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ORIGINAL ARTICLE

*KIT*D816V mutation in blood for the diagnostic screening of systemic mastocytosis and mast cell activation syndromes

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Abstract

Background: Current diagnostic algorithms for systemic mastocytosis (SM) rely on the detection of *KIT*D816V in blood to trigger subsequent bone marrow (BM) investigations.

Methods: Here, we correlated the *KIT*D816V mutational status of paired blood and BM samples from 368 adults diagnosed with mast cell activation syndrome (MCAS) and mastocytosis and determined the potential utility of investigating *KIT*D816V in genomic DNA from blood-purified myeloid cell populations to increase diagnostic sensitivity. In a subset of 69 patients, we further evaluated the kinetics of the *KIT*D816V cell burden during follow-up and its association with disease outcome.

Results: Our results showed a high correlation ($P < .0001$) between the *KIT*D816V mutation burden in blood and BM (74% concordant samples), but with a lower mean of *KIT*D816V-mutated cells in blood ($P = .0004$) and a high rate of discordant $BM^+/blood^-$ samples particularly among clonal MCAS (73%) and BM mastocytosis (51%), but also in cutaneous mastocytosis (9%), indolent SM (15%), and well-differentiated variants of indolent SM (7%). Purification of different compartments of blood-derived myeloid cells was done in 28 patients who were BM mast cell $(MC)^+/blood^-$ for *KIT*D816V, revealing *KIT*D816V-mutated eosinophils (56%), basophils (25%), neutrophils (29%), and/or monocytes (31%) in most (61%) patients. Prognostically, the presence of ≥3.5% *KIT*D816V-mutated cells ($P < .0001$) and an unstable *KIT*D816V mutation cell burden ($P < .0001$) in blood and/or BM were both associated with a significantly shortened progression-free survival (PFS).

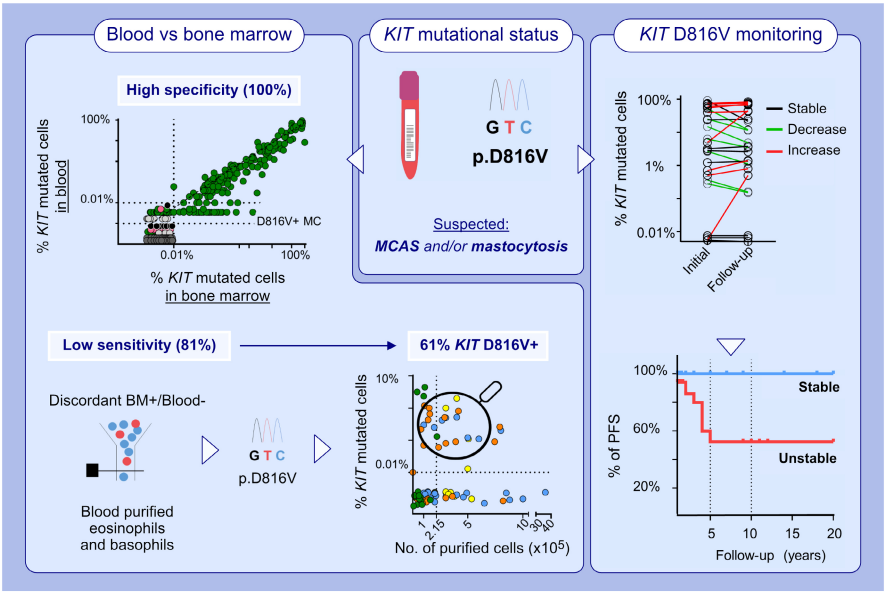
Conclusions: These results confirm the high specificity but limited sensitivity of *KIT*D816V analysis in whole blood for the diagnostic screening of SM and other

Abbreviations: BM, bone marrow; KITD816V, somatic mutation in codon 816 of the stem cell growth factor receptor gene; MC, mast cell; MCAS, mast cell activation syndromes; No., number; PFS, progression-free survival.

María Jara-Acevedo and Alberto Orfao contributed equally to this work and should be both considered as last authors.

primary MCAS, which might be overcome by assessing the mutation in blood-purified myeloid cell populations.

KEYWORDS
basophils, eosinophils, *KIT*D816V, Mastocytosis, MCAS (mast cell activation syndrome)



GRAPHICAL ABSTRACT

This study evaluates the utility of *KIT*D816V mutational status in blood (vs. bone marrow) for the diagnostic screening of SM and MCAS patients, having a high specificity but a limited sensitivity in patients presenting with clonal MCAS without skin lesions. Assessment of *KIT*D816V in blood-purified myeloid cell populations increases the diagnostic sensitivity of this mutation in 61% of patients. Further, an unstable (decrease or increase) *KIT* mutation cell burden during follow-up is a strong predictor of shortened progression-free survival in SM.

1 | INTRODUCTION

Mastocytosis is a clonal hematopoietic stem cell disease that might present both in children and adults with a broad spectrum of clinical manifestations due to the accumulation of variable numbers of clonal mast cells (MC) in the skin, bone marrow (BM) and/or other tissues, and/or the constitutive (or triggered) activation of MC with an increased release of MC mediators.[1–3]

At present, it is well-established that most children with mastocytosis have cutaneous mastocytosis (CM). By contrast, the great majority of adults have systemic mastocytosis (SM), whose diagnosis requires BM investigations as per the recent 2022 World Health Organization (WHO) and International Consensus Classification (ICC) criteria.[4,5] Because most children presenting with mastocytosis in the skin (MIS) have CM, consensus exists in that they should only undergo BM analysis when high suspicion of SM (e.g., underlying organomegaly, altered blood cell counts, persistently elevated tryptase levels and/or severe MC mediator-related symptoms, and extensive skin involvement) and/or signs of disease progression through adulthood exist,[6] which might delay for years the diagnosis of SM in some patients.[7,8] Conversely, most adults presenting with MIS have SM, and thereby, they should undergo BM examination.[2]

By contrast, only a small fraction (<10%) of adults presenting with an MC activation syndrome (MCAS) without skin lesions show BM involvement by clonal MC and SM—for example, typically BM mastocytosis (BMM).[9] For those latter cases, diagnostic algorithms such as the REMA (Spanish Network on Mastocytosis)[10] and NICAS (National Institute for Health and Care Excellence)[11] scores have been proposed to identify patients at higher risk of SM who should undergo BM analysis. In line with recent recommendations by the ECNM-AIM (EU-USA)[12,13] consortium, in the NICAS score, demonstration of a somatic mutation in codon 816 of the stem cell growth factor receptor gene (*KIT*D816V) in whole blood genomic DNA (gDNA) has been proposed as a strong predictor for BM involvement and SM.[14–16]

*KIT*D816V is present in BM of the great majority (>90%) of adult SM patients.[17] Thus, *KIT*D816V has emerged as a robust diagnostic criterion for SM.[18,19] The availability of high-sensitive real-time allele-specific oligonucleotide-qPCR (ASO-qPCR)[18] and digital PCR (dPCR)[20] methods has demonstrated the *KIT*D816V mutation also in blood of the majority of SM patients, including advanced forms of SM (AdvSM), indolent SM (ISM), and to a lesser extent BMM patients.[21,22] However, few studies have investigated the *KIT*D816V mutation in paired BM and blood samples from large series of

children and adults diagnosed with mastocytosis.[14–16,22] Of note, some of these studies have consistently shown that positivity for *KIT*D816V in blood is a surrogate marker of systemic (e.g., BM) involvement in mastocytosis,[16,21,22] the number of mutated cells—e.g., the *KIT*D816V variant allele frequency (VAF)—correlating with the diagnostic subtype of SM[16,23,24] and disease outcome.[16] Those studies also revealed that despite blood positivity for *KIT*D816V is a highly accurate predictor for BM involvement in AdvSM, its sensitivity is clearly lower among ISM and BMM patients.[22] Despite this, no study has yet determined the accuracy of blood assessment of *KIT*D816V in the diagnostic screening of patients suspicious of SM who present with MIS and/or MCAS,[25] and the potential benefit of assessing *KIT*D816V in blood-purified populations of myeloid cells other than circulating MC (which are detected only in around half of SM patients).[26]

Here, we used a high-sensitive molecular approach[18] to determine the *KIT*D816V mutational status in paired blood and BM samples from a series of 368 adults with distinct diagnostic subtypes of MCAS and mastocytosis and the potential utility of investigating *KIT*D816V in gDNA from blood-purified myeloid cell populations vs. whole blood and/or whole BM to increase the diagnostic sensitivity. In a subset of 69 patients, we further evaluated the kinetics of the *KIT*D816V-mutated cell burden during follow-up and its association with disease outcome.

2 | METHODS

2.1 | Patients and samples

A total of 368 patients—168 women and 200 men; median age of 50 years (y), ranging from 11y to 83y—who were referred to the two reference centers of the REMA (Institute of Mastocytosis Studies of Castilla La Mancha (CLMast), Toledo; and Cancer Research Centre, Salamanca, Spain) between July 2008 and July 2021 with a diagnosis of MCAS and MIS, and suspicion of mastocytosis, were (prospectively and retrospectively) studied with exactly the same standardized molecular, immunophenotypic, and cell purification techniques, for improved consistency.[22,26,27] Prior to their enrolment in the study, each patient had given his/her written informed consent to participate according to the Declaration of Helsinki; the study was approved by the local Ethics Committees of the two participating centers (approval codes: PI2021_05_791 and PI2021_07_863).

According to the final diagnosis, retrospectively updated by the WHO/ICC 2022 criteria,[4,5] patients were distributed as follows: nonclonal MCAS (nc-MCAS), 100 patients; clonal MCAS patients not fulfilling the diagnostic criteria for mastocytosis (c-MCAS), 11; CM, 11; SM, 246 patients including 86 BMM, 102 ISM, 7 smoldering SM (SSM), and 37 AdvSM patients—12 aggressive SM (ASM) and 25 SM with an associated hematological neoplasm (SM-AHN). For this later group, the prevalence of the *KIT*D816V mutation in the AHN component was 84%. The remaining 14 SM patients were diagnosed with well-differentiated variant of ISM (ISM^{WD}). Among all diagnostic subtypes, 118 patients were negative for the mutation (including the 100 nc-MCAS patients), 230 cases had *KIT*D816V (62%), 16 patients (4%) showed mutations other than D816V—8 BMM (2 *KIT*M541L, *KIT*D816T, 4 *KIT*D816H, *KIT*D816A), 1 c-MCAS (*KIT*D816Y), 1 CM (*KIT*R815G), and 6 ISM^{WD} (2 *KIT*M541L, *KIT*K509I, *KIT*D419del and 2 *KIT*V532I)—and 4 patients had SM without a *KIT* mutation (2 ISM and 2 SM-AHN).

More detailed clinical and laboratory features of those 220 SM patients who were positive for *KIT*D816V were retrospectively collected from the medical records, including: age, hemoglobin (Hb) levels, blood cell counts, serum baseline tryptase (sBT) and β2-microglobulin (β2M) levels, in addition to the patients' prognostic risk group as defined by the Global Prognostic Score for Mastocytosis (GPSM)[28] (low-risk, 163 patients; intermediate-risk, 32; and high-risk, 25 cases) and the International Prognostic Score for AdvSM (AdvIPSM)[29] (low-risk, 172 patients; intermediate-1, 34; intermediate-2, 12; and high-risk, 2 patients). Median follow-up at the moment of closing the study was of 24 months (range: 2–240 months). Twenty-two SM patients (10%) had undergone disease progression to a more advanced form of SM based on previous criteria.[26]

In every patient, EDTA-anticoagulated paired blood and BM samples were collected at diagnosis. In a subset of 69 SM patients who carried *KIT*D816V and did not receive cytoreductive therapies, additional sequential samples were obtained during follow-up (median: 48 months) for sequential analysis of the *KIT*D816V cell burden. Based on the comparison between the *KIT*D816V cell burden (expressed as percentage of mutated cells) at diagnosis and follow-up, SM patients were classified as having a stable vs. unstable clonal cell burden, when the percentage of *KIT*-mutated cells remained within ±20% of the initial value or it either decreased/increased below/above this cutoff value or decreased/ increased by 5% below/above the initial value for patients with >10% mutated cell at diagnosis.

2.2 | Purification of BM MC and different populations of blood myeloid cells

Purification of BM MC and distinct populations of blood-derived myeloid cells (e.g., eosinophils, basophils, neutrophils, and/or monocytes) was performed in a subgroup of 207 and 28 patients, respectively, the latter including only patients who showed undetectable *KIT*D816V in both whole blood gDNA and whole BM gDNA, with *KIT*D816V-positive purified BM MC. The EuroFlow bulk-lysis and stain-and-then-lyse standard operating procedure for staining of cell surface markers only (available at www.EuroFlow.org) was used prior to cell purification, as described elsewhere.[30,31] For this purpose, BM and blood cells were stained with 7-color combinations of fluorochrome-conjugated—fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), cyanine 5.5 (Cy5.5), PE-cyanine 7 (PE-Cy7), allophycocyanin (APC), APC hilite 7 (APC-H7), and Pacific Blue (PacB)—monoclonal antibodies, aimed at subsequent purification of BM MC (CD117, CD45, CD34, CD3, CD14, CD105, and CD13) and different populations of blood myeloid

cells (CD62L, CD3, CD45, CD16, CD14, CD24, and CD34), respectively. For flow cytometric data acquisition and cell purification, a 4-way FACSAria III fluorescence-activated cell-sorter (Becton/Dickinson Biosciences, San Jose, CA) was used. BM MC were identified as $CD117^{hi}$ $CD45^{+}$ $CD34^{-}$ $CD3^{-}$ $CD14^{-}$ $CD105^{-}$ $CD13^{-}$ cells, and blood neutrophils defined as sideward light scatter $(SSC)^{hi}$ $CD62L^{-}$ $CD3^{-}$ $CD45^{+}$ $CD16^{hi}$ $CD14^{-}$ $CD24^{-}$ $CD34^{-}$ cells, monocytes as SSC^{int} $CD62L^{-}$ $CD3^{-}$ $CD45^{hi}$ $CD16^{-}$ $CD14^{hi}$ $CD24^{-}$ $CD34^{-}$ cells, eosinophils as SSC^{hi} $CD62L^{-}$ $CD3^{-}$ $CD45^{+}$ $CD16^{-}$ $CD14^{-}$ $CD24^{hi}$ $CD34^{-}$ autofluorescent cells, and basophils as SSC^{lo} $CD62L^{hi}$ $CD3^{-}$ $CD45^{+}$ $CD16^{-}$ $CD14^{-}$ $CD24^{-}$ $CD34^{-}$ cells. The purity of each blood-derived cell population was systematically >98%, in the absence of cross-contamination by MC (≤0.001%). In the fractionated blood samples, the overall median frequency (range) of neutrophils, monocytes, eosinophils, and basophils was of 44.3% (33%–60%), 4.7% (3.3%–6.7%), 2.5% (0.12%–4.3%), and 0.9% (0.25%–1.5%), respectively. In 5 patients, all four FACS-sorted myeloid cell populations were simultaneously investigated.

2.3 | Analysis of *KIT*D816V and percentage of *KIT*-mutated cells

gDNA was obtained from whole blood and BM-derived nucleated cells, as well as from FACS-purified blood myeloid cells and BM MC, using the NucleoSpin Tissue XS Kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's protocol. gDNA from paired whole blood and BM samples, and the different FACS-purified populations of blood-derived myeloid cells, were analyzed for *KIT*D816V using an ASO-qPCR method described elsewhere.[18] A peptide nucleic acid-mediated PCR (PNA-PCR) method was used[19,32] to detect the mutation in gDNA from purified BM MC and for the detection of other mutations in the TK2 codons of *KIT*. The detection of other *KIT* mutations was performed by sanger sequencing. ASO-qPCR results were expressed as percentage of mutated cells, after applying the "delta–delta Ct" (ΔΔCt) method[33] based on a calibration curve built with the HMC-$1^{560+/816+}$ cell line as positive control, and multiplying the ΔΔCt value by 100 to derive percent values of affected cells per sample. In every case, VAF values corresponded to half of the percentage of mutated cells, due to the presence of the *KIT* mutation in heterozygosis.

2.4 | Statistical methods

Median (range) and mean (standard deviation) values were calculated for all continuous variables, while for categorical parameters frequencies were used. The degree of correlation between variables was assessed by the Pearson correlation coefficient (Rp) or Spearman's rank correlation coefficient (Rs). To calculate the statistical significance of differences observed between ≥2 groups of patients, the χ^2 or the Fisher tests (for categorical variables) and the parametric paired and un-paired T-tests or the nonparametric Mann–Whitney U and Wilcoxon tests (for continuous variables) were used, respectively. Progression-free survival (PFS) curves were plotted by the Kaplan–Meier method, and the log-rank test was used to assess the statistical significance of differences between PFS curves. Receiver operating characteristic (ROC) curve analysis was used to determine the most accurate cutoffs for PFS and blood-derived purified cell counts providing the highest sensitivity.

For all statistical analyses, the Statistical Package for Social Sciences (SPSS v.26; IBM, Armonk, NY) and the GraphPad Prism software (v.8; GraphPad Software Inc., San Diego, CA) were used. Statistical significance was set at *P*-values <.05.

3 | RESULTS

3.1 | *KIT* mutational status in whole blood vs. BM

All nc-MCAS patients were negative for *KIT*D816V or other *KIT* mutations and consequently considered hereon as the control group. Overall, the *KIT*D816V mutation was detected in BM of 230/268 (86%) c-MCAS and mastocytosis patients. *KIT*D816V-mutated patients in BM included most c-MCAS (8/11; 73%), and the vast majority of SM patients (220/232; 95%), but only 1/11 CM (9%) and 1/14 ISM^{WD} patients (7%) (Table 1). The single patient presenting with CM in whom the *KIT*D816V mutation was detected in purified BM MC did not fulfill the criteria for SM because only two minor diagnostic criteria for SM were present in this patient: aberrant CD25+ and *KIT*D816V-mutated BM MC. Despite in 199/268 (74%) c-MCAS plus mastocytosis patients, the *KIT*D816V mutation was detected in gDNA from both whole BM and purified BM MC, in 31/230 patients (13%) *KIT*D816V could only be detected in purified BM MC, while it was negative in whole BM gDNA. These included 8/11 (73%) c-MCAS, 1/11 (9%) CM, 1/14 ISM^{WD} (7%), and 21/232 (9%) patients with classical variants of SM (Table 1). Among SM patients, the number of cases that showed *KIT*D816V-positive (purified) BM MC in the absence of the mutation in whole BM gDNA progressively decreased from BMM (16/86; 19%) to ISM (4/102; 4%) and SSM plus AdvSM (1/44; 2%). Upon comparing the *KIT*D816V mutational status and cell burden (percentage of mutated cells) in blood vs. BM, we found a high degree of correlation ($R\rho = 0.9051$; $P < .0001$; Figure 1A) and concordance (74%; Table 1) between both types of samples. These included 10/11 *KIT*D816V-negative CM (91%), 13/14 *KIT*D816V-negative ISM^{WD} (93%), and 159/232(69%) *KIT*D816V-positive SM cases, but only 3/11 *KIT*-negative c-MCAS (27%) and 12/232 *KIT*D816V-negative (5%) SM cases. Of note, higher median numbers of *KIT*D816V-mutated cells were found in BM vs. blood for SM patients (8% vs 6%, respectively; $P = .0004$; Supplementary Figure S1). More detailed analysis of the discordant (BM $KITD816V^{+}$ vs. blood $KITD816V^{-}$) SM cases (61/232; 26%) showed predominance of discordant cases among BMM (44/86; 51%) and to a lesser extent also ISM (15/102; 15%), with no or a few discrepant cases among SSM (0/7; 0%) and AdvSM (2/37; 5%) patients (Table 1; Figure 1B).

Further direct comparison between paired blood and BM samples of SM patients showed increasing median percentages of

TABLE 1 Prevalence of the *KIT*D816V mutation in blood and BM of patients with MCAS and mastocytosis distributed according to the diagnostic subtype of these diseases ($n = 268$)

		*KIT*D816V mutational status				
		Bone marrow				
		Purified BM MC/Whole BM				
Disease subtype	Blood	−/− ($n = 38$)	+/− ($n = 31$)	+/+ ($n = 199$)	% Discordant with blood	P
c-MCAS ($n = 11$)	−	3 (27%)	8 (73%)	0 (0%)	8/11 (73%)	.006
	+	0 (0%)	0 (0%)	0 (0%)		
CM ($n = 11$)	−	10 (91%)	1 (9%)	0 (0%)	1/11 (9%)	.09
	+	0 (0%)	0 (0%)	0 (0%)		
SM ($n = 232$)	−	12 (5%)	21 (9%)	40 (17%)	61/232 (26%)	<.0001
	+	0 (0%)	0 (0%)	159 (69%)		
BMM ($n = 86$)	−	8 (9%)	16 (19%)	28 (33%)	44/86 (51%)	<.0001
	+	0 (0%)	0 (0%)	34 (39%)		
ISM ($n = 102$)	−	2 (2%)	4 (4%)	11 (11%)	15/102 (15%)	<.0001
	+	0 (0%)	0 (0%)	85 (83%)		
SSM ($n = 7$)	−	0 (0%)	0 (0%)	0 (0%)	0/7 (0%)	NA
	+	0 (0%)	0 (0%)	7 (100%)		
AdvSM ($n = 37$)	−	2 (5%)	1 (3%)	1 (3%)	2/37 (5%)	<.0001
	+	0 (0%)	0 (0%)	33 (89%)		
ASM ($n = 12$)	−	0 (0%)	0 (0%)	0 (0%)	0/12 (0%)	NA
	+	0 (0%)	0 (0%)	12 (100%)		
SM-AHN ($n = 25$)	−	2 (8%)	1 (4%)	1 (4%)	2/25 (8%)	.0001
	+	0 (0%)	0 (0%)	21 (84%)		
ISM^{WD} ($n = 14$)	−	13 (93%)	1 (7%)	0 (0%)	1/14 (7%)	.07
	+	0 (0%)	0 (0%)	0 (0%)		
Total ($n = 268$)	−	38 (14%)	31 (12%)	40 (15%)	71/268 (26%)	<.0001
	+	0 (0%)	0 (0%)	159 (59%)		

Note: Results expressed as number of patients (percentage) from all patients in the group investigated or as number of patients/total patients (percentage).

Abbreviations: AdvSM, advanced systemic mastocytosis; BM, bone marrow; BMM, bone marrow mastocytosis; CM, cutaneous mastocytosis; c-MCAS, monoclonal mast cell activation syndrome not fulfilling the diagnostic criteria for mastocytosis; ISM, indolent systemic mastocytosis; ISM^{WD}, well-differentiated variant of ISM; MC, mast cell; NA, not appropriate; SM, systemic mastocytosis; SSM, smoldering systemic mastocytosis.

*KIT*D816V-mutated cells in both types of samples from BMM to ISM, ($P \leq .002$), from ISM and BMM to SSM ($P < .0001$), and to AdvSM patients ($P < .0001$). Notably, comparison between ISM and SSM patients showed clear differences, with median (range) percentage of *KIT*D816V-positive cells in PB of 1.5% (0–30.3) vs. 42% (0.69–92) ($P < .0001$) and in BM of 2.8% (0–38) vs. 44% (3.96–84.7) ($P < .0001$) (Supplementary Figure S1). Overall, this was associated with a high degree of correlation between the percentage of *KIT*D816V-mutated cells in BM vs. blood both when all SM patients ($R\rho = 0.9037$; $P < .0001$; Figure 1B), and only the major subset *KIT*D816V-positive patients ($R\rho = 0.9030$; $P < .0001$; Figure 1C) were considered. However, lower median values of mutated cells were also systematically found in blood vs. BM for virtually all diagnostic subtypes of SM: BMM, 0.04% vs 0.14% ($P < .0001$; $R\rho = 0.7225$; $P < .0001$); ISM, 1.5% vs 2.8% ($P < .0001$; $R\rho = 0.9143$; $P < .0001$); ASM, 17.6% vs 27% ($P = .003$; $R\rho = 0.8741$, $P = .0007$); and SM-AHN, 31% vs 39%($P = .006$; $R\rho = 0.8169$; $P < .0001$), except for SSM patients who had similar median percentages of *KIT*D816V-mutated cells in blood (42%) and BM (44%) ($P = .78$; $Rs = 0.7857$; $P = .048$) (Figure 1C; Supplementary Figure S1). Interestingly, a correlation existed between the percentage of *KIT*D816V-mutated cells in blood and BM, and sBT levels, (BM $R\rho = 0.3700$; blood $R\rho = 0.3470$; $P < .0001$; Supplementary Figure S2), even when the analysis was restricted to ISM ($R\rho = 0.4659$; $P < .0001$) and BMM ($R\rho = 0.3844$; $P = .0003$) patients.

3.2 | *KIT*D816V mutational status of different compartments of myeloid cells in blood

In a subset of 28 patients who showed *KIT*D816V-mutated BM MC, but in whom the mutation was not detected in gDNA from either whole blood or whole BM, we further investigated *KIT*D816V in

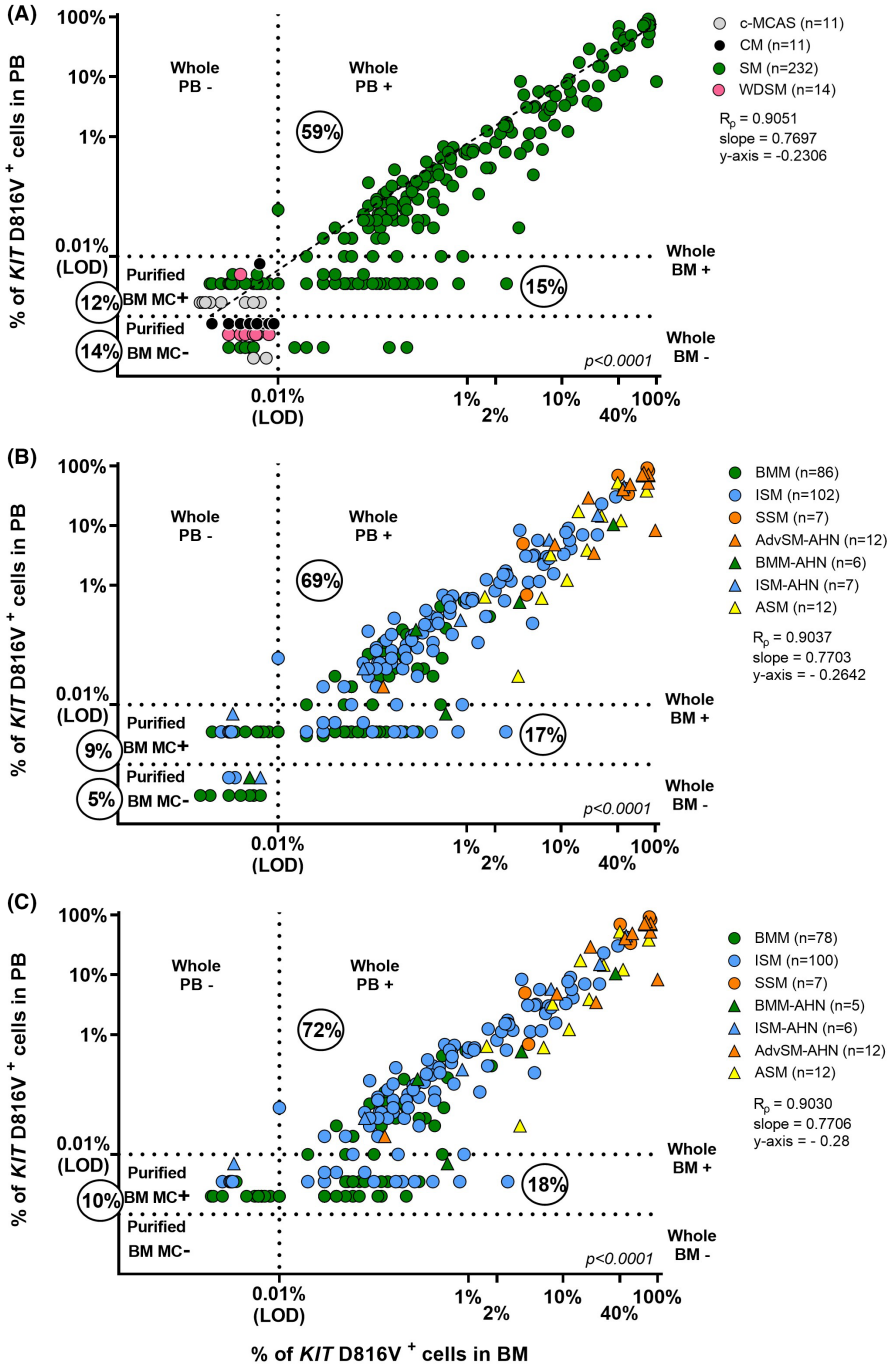


FIGURE 1 Correlation between the percentage of *KIT*D816V-mutated cells in blood vs. BM of MCAS and mastocytosis patients. In panel A, patients with nonclonal and clonal mast cell activation syndromes and mastocytosis ($n = 268$) are shown, whereas in panel B, only SM cases grouped according to the different diagnostic subtypes of SM are displayed ($n = 232$). In panel C, only those SM patients that showed the *KIT*D816V mutation are depicted ($n = 220$). The distribution of patients according to their *KIT*D816V mutational status in blood vs. BM is represented as percent values inside circles for cases falling in each quadrant of the plot.; AdvSM, advanced systemic mastocytosis; AHN, associated hematological neoplasm; BM, bone marrow; BMM, bone marrow mastocytosis; CM, cutaneous mastocytosis; c-MCAS, monoclonal mast cell activation syndrome not fulfilling criteria for mastocytosis; ISM, indolent systemic mastocytosis; ISM^{WD}, indolent systemic mastocytosis, well-differentiated variant. LOD, limit of detection; MC, mast cell; PB, peripheral blood; SM, systemic mastocytosis; SSM, smoldering systemic mastocytosis

highly purified blood-derived (median number of purified cells; range) neutrophils (345,000; 200,000-535,000), monocytes (330,000; 100,000-880,000), eosinophils (254,000; 1165-790,000), and basophils (72,000; 8000-220,000). Overall, 17/28 (61%) of these BM MC *KIT*D816V-positive but whole blood/BM *KIT*D816V-negative patients became positive for the mutation when gDNA from distinct blood-derived purified myeloid cell populations were investigated, with a median frequency (range) of *KIT*-mutated cells within the affected cell compartments of 0.70% (0.013% - 4.45%) (Table 2). Among the purified blood myeloid cells, eosinophils showed the highest rate of positivity for *KIT*D816V (56%) compared with neutrophils (29%), monocytes (31%) and basophils (25%) (Table 2). Despite this, among the four blood compartments of myeloid cells investigated, basophils showed the highest percentage of mutated cells among *KIT*D816V$^+$ purified cell samples: 2.52% (vs. 0.39% for eosinophils, $P = .01$) (Table 2). Such apparent discrepancy could be due to the more limited number of purified basophils (vs. eosinophils and other myeloid cells), as the rate of positivity for *KIT*D816V was significantly higher for samples containing ≥215,000 purified cells: 27% vs 50% ($P = .039$) (Table 2; Supplementary Figure S3). Of note, similar results were observed for those 5 patients in whom all four myeloid cell populations were simultaneously assessed (data not shown).

TABLE 2 Distribution of *KIT*D816V-mutated cells within different populations of myeloid cells purified from blood of SM patients with discordant results between purified BM MC gDNA (*KIT*D816V^{+}) and both whole blood and whole BM gDNA (*KIT*D816V^{-}) ($n = 28$)

	*KIT*D816V +				
			No of purified blood cells		
Cell type	No of patients	% of mutated cells	< 215,000	≥ 215,000	P
Neutrophils ($n = 14$)	4/14 (29%)	0.78% (0.013–2)	0/3 (0%)	4/11 (36%)	.51
Monocytes ($n = 26$)	8/26 (31%)	0.30% (0.11–0.52)	2/12 (17%)	6/14 (43%)	.21
Eosinophils ($n = 25$)	14/25 (56%)	*0.39% (0.01–1.18)	7/15 (47%)	7/10 (70%)	.41
Basophils ($n = 16$)	4/16 (25%)	*2.52 (0.13–4.45)	3/15 (20%)	1/1 (100%)	.25
P	.13	.01	.16	.29	
Total	17/28 (61%)	0.70% (0.013–4.45)	12/45 (27%)	18/36 (50%)	.039

Note: Results expressed as number of patients (percentage) from all patients in the group investigated or as median (range) percentage of KITD816V-mutated cells. In 5/28 patients, all four cell populations from the same PB sample were analyzed.

Abbreviations: BM, bone marrow; gDNA, genomic DNA; MC, mast cell; SM, systemic mastocytosis.

*Statistically significant differences found between these groups.

3.3 | Impact of the number of *KIT*D816V-mutated cells in blood and/or BM on the outcome of SM patients

ROC curve analysis identified the presence of ≥3.5% *KIT*D816V-mutated cells in blood and/or BM as the most accurate cutoff for risk stratification of SM patients at diagnosis (accuracy, 86% and 81%; specificity, 87.9% and 80.7%; and sensitivity, 68.4% and 84.2%, for blood and BM, respectively). This translated into the identification of patients at low-risk vs. high-risk of disease progression with median (95% confidence interval; CI) 10y PFS rates of 92%-(81%–97%) vs 48% (23%–69%) ($P < .0001$) and of 98% (91%–100%) vs. 43% (21%–63%) ($P < .0001$) for cases with a mutation burden below and above this cutoff in blood and/or BM, respectively (Figure 2A). Noteworthy, the 3.5% cutoff for *KIT*D816V-mutated cells also showed a discriminatory value for PFS when nonadvanced SM patients were separately considered ($P = .003$, Supplementary Figure S4). In addition, the percentage of *KIT*D816V-mutated cells significantly increased from low-risk to intermediate-risk and high-risk SM patients as defined by both the GPSM ($P < .0001$) and AdvIPSM ($P < .0001$) models (Figure 2B), at the same time it allowed an improved prognostic stratification of patients included in individual risk groups as defined by both scores (Figure 2C). Thus, decreasing median (95% CI) 10y-PFS rates were observed for (1) low-risk and intermediate-risk patients who presented <3.5% mutated cells in both blood and BM, compared with (2) SM patients who had low-risk and intermediate-risk disease but ≥3.5% mutated cells in blood and/or BM plus high-risk patients with <3.5% mutated cells, and particularly (3) high-risk patients ≥3.5 *KIT*D816V-mutated cells in blood or BM as defined by the GPSM with 98% (91%–100%) vs 62% (17%–88%) vs 29% (10%–51%), respectively ($P < .0001$) and the AdvIPSM with 98% (91%–100%) vs 53% (28%–73%) vs 0%, respectively ($P < .0001$) (Figure 2C).

3.4 | Sequential analysis of the number of *KIT*D816V-mutated cells

Sequential analysis of the number of *KIT*D816V-mutated cells showed a stable profile in 35/69 (51%) patients investigated. By contrast, an unstable (variable) number of *KIT*D816V-mutated cells, associated with either decreased or increased percentages of mutated cells, was observed in 6/69 (9%) and 28/69 (40%) patients, respectively (Figure 3A). Interestingly, a stable *KIT*D816V mutation burden was more frequently found among BMM (58%) and ISM patients (51%), compared with AdvSM cases (38%). However, half of patients with unstable (varying) percentages of mutated cells was still observed among patients within all diagnostic subtypes of SM (Table 3).

Once we compared the outcome of patients with stable vs. unstable tumor loads as defined by the percentage of *KIT*D816V-mutated cells, none of the patients who had a stable profile (0/35; 0%) (Table 3) had undergone disease progression at 5y and at 10y. By contrast, 9/34 (26%) of SM patients (Table 3) who had variable (decreasing or increasing) numbers of *KIT*-mutated cells showed disease progression in association with shortened 5y-PFS rates of 56.4% (95%CI: 30%–77%; $P = .005$) (Figure 3B). Similar differences were observed when we restricted the analysis to ISM and SSM patients: 5y-PFS rates of 43% (95%CI: 16%–68%) vs. 100% for patients with unstable vs. stable percentages of *KIT*D816V-mutated cells ($P = .030$) (Figure 3B).

4 | DISCUSSION

*KIT*D816V is a hallmark of adult SM, which is present at variable amounts in BM of ≃90% of patients,[22,34] as also confirmed here. Previous studies have repeatedly shown that *KIT*D816V can also be found in blood of the majority of SM patients.[18,21,22,35,36] Thus,

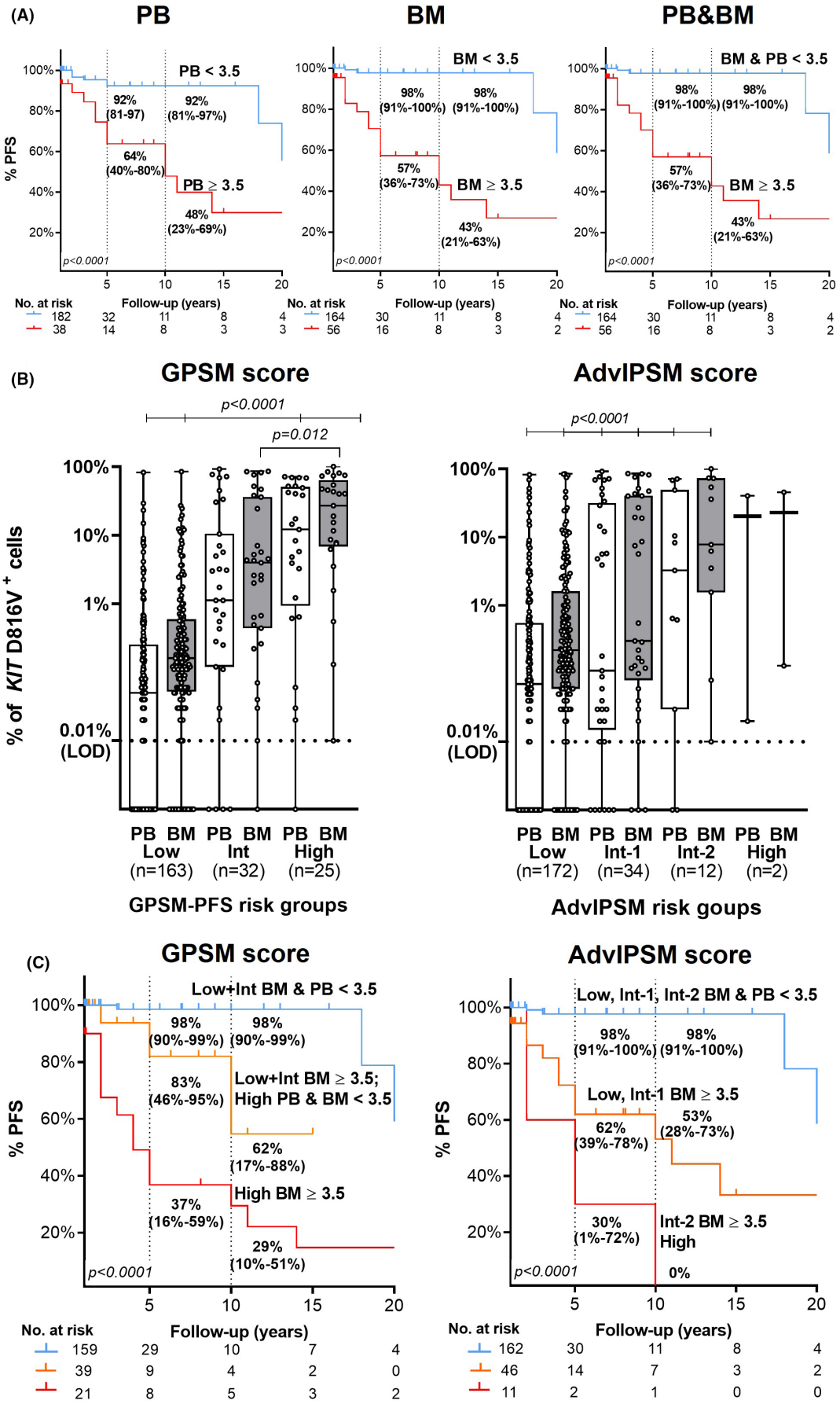


FIGURE 2 Relationship between the *KIT*D816V-mutated cell burden in blood and/or BM and the prognosis and outcome of SM patients. Panel A depicts the prognostic impact of the blood and/or BM *KIT*D816V cell burden on PFS of SM patients. In panel B, the blood and BM *KIT*D816V cell burden of SM patients grouped according to the GPSM and AdvIPSM models is shown, while panel C depicts the impact on PFS of SM patients, of the combination of the BM and blood *KIT*D816V cell burden and both the GPSM and AdvIPSM risk groups. For each patient group, 5-year and 10-year PFS rates (95% confidence interval) are shown as dotted vertical lines in panels A and C; while in panel B, boxes extend from the 25th to the 75th percentile values, while the line in the middle and vertical lines correspond to median values and the corresponding 5th and 95th percentiles, respectively. BM, bone marrow; GPSM, global prognostic score for systemic mastocytosis; Int, intermediate; IPSM, international prognostic scoring for systemic mastocytosis; PB, peripheral blood; PFS, progression-free survival

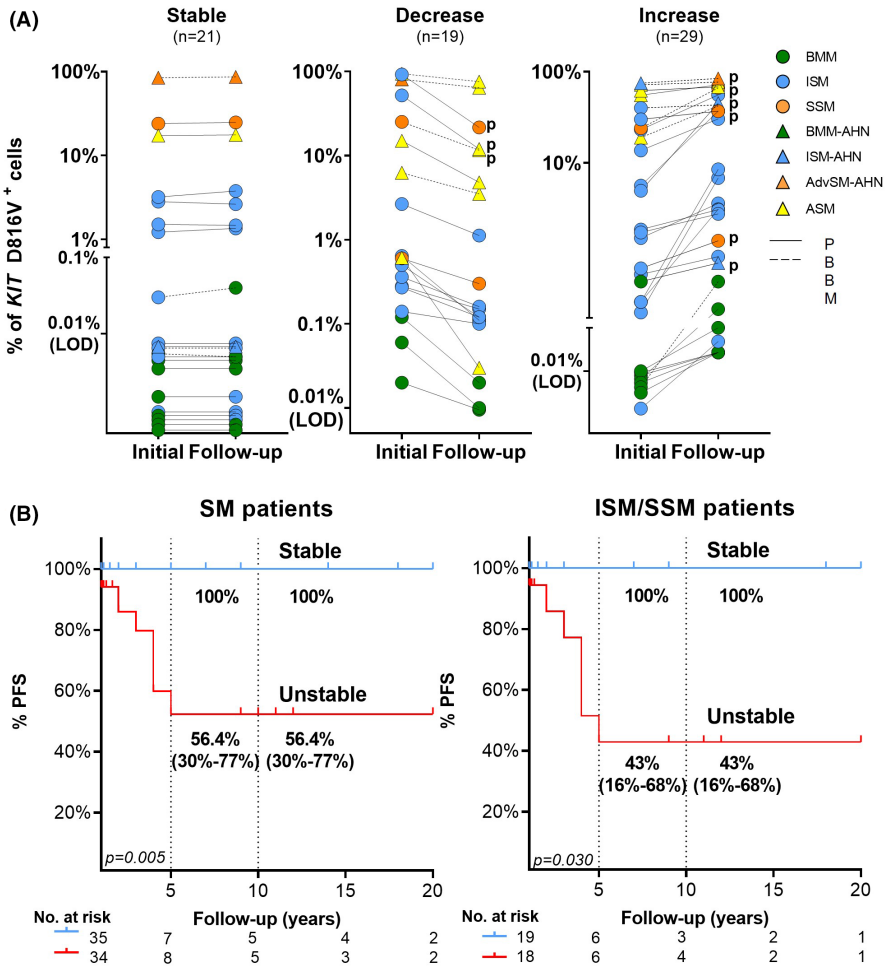


FIGURE 3 Sequential percentage of *KIT*D816V-mutated cells in SM patients ($n = 69$) studied at diagnosis and follow-up: impact on PFS. In panel A, patients are grouped according to its kinetics while panel B depicts the impact of a stable vs. unstable *KIT*D816V tumor cell burden on PFS of SM and ISM/SSM patients. In panel A, dotted lines depict BM samples ($n = 14$) while continuous lines depict blood samples ($n = 55$). Bold "p" depicts patients with an unstable *KIT*D816V cell load who progressed to more advanced forms of the disease. For each patient group, 5-year and 10-year PFS rates (95% confidence interval) are shown as dotted lines in panel B. AdvSM, advanced systemic mastocytosis; AHN, associated hematological neoplasm; BM, bone marrow; BMM, bone marrow mastocytosis; ISM, indolent systemic mastocytosis; PB, peripheral blood; SM, systemic mastocytosis; PFS, progression-free survival; SSM, smoldering systemic mastocytosis

high-sensitive molecular detection of this *KIT* mutation[18] in blood has been recently proposed to be a robust biomarker for BM involvement to be used in the diagnostic work-up of patients presenting with MIS and/or MCAS.[13] However, previous reports also indicate that some SM patients who carry *KIT*D816V restricted to BM MC (particularly those presenting with MCAS) might be negative for the mutation in blood, leading to false-negative results, and consequently, to delayed diagnosis or even misdiagnosis of the disease.[21,22]

Here, we used a high-sensitive molecular technique[18] to investigate *KIT*D816V in gDNA from paired whole blood and whole BM samples, in addition to gDNA from purified BM MC, from a large cohort of patients presenting with either MCAS or MIS, who were suspicious of having mastocytosis. Our results showed that despite an overall high concordance rate between the *KIT*D816V status in blood and BM, a major fraction of c-MCAS cases, and to a less extent also of BMM and ISM patients presenting with MCAS and/or MIS, who tested negative by ASO-qPCR for *KIT*D816V in whole blood and whole BM gDNA, still harbor the mutation at relatively high levels (above the >1% sensitivity limit of detection of the PNA-PCR method[19]) in purified BM MC. These findings reinforce the utility of purifying BM MC for further assessment of *KIT*D816V in patients with high suspicion of SM, as previously suggested.[35] Most interestingly, our data also showed that in the majority of c-MCAS and BMM patients, and in a significant fraction of ISM cases, together with a few CM and ISM^{WD} patients who harbored *KIT*D816V in whole BM and/or purified BM MC, this mutation might be undetected in whole blood gDNA by ASO-qPCR, probably due, at least in part, to the overall lower burden of *KIT*D816V-mutated cells in blood. Altogether, these findings support the notion that, while whole blood testing for *KIT*D816V using high-sensitive molecular assays is a highly accurate surrogate marker for BM involvement in AdvSM, SSM, and to a

TABLE 3 Relationship between the kinetics of the percentage of *KIT*D816V-mutated cells during follow-up and disease progression

	% *KIT*D816V + cells at follow-up		Disease progression		
Diagnostic subtype of SM	**Stable *KIT*-mutated cell burden**	**Unstable *KIT*-mutated cell burden**	**Stable *KIT*-mutated cell burden**	**Unstable *KIT*-mutated cell burden**	**P**
BMM ($n = 19$)	11/19 (58%)	8/19 (42%)	0/11 (0%)	*1/8 (12.5%)	.42
ISM/SSM ($n = 37$)	19/37 (51%)	18/37 (49%)	0/19 (0%)	*7/18 (39%)	.003
ISM ($n = 33$)	17/33 (51%)	16/33 (49%)	0/17 (0%)	6/16 (38%)	.005
SSM ($n = 4$)	2/4 (50%)	2/4 (50%)	0/2 (0%)	1/2 (50%)	1.00
AdvSM ($n = 13$)	5/13 (38%)	8/13 (62%)	0/5 (0%)	*1/8 (12.5%)	.41
ASM ($n = 10$)	3/10 (30%)	7/10 (70%)	0/0 (0%)	0/0 (0%)	NA
SM-AHN ($n = 3$)	2/3 (100%)	1/3 (0%)	0/2 (0%)	1/1 (100%)	.33
P	.76		–	.22	
Total	35/69 (51%)	34/69 (49%)	0/35 (0%)	9/34 (26%)	.001

Note: Results expressed as number of patients (percentage) from all patients in the group. * Disease progression for each diagnostic subtype involved transformation from BMM to ISM ($n = 1$), from SSM to ASM ($n = 1$), from ISM to ASM ($n = 2$), from ISM to ISM-AHN ($n = 1$), from ISM to SSM ($n = 3$), and from ISM-AHN to SSM + AHN ($n = 1$).

Abbreviations: AdvSM, advanced systemic mastocytosis; BMM, bone marrow mastocytosis; ISM, indolent systemic mastocytosis; NA, not appropriate; SSM, smoldering systemic mastocytosis.

less extent also in ISM, its diagnostic utility decreases substantially among patients presenting with a low burden of *KIT*D816V cells in blood (e.g., c-MCAS and BMM presenting without skin lesions) due to the high rate of false-negative results observed in whole blood vs. whole BM plus purified BM MC, as previously suggested by our[22] and other groups.[21] Altogether, this points out the need for a greater sensitivity of detection of the mutation in patients presenting with a very low burden of *KIT*D816V-mutated *cells*.[37]

Recent studies have found circulating clonal MC in blood of virtually all AdvSM patients and almost half of ISM patients, while they were absent in secondary/idiopathic nc-MCAS, CM and the great majority (>90%) of BMM patients.[26,38] Thus, the overall[32] frequency of ISM and BMM patients that show blood circulating clonal MC is far below that of patients that displayed here positivity for *KIT*D816V in blood by ASO-qPCR. These findings point out the potential existence in blood of SM patients of circulating *KIT*D816V cells other than MC probably due to multilineage involvement of hematopoiesis by *KIT*D816V.[19,27,36] Based on this hypothesis, in a subset of patients presenting with a very low tumor burden, as defined by an undetectable *KIT*D816V in both whole blood and whole BM gDNA together with the presence of this mutation in purified BM MC, we purified distinct myeloid cell populations from blood for subsequent investigation of *KIT*D816V by ASO-qPCR. Through this approach, in almost two-thirds of these patients who were *KIT*D816V-negative in whole blood and whole BM gDNA, we could confirm the presence of the *KIT*D816V in blood-purified neutrophils, monocytes, eosinophils, and/or basophils. Among these cell populations, eosinophils were the most frequently mutated cells, while basophils showed the highest percentages of *KIT*D816V$^+$ cells. Such apparent discrepancy might be due to the lower frequency of basophils in blood and, consequently, the lower number of purified basophils (vs. eosinophils and other purified myeloid cell types) per patient. In line with this hypothesis, we found that purification of higher (≥215,000) vs. lower (<215,000) numbers of blood-derived myeloid cells was associated with a higher rate of *KIT*D816V-positive cases. Altogether, these findings support previous *in vitro*[39] and *ex vivo*[40] studies, which suggest a close ontogenic relationship during hematopoiesis between *KIT*D816V MC and both basophils and eosinophils. From a practical point of view, our data also highlight the need for more sensitive blood assays for the detection of *KIT*D816V in patients suspicious of SM, particularly in those presenting with MCAS, suggesting this might be achieved through analysis of *KIT*D816V in enough numbers of highly purified blood eosinophils and/or basophils. For this purpose, the availability of fast, unexpensive, and user-friendly cell isolation procedures[41–45] is still lacking. In this regard, the use of classical dextran-based gradient density cell-enrichment methods should be re-evaluated for isolation of blood eosinophils, despite lower and variable levels of purity have been reported with these techniques[46] even when compared to MACS-purification methods.[47] Alternatively, immunomagnetic-based cell isolation approaches also need to be prospectively evaluated for purification of blood eosinophils and basophils in routine diagnostics for patients presenting with MCAS and suspected of having mastocytosis in routine diagnostics.

In line with previous observations,[24] the percentage of *KIT*D816V cells in blood and BM increased from BMM to ISM and both SSM and AdvSM patients. In this regard, a higher mutation burden in blood and/or BM was associated here with a poorer outcome (e.g., shorter PFS), also when we restricted the analysis to specific prognostic risk groups of SM, as defined by the GPSM[28] and the Adv-IPSM[29] prognostic models, particularly to low-risk and intermediate-risk patients. Overall, these data confirm and extend on previous observations, suggesting that a *KIT*D816V VAF of >1%[35] or >2%,[24] vs. >1.75% (>3.5% mutated cells) as found here, is a reliable prognostic factor[27,48,49] which can even refine prognostic stratification of low-risk and intermediate-risk SM patients[27,48] as defined by the GPSM[28] and AdvIPSM[29] models.

Based on the prognostic impact of the *KIT*D816V burden, we subsequently sought to investigate the potential impact on patient outcome of distinct kinetics of the percentage of *KIT*-mutated cells observed over time. Thus, sequential analysis of the *KIT*D816V-mutated cell burden of a subset of 69 SM patients revealed an unstable profile in almost half of the patients. Interestingly, a decreased or increased mutation burden was associated with a higher rate of progression to more advanced disease and a significantly shortened PFS, in contrast to what was observed in SM cases with a stable mutation profile who systematically showed stable disease. These results point out for the first time the potential utility of incorporating sequential assessment of *KIT*D816V (in the blood) for routine follow-up of adult SM patients and identifying patients at high risk of disease progression and who might benefit from early treatment interventions. Further studies in larger series of patients are needed to confirm these findings.

In summary, here we demonstrate the high specificity but limited sensitivity of the detection of *KIT*D816V in whole blood for the diagnostic screening of SM, particularly among primary MCAS (e.g., c-MCAS and BMM) patients, and propose an ultra-sensitive molecular approach to overcome these limitations through the analysis of the mutation in purified blood eosinophils and/or basophils. Overall, this will contribute to earlier and more accurate diagnosis of clonal MC diseases in patients presenting with MCAS via minimally invasive blood analysis of *KIT*D816V. In addition, our data also support the use of *KIT*D816V as a new biomarker for disease monitoring and early identification of SM patients at high risk of progression to more advanced disease.

AUTHOR CONTRIBUTIONS

PN-N performed experiments, analyzed the data, interpreted the results, made the figures, and wrote the paper; AP-P, AM, CC, and OGL performed experiments and critically reviewed the paper; IA-T, AM, AH, and LS-M recruited and followed the patients, collected samples, and critically reviewed the paper; ACG-M critically reviewed the paper; MJ-A designed the study, performed experiments and analyzed the data, interpreted the results, supervised the study, and wrote the paper; and AO designed and supervised the study and wrote the paper. All authors have reviewed and approved the paper, prior to submission.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

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

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