact of mutations on response and progression of patients with AdvSM treated with midostaurin. These authors found that patients with mutations in the S/A/R gene panel had a significantly lower overall response rate and a significantly shorter OS as

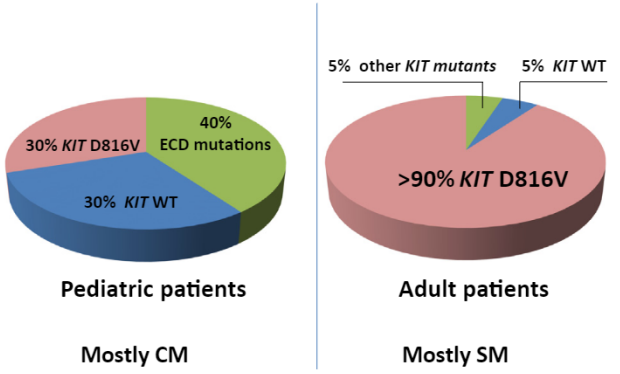

FIGURE 2. Comparison of the frequency of the different *KIT* mutants found in pediatric *vs* adult patients with mastocytosis. In pediatric patients (left panel), analysis of the *KIT* structure in lesional skin biopsies has revealed that the *KIT* p.D816V mutant can be found in approximately 30% of the cases, whereas approximately 40% of the patients harbor non-*KIT* p.D816V mutants, mostly located in the ECD of KIT. Finally, approximately 30% of pediatric patients are *KIT* WT. In contrast, in adults (lower panel), the great majority of patients with ISM, SSM, ASM, and SM-AHN harbor the *KIT* p.D816V mutation, which can be usually detected both in BM and/or in PB. In contrast patients with MCL and MCS are less frequently positive for this mutation and may harbor other non-*KIT* p.D816V mutants, or no mutation in *KIT* (*KIT* WT). Besides, in WDSM, only a minority of patients present with the *KIT* p.D816V mutation or with other non-D816V mutants, whereas most patients are *KIT* WT. *CM,* Cutaneous mastocytosis; *MCS*, mast cell sarcoma; *WDSM*, well-differentiated SM; *WT,* wild-type.

compared with S/A/R-negative patients. In addition, they observed that acquisition of additional mutations in myeloid-neoplasm–associated genes and an increase in the allele burden of preexisting mutations were both associated with disease progression.[48]

Molecular prognostic scoring systems

New prognostic tools have recently emerged for SM. Over the years, several biological parameters (eg, hematological cell counts, MC immunophenotype, and serum markers) as well as the patient age have been shown to be associated with the outcome of SM.[58-60] More recently, NGS allowed the identification of new molecular markers (or combinations of molecular markers) linked to disease progression and patient survival (eg, the S/A/R gene panel).[61] With the aim of increasing the accuracy of the assessment of the risk of progression, as well as of patient survival, several groups have attempted to propose prognostic score models, which integrated these new molecular markers together with the *KIT* p.D816V VAF and clinical and laboratory data.[40,56,62,63] These scoring systems are presented in Table III.

STATE OF THE ART OF GENETIC ANALYSIS IN SM AND RECOMMENDATIONS OF THE EU-US COOPERATIVE GROUP

Materials for molecular analysis

For identification of the *KIT* p.D816V mutation, different biological samples can be used, such as BM, PB, and tissue biopsies from skin lesions, or any other affected organ (ie, the gastrointestinal tract). Despite this, in case of suspicion of SM, the WP4 consensus reference test (or "criterion standard") remains the BM-aspirated sample collected in a tube anticoagulated with EDTA. Although fresh samples are preferred, the use of frozen material is possible, under suitable storage conditions (−70°C for RNA and −20°C for cDNA and DNA). In addition, some techniques might allow the search of *KIT* p.D816V or of other *KIT* mutants in formalin-fixed paraffin-embedded tissues.[65,66] The EU-US Cooperative Group acknowledges the use of enriched MC (FACS-sorted or microdissected)[33,35,67] as a valuable tool in selected SM cases with low levels of infiltration by immunophenotypically aberrant MCs that can be used in specialized centers but does not consider it as a universally recommended state-of-the-art procedure for molecular analysis in SM.

Detection of *KIT* mutations

Several nonquantitative (or semi-quantitative) techniques have been used in the past (and are still in use in some laboratories) to detect the *KIT* p.D816V mutation, including RT-PCR plus restriction fragment length polymorphism,[68] nested RT-PCR followed by D-HPLC of PCR amplicons,[42] or peptide nucleic acid–mediated PCR.[26,65] It is beyond the scope of this review to cover in detail each of these techniques, which are collectively characterized by a relatively low sensitivity and an increased frequency of false-negative results in cases presenting with low MC burden and MC-restricted *KIT* mutations. Their specific advantages and limitations are presented in Table IV, together with each of the other quantitative techniques discussed below. In the past decade, multiple studies have shown that the use of highly sensitive allele-specific quantitative PCR (ASO-qPCR) performed on DNA as proposed by Kristensen et al[70,72] can detect the presence of the *KIT* p.D816V mutation in virtually all typical adult (eg, ISM with skin lesions, SSM, and AdvSM) patients with SM, BM, and even PB.[26,39] More recently, a study that used ddPCR performed in PB and BM samples was conducted on different variants of SM, and compared the results of ddPCR to those of ASO-qPCR, showing that both techniques are equally sensitive.[66] Both techniques, ASO-qPCR and ddPCR, however, are restricted to the mutation *KIT* p.D816V. Although an ultrasensitive NGS protocol has been described to detect *KIT* p.D816V,[71] standard NGS assays widely used for the diagnostic workup of myeloid neoplasms typically show low sensitivity with a limit of detection under the best conditions of approximately 1% to 5% VAF, which is not sufficient to detect the *KIT* D816V mutation in a large fraction of adult patients with SM, and particularly of patients with ISM and bone marrow mastocytosis.

In the case of a negative test result for *KIT* p.D816V (5%-10% of SM cases), the EU-US Cooperative Group proposed several explanations: (a) the patient may have the *KIT* p.D816V mutation, but the mutation burden is below the detection limit of the method (false-negative), (b) the patient has no *KIT* mutation (*KIT* wild-type), or (c) the patient has mutations other than p.D816V, either at codon 816 or another codon of *KIT*, that are not detected by the ASO-qPCR/ddPCR method.

Based on all the above, and the state-of-the-art knowledge and collective experience, the WP4 discussions led to the EU-US Cooperative Group recommendation that in case of suspicion of SM, a highly sensitive method such as ASO-qPCR or ddPCR

TABLE I. Overview of the frequency of the different *KIT* mutants found in adult SM and in pediatric CM cases

KIT mutant	Frequency in adult SM	Frequency in pediatric CM	Affected exon	Position in the receptor
Y269C	<3%	<3%	5	
E414D	<3%	<3%		
Del417-419insF	<3%	<3%		
Del417-419insI	<3%	<3%		
Del417-419insNA	<3%	<3%	8	
Del417-419insY	<3%	<3%		
Del419	<3%	17%*		
InsFF419	<3%	<3%		
C443Y	<3%	<3%		ECD
S451C	<3%	<3%*		
S476I	<3%	<3%		
ITD501-502	<3%	<3%		
501_502InsAF	<3%	<3%		
ITD502-503	<3%	±5%	9	
503_504insAY	<3%	<3%		
ITD504	<3%	<3%		
ITD505-508	<3%	<3%		
K509I	<3%	<3%*		
Q515H	<3%	<3%		
F522C	<3%	<3%*		TMD
A533D	<3%	<3%*	10	
V540L	<3%	<3%		
M541L	<3%	<3%		
K550N	<3%	<3%		
W557R	<3%	<3%		JMD
V559A	<3%	<3%*		
V559I	<3%	<3%		
Del559-560	<3%	<3%*	11	
V560G	<3%	<3%		
Del564-576	<3%	<3%		
D572A	<3%	<3%		
L576P	<3%	<3%*		KD1
R634W	<3%	<3%*		KID (ATP-binding site)
K642E	<3%	<3%	13	
V654A	<3%	<3%		
L799F	<3%	<3%		
InsV815-816	<3%	<3%		
D816A	<3%	<3%		
D816F	<3%	<3%	17	
D816H	<3%	<3%		
D816I	<3%	<3%		
D816V	>80% (>90% for ISM)	±30%		KD2 (phosphotransferase domain)
D816Y	<3%	<3%		
I817V	<3%	<3%		
N819Y	<3%	<3%		
D820G	<3%	<3%		
N822I	<3%	<3%*		
N822K	<3%	<3%		
N822Y	<3%	<3%*		
M835K	<3%	<3%*		
E839K	< 3%	<3%	18	
S840N	<3%	<3%		C-terminal trail
S849I	<3%	<3%*		
E885D	<3%	<3%	19	

CM, Cutaneous mastocytosis; *Dup*, duplication; *Ins*, insertion; *ITD*, internal tandem duplication; *JMD*, juxtamembrane domain; *KD*, kinase domain; *KID*, kinase interdomain; *TMD*, transmembrane domain.

**KIT* mutant found to be germline in familial cases of CM.

TABLE II. Distribution of *KIT* mutations within different categories of SM

Categories of disease	Type and frequency of *KIT* mutations
ISM	• ~94% *KIT* p.D816V • A few patients are *KIT* wild-type or may present noncodon 816 *KIT* mutations
SSM	• >90% *KIT* p.D816V
ASM	• >90% *KIT* p.D816V • A few patients may present noncodon 816 *KIT* mutations
SM-AHN	• >90% *KIT* p.D816V • The AHN may present its own recurrent genetic lesion (eg, *BCR/ABL* and *JAK2* V617F)
MCL	• *KIT* D816V mutant is slightly less frequent than in ISM, but other mutations at codon 816, such as D816H or D816Y, have been repeatedly reported. In total, the frequency of mutations at codon 816 is ~90% in MCL • Several MCL cases have been reported to present *KIT* mutations in ECD or JMD or no *KIT* mutations
MC sarcoma	• Only 21% of cases with *KIT* p.D816V mutation described to date • Other noncodon 816 *KIT* mutations can be found

JAK2, Janus kinase 2; *JMD*, juxtamembrane domain.

TABLE III. Different risk-scoring systems for prognostic stratification of SM that include the *KIT* D816V and/or additional genetic alterations among other parameters

Name of the scoring system and reference	MAPS (Mayo Alliance Prognostic System)[56]	REMA score (Spanish Network for Mastocytosis)[40]	REMA score (Spanish Network for Mastocytosis)[40]	MARS (Mutation-Adjusted Risk Score)[62]	GPSM (Global Prognostic score for Systemic Mastocytosis)[63]
Category of SM concerned	All	ISM only	ISM only	AdvSM only	All
End point	OS	PFS	OS	LFS, OS	OS
WHO class of SM	—	—	—	+	—
Age	+ (≥60 y)	—	—	+ (≥60 y)	—
Anemia	+ (≤110 g/L)	—	—	+ (≤100 g/L)	+ (≤110 g/L)
Thrombocytopenia	+ (<100 × 10^9 /L)	—	—	+ (<150 × 10^9/L)	+ (≤100 × 10^9/L)
Serum β2m	+ (≥2.5 μg/mL)	—	—	—	+ (≥2.5 μg/mL)
Serum ALP	—	—	—	+ (>ULN)	+ (≥140 IU/L)
KIT p.D816V mutation in BM	—	+ (VAF ≥ 1%)	—	—	—
Non-*KIT* mutations (NGS)	+ (*A/R/NRAS*)	+ *A/R/D* (VAF ≥30%)	+ *A/R/D* (VAF ≥30%)	+ (*S/A/R*)	+ (*S/A/R/D*)

A, *ASXL1*; *ALP*, alkaline phosphatase; *β2m*, β2 microglobulin; *D*, *DNMT3A*; *IU*, international unit; *LFS*, leukemia-free survival; *PFS*, progression-free survival; *R*, *RUNX1*; *S*, *SRSF2*; *ULN*, upper limit of normal; *WHO*, World Health Organization.
Adapted from Martelli et al.[64]

should be used for the detection of the *KIT* p.D816V mutant in PB, in combination with BST levels measurement and/or informative hematological and biochemical parameters. A negative result in PB does not (completely) exclude the presence of a *KIT* mutation and SM. Therefore, the analysis should be repeated on high-quality (undiluted) BM samples in patients with bone marrow mastocytosis and other forms of SM, if strong clinical/biological evidence pleading for the diagnosis of SM exists—in line with previously published recommendations of the ECNM.[3] In addition, a negative test result for *KIT* p.D816V, if performed with the most sensitive technique in cases with significant BM MC infiltration, should be followed by additional molecular analysis to rule out *KIT* mutations other than p.D816V. Peptide nucleic acid–mediated PCR for detection of other mutations at codon 816 (and its neighbor codons) may be considered. However, sequencing of the complete coding sequence of *KIT* is recommended to detect or rule out rare mutations outside of this hotspot in exon 17, as is the case in well-differentiated SM.[73] When *KIT* p.D816V has already been excluded by a sensitive molecular method, the EU-US Cooperative Group proposes to apply standard NGS protocols available in most centers while being aware of the limitations of these protocols (inability to detect currently *KIT* mutant at a VAF ≤1%-5%).

Quantification of *KIT* p.D816V mutant allele burden

A number of techniques used for the detection of *KIT* p.D816V are also suitable to quantify the *KIT* mutation VAF. In most studies, ASO-qPCR was used for quantification of the *KIT* p.D816V VAF.[41] Greiner et al[66] validated ddPCR and found a

TABLE IV. Advantages, limitations, and sensitivity of the different tests available to detect *KIT* mutations at codon 816

Technique	RT-PCR plus restriction fragment length polymorphism (RFLP)	Nested RT-PCR followed by D-HPLC of PCR amplicons	PNA-mediated PCR (PNA-PCR)	ASO-qPCR on DNA or RNA/cDNA	ddPCR	NGS
Advantages	- Simple - Fast - Reliable - Cost-saving	- Detects different *KIT* mutations at position 816	- Allows detection of *KIT* mutations at position 816 or at adjacent positions - Recommended for FFPE tissues	- Simple-fast-cost-saving-highly sensitive test - Quantitative: allows the quantification of the *KIT* p.D816V allele burden in blood or BM at diagnosis or in the follow-up	- Simple-fast-cost-saving-highly sensitive test - Quantitative: allows the quantification of the *KIT* p.D816V allele burden in blood or BM at diagnosis or in the follow-up - Works well on FFPE tissues	- Full *KIT* codons analysis - Quantitative results - Allows detection of non-*KIT* mutations
Disadvantages	- Detects only *KIT* D816V mutant - Not quantitative	- Relatively low sensitivity - Not quantitative - Time-consuming - Needs special facilities (HPLC)	- Not quantitative - Intermediate sensitivity	- Detects only *KIT* p.D816V mutant - Needs standardization, validation, and interlaboratory harmonization	- Detects only *KIT* p.D816V mutant - Needs standardization, validation, and interlaboratory harmonization	- Relatively high cost - Relatively time-consuming - Low sensitivity
Sensitivity	~0.05 %	0.5%-1.0%	~0.1%	~0.01%	~0.01%	~0.2% with TU-NGS ~1%-5% with "classical" NGS
Reference	Feix et al[69]	Erben et al[42]	Sotlar et al[65]	Kristensen et al[70,71]	Greiner et al[66]	TU-NGS: Kristensen et al[71] Classical NGS: Reiter et al[61]

FFPE, Formalin-fixed paraffin-embedded; *PNA*, peptide nucleic acid; *TU*, targeted ultradeep.

high concordance with ASO-qPCR without systematic deviation of results. The faculty considers both methods suitable for quantification of *KIT* p.D816V mutant allele burden from DNA. ddPCR might have an advantage for interlaboratory standardization because it does not rely on an internal calibrator material.[66] In contrast, mRNA-based analysis of the EAB is considered as a different biomarker that is not interchangeable with DNA-based results.[42] Particularly, Naumann et al[74] have recently demonstrated on a cohort of 161 patients (ISM, n = 40; AdvSM, n = 121) that if *KIT* p.D816V VAF and EAB strongly correlated in patients with ISM, the same cannot be said for patients with AdvSM. Interestingly, the same authors identified an EAB/VAF ratio of more than 2 as predictive for an advanced phenotype and a significantly inferior OS, suggesting that the transcriptional activity of *KIT* p.D816V may play an important role in the pathophysiology of SM.[74] Thereby, differences between methodologies and specimens need to be considered when interpreting the mutant allele burden.[45]

To monitor the *KIT* p.D816V VAF, the EU-US Cooperative Group considers that the frequency of measurement depends on the stage or type of mastocytosis. For instance, among patients with ISM, a VAF measurement has to be performed at the time of diagnosis, but follow-up measurements might be required only if there are signs of disease progression. In more advanced SM cases, including patients (more frequently) enrolled in clinical studies and therapeutic trials, the EU-US Cooperative Group considers that the measurement of the *KIT* p.D816V VAF should be repeated, particularly in case of (1) patients with AdvSM undergoing targeted therapy to monitor the efficacy of the treatment and (2) patients with ISM and SSM who show signs of disease progression.

NGS-based analysis of non-*KIT* mutations and cytogenetic analyses

NGS is the preferred technique for detection of somatic mutations in genes other than *KIT*, which are recurrently altered in SM. The development and routine use of NGS in patients with SM has allowed investigators to better define the relationship between the presence of additional molecular abnormalities and the clinical heterogeneity of the different subtypes of SM.[75] In this regard, the EU-US Cooperative Group reached consensus recommendations during the 2020 WoCo on the combined use of highly sensitive quantitative techniques for the detection and quantification of the *KIT* p.D816V mutant and of NGS to characterize additional non-*KIT* mutations. This combined use allows the categorization of patients with SM into 3 categories: (a) patients for whom the *KIT* p.D816V mutation is limited to the MC compartment, which is the case for most ISM cases with medium- to long-term stable disease; (b) patients with multilineage involvement of hematopoiesis by the *KIT* p.D816V mutation, as demonstrated in patients with AdvSM, patients with SSM, and a fraction of patients with ISM more prone to undergo progression of the disease; and (c) a third category of patients with additional mutations in genes other than *KIT*, including a small fraction of patients with ISM and most patients with AdvSM.

Additional issues that are critical for optimal patient care and treatment of SM include, among other, technical standardization and improved laboratory practices at the technical level. Regarding standardization, a question remains about the type of patients who may benefit from NGS testing for a panel of

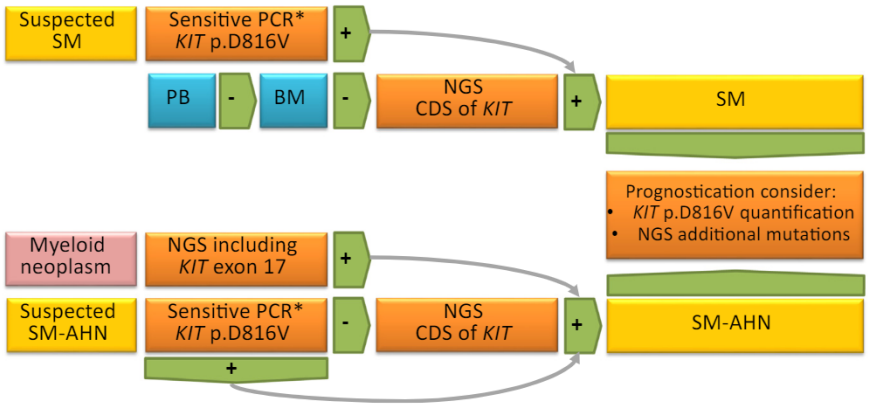

FIGURE 3. Diagnostic molecular algorithms in mastocytosis. In cases with suspected SM (upper panel), a sensitive PCR-based analysis is recommended to screen for the presence of *KIT* p.D816V. The LOD of the assay shall be less than or equal to 0.01% VAF as has been shown to be consistently reached by both ASO-qPCR and ddPCR. PB can be used for screening, but the analysis shall be repeated in BM in negative cases with high likelihood of SM. Subsequently, the CDS of *KIT* shall be analyzed by NGS from BM in cases confirmed to be negative for *KIT* p.D816V. In cases with myeloid neoplasms (lower panel), NGS assays including exon 17 of *KIT* can be used but are not sufficient to rule out SM-AHN due to the typically low sensitivity (high LOD) of the assay. Thus, sensitive PCR-based analysis for *KIT* p.D816V (potentially followed by NGS analysis of the CDS of *KIT* in cases negative for D816V) is recommended additionally for cases with suspected SM-AHN. In addition, the quantification of the *KIT* p.D816V VAF and the NGS-based analysis for additional non-*KIT* mutations are recommended for prognostication both in cases with SM-AHN and in SM in general. *CDS*, Complete coding sequence; *LOD*, limit of detection. *LOD ≤0.01% VAF, for example, ASO-qPCR or ddPCR.

myeloid-neoplasm–associated mutated genes. In this regard, consensus existed in that all patients with AdvSM are candidates for such test, whereas consensus was not totally reached for patients with ISM. However, with the generalization of such testing leading to decreased costs per test, the EU-US Cooperative Group strongly recommends that such analysis become standard for all patients with SM in the near future, or at least for those presenting with an increased VAF (>2%) of *KIT* p.D816V. Another question that remains a matter of discussion relates to the minimal set of genes to be tested. In this regard, consensus was reached on the need for routine testing for at least the *ASXL1*, *SRSF2*, *RUNX1*, *DNMT3A*, and *NRAS* gene mutations, given their strong adverse impact on the outcome of the disease. However, because of the generalization of NGS testing for larger panels of genes and even whole-exome sequencing, the EU-US Cooperative Group strongly recommends the use of extended panels that include additional genes recurrently mutated in myeloid neoplasms, such as *JAK2* and others, for an adequate workup for correct diagnosis of AdvSM.[76] Of note, it has to be emphasized that most of the genetic testing, including NGS and *KIT* mutation, studies are now-a-days performed in routine hematology centers, and that all patients diagnosed with SM should be able to be explored by combining these 2 types of molecular analyzes. Finally, because it has been reported that in patients with AdvSM, and particularly in patients with SM-AHN, cytogenetic aberrations are found in a significant proportion of patients and impact also the prognosis, particularly in case of poor-risk karyotype,[77,78] the EU-US Cooperative Group strongly recommends to perform a karyotype analysis in all patients with AdvSM.

CONCLUDING REMARKS AND PERSPECTIVES

The pathogenesis of SM is strongly associated with the presence of activating mutations of *KIT*, the *KIT* p.D816V mutation being by far the most frequent *KIT* mutant found in patients with SM. In recent years, new techniques such as ASO-qPCR or ddPCR have been developed that allow highly sensitive detection and quantification of *KIT* p.D816V. Such techniques have been now validated in multiple studies and are thus strongly recommended as the reference techniques for the diagnostic workup of patients with SM by the EU-US Cooperative Group based on the discussions and consensus reached during the 2020 WoCo held in Vienna. In line with what had been previously recommended by the ECNM,[3] it can be combined with the measurement of BST levels to screen in PB for the presence of SM before a BM study in suspicious patients (Figure 3). In addition to its diagnostic utility, several groups have reported that the determination of the *KIT* p.D816V VAF can also be used for prognostication. Indeed, relationships have been demonstrated between the *KIT* p.D816V VAF, the type of SM, and patient outcome and survival.[42,44] Furthermore, these new highly sensitive molecular techniques are equally suited for the follow-up of patients and for monitoring cytoreductive therapies, in particular for patients with AdvSM treated with KIT-targeting TK inhibitor.[48] In addition, analysis of non-*KIT* somatic mutations based on NGS panels of genes recurrently mutated in SM and other myeloid neoplasms is strongly recommended, at least for patients with AdvSM, including serial monitoring of patients on KIT inhibitors.[49-53,55] Furthermore, given recently published data,[78,79] a karyotypic analysis is recommended for those patients with AdvSM.

Finally, with regard to standardization of techniques, both internal and external quality controls are of utmost relevance. Regarding *KIT* p.D81V, there is 1 external quality control available, the UK-NEQAS program, which consists in 3 sets of samples per year and where *KIT* p.D816V VAF measurement is included. The EU-US Cooperative Group recommends thus to all the laboratories where *KIT* p.D816V VAF is measured not

only to establish solid internal quality controls but also to use such type of external quality controls.

Summary points

- There is still a need of harmonization of the technique(s) used for the detection/quantification of the *KIT* p.D816V mutant. However, the EU-US Cooperative Group strongly recommends using new quantitative and sensitive techniques such as ASO-qPCR or ddPCR for such purpose.
- Together with *KIT* mutation analysis, NGS has allowed to better define the relationships between the presence of additional mutations and the clinical heterogeneity of SM.
- This combination has recently allowed to divide patients with SM into 3 categories: (1) most patients with ISM will not progress and only their MC compartment harbors the *KIT* p.D816V mutation, (2) most patients with pure ASM and some patients with ISM or SSM present with a multilineage involvement by *KIT* p.D816V and may progress, and (3) patients with advanced forms of SM, more often those with an AHN (SM-AHN), harbor not only the *KIT* p.D816V mutation but also other acquired genetic alteration(s) that condition their prognosis.
- Following these advances in the molecular understanding of SM, several hybrid molecular/clinical prognostic scoring have been published. However, despite being promising, there is still a need to improve their stratification capabilities.

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