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Evaluation of the WHO criteria for the classification of patients with mastocytosis

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Diagnosis and classification of mastocytosis is currently based on the World Health Organization (WHO) criteria. Here, we evaluate the utility of the WHO criteria for the diagnosis and classification of a large series of mastocytosis patients ($n = 133$), and propose a new algorithm that could be routinely applied for refined diagnosis and classification of the disease. Our results confirm the utility of the WHO criteria and provide evidence for the need of additional information for (1) a more precise diagnosis of mastocytosis, (2) specific identification of new forms of the disease, (3) the differential diagnosis between cutaneous mastocytosis *vs* systemic mastocytosis, and (4) improved distinction between indolent systemic mastocytosis and aggressive systemic mastocytosis. Based on our results, a new algorithm is proposed for a better diagnostic definition and prognostic classification of mastocytosis, as confirmed prospectively in an independent validation series of 117 mastocytosis patients.
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During the last two decades, major advances have been achieved in the diagnosis and classification of mastocytosis. In 1991, a comprehensive clinicomorphological classification identified two major categories of mastocytosis associated with good and poor prognosis, respectively; noteworthy, indolent systemic mastocytosis without skin lesions was included in this classification as a clinical entity.[1] Ten years later, an updated consensus classification of mastocytosis was proposed by the World Health Organization (WHO)[2–4] in which, in addition to major conventional histopathological criterion (multifocal dense aggregates of ≥ 15 mast cells in bone marrow and/or other extracutaneous tissues), four minor morphological (atypical mast cells in smears or biopsy sections of bone marrow or other extracutaneous organs), immunophenotypical ($CD25^+$ and/or $CD2^+$ mast cells), molecular (D816V KIT mutation) and biochemical (serum tryptase levels persistently >20 ng/ml) criteria are recommended for the diagnosis of systemic mastocytosis. According to the WHO, additional clinical investigations should be performed to define the exact subtype of the disease. Overall, seven categories of mastocytosis are defined in the WHO classification: cutaneous mastocytosis, extracutaneous mastocytoma, indolent systemic mastocytosis, aggressive systemic mastocytosis, systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease, mast cell leukemia, and mast cell sarcoma.

More recently, diagnostic guidelines, algorithms, and recommendations to facilitate implementation of the WHO criteria have been proposed and

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preliminary descriptions of new provisional subvariants have been described;[5–12] these include well-differentiated systemic mastocytosis[13,14] and clonal mast cell-activation syndromes in the absence of skin lesions, also termed as monoclonal mast cell-activation syndrome[12,15] or clonal mast cell-activation disorders,[16,17] the later only partially fulfilling the criteria for systemic mastocytosis.

Overall, the great majority of mastocytosis cases belong to the good-prognosis categories of the disease (eg cutaneous mastocytosis, indolent systemic mastocytosis, and well-differentiated systemic mastocytosis) and they are typically characterized by low mast cell burden, particularly at early stages of the disease. However, recent results suggest that these categories remain heterogeneous and some patients experience disease progression, particularly those with elevated serum β_2-microglobulin levels and D816V *KIT* mutation involving multiple myeloid or myeloid plus lymphoid hematopoietic lineages.[18] These results indicate that refined criteria for an improved prognostic stratification of systemic mastocytosis are needed, particularly for cases at early phases of the disease.

Despite all the above, so far the utility of the WHO classification for the diagnosis and classification of mastocytosis has been prospectively evaluated in only one study, which suggests that the WHO criteria for systemic mastocytosis may still be associated with some false-negative cases.[19] A total of 59 patients with clinically suspected systemic mastocytosis, underwent comprehensive evaluation and 53 of them (90%) met the diagnostic criteria for systemic mastocytosis. In the six patients in which bone marrow examination could not confirm systemic mastocytosis, atypical mast cell morphology was identified in five, aberrant immunophenotype in five, KIT mutation in two, and elevated serum tryptase in two. None of these cases met the major criteria; one of the patients had systemic mastocytosis of the spleen. The results showed the relative values of traditional morphologic criteria (ie major criterion) and the results of ancillary testing (ie minor criteria), suggesting that the WHO system is neither completely sensitive nor specific for systemic mastocytosis.[19]

Here, we prospectively evaluate the utility of different clinical, biological, immunophenotypical, and molecular features of the disease (including all WHO major and minor diagnostic criteria), in a cohort of 133 patients uniformly diagnosed and followed at the Spanish Network on Mastocytosis (REMA). Based on our findings, a new algorithm is proposed, which may contribute to improve refined diagnosis and classification of mastocytosis. Accordingly, the proposed refined classification of mastocytosis provides better diagnosis and long-term prognosis classification of mastocytosis, as confirmed prospectively in an independent validation series of 117 mastocytosis patients.

Patients and methods

Patients and Controls

A total of 250 patients with suspected mastocytosis were studied. These included 133 patients—69 women and 64 men; median age of 42 years, range: 9–77 years—who were consecutively referred to the Instituto de Mastocitosis de Castilla La Mancha (CLMast) (Reference Centre of the Spanish Network on Mastocytosis; REMA) from January 1996 till September 2007 (*Test group*), plus 117 patients (68 women and 49 men; mean age of 44 years, range: 21–71 years), who were consecutively referred to the same center (CLMast) from September 2007 to December 2009 (*Validation group*). None of them had received cytoreductive therapy before inclusion in the study.

A control group of 855 bone marrow samples from either healthy subjects ($n=57$; 7%) or patients with different hematological and non-hematological disorders ($n=798$; 93%) other than mastocytosis (Table 1)—median age of 69 years (range: 17–93 years)—was analyzed in parallel. All participants gave their written informed consent to participate in the study, and the study was approved by the Hospital's Ethics Committee.

Diagnostic Work-Up for Mastocytosis

All mastocytosis patients had a complete physical examination, blood cell count and differential,

Table 1 Control subjects: distribution of healthy subjects and control patients included in this study according to diagnosis

Diagnosis	*No. of cases*
Healthy controls	57 (7%)
Patients with lymphoid neoplasias	448 (52%)
Acute lymphoblastic leukemia	15
Chronic lymphocytic leukemia	74
Hodgkin lymphoma	25
Non-Hodgkin's lymphoma	159
Waldenström's macroglobulinemia	23
Multiple myeloma	87
Plasma cell leukemia	2
Monoclonal gammopathy of undetermined significance	63
Patients with myeloid malignancies	142 (17%)
Acute myeloid leukemia	33
Chronic myeloid leukemia	39
Myelodysplastic syndrome	45
Myeloproliferative disorders	25
Patients with other non-neoplastic diseases	113 (13%)
Anemia	42
Hypocellular bone marrow	36
Immune thrombocytopenic purpura	6
Polyclonal lymphocytosis	15
Hypereosinophilia/chronic eosinophilic leukemia	14
Individuals with other disease conditions	95 (11%)
Anaphylaxis	15
Reactive bone marrow	53
Solid tumor	27
Total	855 (100%)

routine serum biochemistry tests, abdominal ultrasonography and/or computed tomography-scan, dual energy X-ray absorptiometry, and skeletal X-ray survey. Presence of osteoporosis was defined following well-established criteria[20,21] and the presence of bone sclerosis—as assessed by skeletal X-ray survey and/or computed tomography-scan—was also recorded. Skin biopsy was performed in all cases with cutaneous lesions. Serum tryptase (CAP, Phadia, Uppsala, Sweden) was measured in all patients at the time of bone marrow biopsy.

Bone marrow evaluation was performed following previously established criteria for morphology,[6] histopathology, immunohistochemistry,[5,22,23] flow cytometry immunophenotyping,[11,24,25] detection of *KIT* mutations,[26,27] and bone marrow mast cell clonality as previously reported in detail.

For morphological evaluation, bone marrow smears were stained with Wright–Giemsa and toluidine blue and analyzed by three independent pathologists using light microscopy. In each case, 25–100 mast cells were analyzed and classified as described elsewhere.[6] In addition, the presence of mast cells aggregates in bone marrow particles (as assessed in toluidine blue-stained samples), presence of focal or diffuse eosinophilia, as well as dysplastic features, were also examined and recorded.

Bone marrow biopsy sections were stained with hematoxylin–eosin, giemsa, tryptase, and c-kit stains, and analyzed by three independent pathologists for overall cellularity, mast cell number and morphology, presence of compact mast cells aggregates, grade and type of mast cell infiltration, presence of fibrosis and/or bone sclerosis, and of lymphoid aggregates.

Immunophenotypical analysis of bone marrow mast cells was performed following the REMA guidelines.[11,24,25] Both external and internal quality controls were routinely applied and all bone marrow samples were studied in parallel at two different laboratories of the REMA. Briefly, bone marrow samples were analyzed by direct immunofluorescence using ≥ 3 color flow cytometer, after staining of bone marrow cells with fluorochrome-conjugated monoclonal antibodies. The CELLQuest PRO (BD Biosciences) and Paint-A-Gate PRO software programs (BD Biosciences) were used for data acquisition and analysis as described elsewhere.[11,24,28,29]

KIT D816V mutation and other KIT mutations localized at codons 814–819 (exon 17) were detected on highly purified ($\geq$97% purity) bone marrow cell populations, as previously described.[27,30] In turn, identification of KIT mutations at exons 2, 9, 10, 11, 13, 14, and 15 was performed on genomic DNA by direct sequencing of the amplified PCR products in both directions, using the dye-deoxy terminator method, in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). To evaluate clonality in female patients without KIT mutation, the pattern of inactivation of chromosome X was studied by the human-androgen receptor-α gene (HUMARA) assay.[31]

Statistical Methods

For all statistical analyses, the SPSS 15.0 software (Chicago, IL, USA) was used. Median values, mean, s.d., and range were calculated for all continuous variables in each group; for categorical variables, frequencies were used. Comparisons between groups were performed with either the non-parametric Kruskal–Wallis and Mann–Whitney *U*-tests (for continuous variables) or the Pearson χ^2 and Fisher's exact tests (for categorical variables). *P*-values <0.05 were considered to be associated with statistical significance. Receiver operating curves were used to assess the sensitivity and specificity of each variable or combination of variables, for the diagnosis and classification of individual cases.

A hierarchical logical inference procedure was used through intensive computation to build 'diagnostic' algorithms, consisting of a combination of propositions associated by logical operators attaining a web of conjunctions, disjunctions, and conditionals. Briefly, an ensemble of previously diagnosed cases was used by way of inductive learning, to infer a general rule that satisfied all cases.

Results

WHO Criteria Applied to Controls and Mastocytosis (*Test Group*)

None of the 855 control bone marrow samples met the diagnostic criteria for systemic or cutaneous mastocytosis (Table 2). However, six cases fulfilled one ($n=4$) or two ($n=2$) minor criteria. These included three cases with morphologically abnormal bone marrow mast cells: two FIP1L1/PDGFRA-positive chronic eosinophilic leukemia, also exhibiting $CD25^{bright+}$ bone marrow mast cells and one myelodysplastic syndrome. The remaining three patients corresponded to control cases with increased serum tryptase and recurrent anaphylaxis (severe systemic mast cell-activation syndromes) without other criteria for systemic mastocytosis (non-clonal idiopathic or secondary mast cell-activation syndrome).

Based on the WHO criteria, from the 133 mastocytosis patients of the test group, 20 (15%) were classified as cutaneous mastocytosis and 112 (85%) as systemic mastocytosis: indolent systemic mastocytosis, 93 (70%), aggressive systemic mastocytosis, 11 (8%), systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease, 6 (5%), and mast cell leukemia, 2 (1%); one case was unclassifiable (Table 2). This later patient was referred because of anaphylaxis episodes and he had neither skin lesions nor bone marrow mast cell

Table 2 Distribution of mastocytosis patients and controls according to the WHO diagnostic criteria

WHO subtype of mastocytosis	*No. of cases (%)*	*Compact bone marrow mast cell aggregates*	*Morphologically atypical bone marrow mast cell*	*CD25⁺ and/or CD2⁺ bone marrow mast cell*	*KIT D816V⁺ bone marrow mast cell*	*Serum tryptase >20 ng/ml*
Total mastocytosis	133	100/133 (75%)	107/133 (80%)	115/133 (86%)	108/133 (81%)	83/133 (62%)
Cutaneous mastocytosis	20 (15%)	30%	5%	20%	10%	0%
Indolent systemic mastocytosis	93 (70%)	81%	95%	98%	92%	72%
Systemic mastocytosis associated to an hematological non-mast cell disease	6 (5%)	100%	83%	100%	100%	66%
Aggressive systemic mastocytosis	11 (8%)	100%	100%	100%	100%	90%
Mast cell leukemia	2 (1%)	100%	100%	100%	100%	100%
Unclassifiable	1 (1%)	0%	0%	100%	100%	0%
Controls	855	0%	4%	1%	0%	3%

Results are expressed as number of positive cases from all cases analyzed and percentage between brackets.

aggregates; however, he had aberrant $CD25^{bright+}$ bone marrow mast cells with the D816V *KIT* mutation, suggesting it could correspond to an early phase of indolent systemic mastocytosis without skin lesions. The frequency of cases fulfilling the different WHO major and minor criteria for systemic mastocytosis are shown in Table 2. In detail, dense aggregates of ≥ 15 bone marrow mast cells were found in all systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease, aggressive systemic mastocytosis, and mast cell leukemia cases, but in only 30 and 81% of cutaneous mastocytosis and indolent systemic mastocytosis cases, respectively (Table 3). Round-shaped bone marrow mast cells predominated in cutaneous mastocytosis (90%), while spindle mast cells or a mixed morphology was more frequent in systemic mastocytosis ($P<0.0001$; Table 3). All systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease, aggressive systemic mastocytosis, and mast cell leukemia cases and most (98%) indolent systemic mastocytosis patients showed $CD25^{bright+}$ bone marrow mast cells (Table 2). Noteworthy, expression of $CD25^{bright+}$ was also detected on bone marrow mast cells from 4/20 (20%) cutaneous mastocytosis and the unclassifiable case. Eighty percent of systemic mastocytosis cases also coexpressed CD2 on bone marrow mast cells: 73/93 indolent systemic mastocytosis (78%), 5/6 systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease (83%), 9/10 aggressive systemic mastocytosis (90%), and 0/1 mast cell leukemia (Table 2). Most patients with mastocytosis ($n=108/133$; 81%) carried the D816V *KIT* mutation in bone marrow mast cells: 10% of cutaneous mastocytosis, 92% of indolent systemic mastocytosis, and all (100%) systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease, aggressive systemic mastocytosis, and mast cell leukemia patients (Table 2). Of note, the two *KIT* mutation-positive cutaneous mastocytosis cases also showed $CD25^{bright+}$ bone marrow mast cells. Serum tryptase >20 ng/ml was detected in 72% of indolent systemic mastocytosis cases (median: 27.1 ng/ml). All patients in the aggressive groups had highly elevated serum tryptase—median of 195 ng/ml for aggressive systemic mastocytosis ($P<0.0001$ *vs* indolent systemic mastocytosis) and 587 ng/ml for mast cell leukemia ($P<0.001$ *vs* indolent systemic mastocytosis)—except for one aggressive systemic mastocytosis patient who showed normal serum tryptase levels and was classified as aggressive systemic mastocytosis because of the presence of organomegalies with organ failure, malabsorption, and cytopenias. 'C'-findings were found in 10% of mastocytosis patients including skeletal involvement (4%), bone marrow dysfunction with cytopenia(s) (7.5%), and malabsorption with weight loss (3%). Hepatosplenomegaly with or without signs of organ failure was found in 4 and 17% of the cases, respectively (the former was seen in 4/11 (36%) aggressive systemic mastocytosis and 2/2 mast cell leukemia cases). In all systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease cases, the specific subtype of mastocytosis corresponded to an indolent systemic mastocytosis and the associated hematological diseases were myelodisplastic syndrome ($n=1$), acute myeloid leukemia ($n=1$), non-Hodgkin's lymphoma ($n=1$), polycythemia vera ($n=2$), and essential thrombocythemia ($n=1$).

Differential Diagnosis of Cutaneous *vs* Systemic Mastocytosis

Among those 20 patients classified as cutaneous mastocytosis by the WHO, three different subgroups were identified (Table 4). The first subgroup was

Table 3 Cytomorphological and histopathological characteristics of bone marrow samples from mastocytosis patients grouped according to the WHO subtype of the disease *vs* controls

(A) Cytomorphological characteristics

WHO subtype of mastocytosis	*Increased mast cells*	*Round; spindle; mixed* mast cells*	*Mast cell aggregates*	*Eosinophilia*	*Promastocytes*
Mastocytosis	114/133 (86%)	25; 45; 63	100/128 (78%)	49/133 (37%)	32/133 (24%)
Cutaneous mastocytosis	10/20 (50%)	18; 1; 1	8/20 (44%)	4/20 (20%)	0/20 (0%)
Indolent systemic mastocytosis	85/93 (91%)	4; 38; 51	74/91 (81%)	38/93 (41%)	24/93 (26%)
Systemic mastocytosis associated to an hematological non-mast cell disease	6/6 (100%)	1; 1; 4	6/6 (100%)	1/6 (17%)	1/6 (17%)
Aggressive systemic mastocytosis	11/11 (100%)	0; 4; 7	11/11 (100%)	5/11 (45%)	5/11 (45%)
Mast cell leukemia	2/2 (100%)	1; 1; 0	2/2 (100%)	0/2 (0%)	2/2 (100%)
Unclassifiable	0/1 (0%)	1; 0; 0	0/1 (0%)	1/1 (100%)	0/1 (0%)
Controls	83/83 (100%)	27; 3; 53	6/83 (7%)	14/83 (17%)	0/83 (0%)

(B) Histopathological characteristics

WHO subtype of mastocytosis	*Compact mast cell aggregates*	*Subdiagnostic mast cell aggregates*	*Bone marrow fibrosis*	*Bone marrow sclerosis*	*Lymphoid aggregates*
Mastocytosis	100/133 (75%)	12/32 (37%)	46/132 (35%)	18/132 (14%)	58/131 (44%)
Cutaneous mastocytosis	6/20 (30%)	1/14 (7%)	0/20 (0%)	0/20 (0%)	3/20 (15%)
Indolent systemic mastocytosis	75/93 (81%)	11/17 (65%)	29/92 (31%)	11/92 (12%)	48/91 (53%)
Systemic mastocytosis associated to an hematological non-mast cell disease	6/6 (100%)	0/1 (0%)	5/6 (83%)	0/6 (0%)	3/6 (50%)
Aggressive systemic mastocytosis	11/11 (100%)	NA	11/11 (100%)	7/11 (64%)	4/11 (36%)
Mast cell leukemia	2/2 (100%)	NA	1/2 (50%)	0/2 (0%)	0/2 (0%)
Unclassifiable	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)
Controls	0/46 (0%)	1/46 (2%)	1/46 (2%)	1/46 (2%)	9/46 (20%)

NA: not applied.
Results are expressed as number of positive cases/from all cases analyzed and percentage between brackets or as *number of cases.

composed of patients with only mastocytosis in the skin but no major or minor criteria for systemic mastocytosis—$n=9$; 8 women (89%). The second subgroup, included six patients with mastocytosis in the skin associated with 'tryptase-positive round cell infiltration of bone marrow'[32] (TROCI) by c-kit+ round-shaped and fully granulated mast cells with a mature $CD25^-/CD2^-$ phenotype[33] in the absence of D816V *KIT* mutation. Although these cases fulfilled only the major WHO diagnostic criterion for systemic mastocitosis, they all showed typical clinical, cytogenetic, and molecular features of well-differentiated systemic mastocytosis.[13,14] Finally, the third subgroup consisted of five patients with mastocytosis in the skin, no major diagnostic criterion for systemic mastocytosis but the presence of either 1 or 2 minor criteria; 4/5 cases had a low bone marrow mast cell burden with normal serum baseline tryptase and coexistence of a double population of clonal $CD25^{bright+}$ and polyclonal $CD25^-$ mast cells and either the D816V *KIT* mutation ($n=2$) or morphologically atypical bone marrow mast cell ($n=1$), suggesting that these cases could correspond to early stages of indolent systemic mastocytosis; the fifth case presented with mastocytosis in the skin and clonal bone marrow mast cell together with round-shaped mast cells, suggesting well-differentiated systemic mastocytosis with low bone marrow mast cell burden.

Classification of Systemic Mastocytosis

Among indolent systemic mastocytosis cases as per the WHO, three subgroups of patients could be clearly distinguished; two major groups identified by the presence ($n=77$; indolent systemic mastocytosis with skin lesions) *vs* absence ($n=16$; indolent systemic mastocytosis without skin lesions) of mastocytosis in the skin. And a small group identified by the presence of the so-called 'tryptase-positive round cell infiltration of bone marrow' (compact tryptase-positive round cell infiltrates of the bone marrow), c-kit+ round-shaped and fully granulated mast cells displaying a mature $CD25^-/CD2^-$ phenotype. The two cases in this later group showed features compatible with well-differentiated systemic mastocytosis; one was associated with the D816V *KIT* mutation and the other patient showed serum baseline tryptase >20 ng/ml.

Among the two major groups of indolent systemic mastocytosis (with and without skin lesions), the frequency of diagnostic criteria was similar, except for the presence of clonal bone marrow mast cells (96% for indolent systemic mastocytosis with skin lesions *vs* 88% for indolent systemic mastocytosis without skin lesions, $P=0.16$) and of dense bone marrow mast cell aggregates (78% in indolent systemic mastocytosis with skin lesions, 71% in indolent systemic mastocytosis without skin

Table 4 Characteristics of patients classified as cutaneous mastocytosis by the WHO criteria

Subgroups of cutaneous mastocytosis	*No. of cases*	*Skin lesions*	*Anaphylaxis*	*Compact bone marrow aggregates*	*Morphologically atypical bone marrow mast cells*	*CD25^{++} bone marrow mast cells*	*Clonal bone marrow mast cells*[a]	*Serum tryptase >20 µg/l*	*Diagnosis according to the new algorithm (Figure 1)*
No criteria for systemic mastocytosis	9	9/9	1/9	0/9	0/9	0/9	0/9	0/9	*Cutaneous mastocytosis*
Major criterion only	6	6/6	1/6	6/6	0/6	0/6	0/6	0/6	*Well-differentiated systemic mastocytosis*
1–2 minor criteria only	5	5/5	2/5	0/5	1/5	4/5	3/5	0/5	*Indolent systemic mastocytosis or well-differentiated systemic mastocytosis*
Total cutaneous mastocytosis	20	20/20	4/20	6/20	1/20	4/20	3/20	0/20	

Results are expressed as number of positive cases from all cases analyzed.
[a]Clonal bone marrow mast cells: including all *KIT* mutations other than D816V or a clonal HUMARA test.

Table 5 Distribution of mastocytosis patients ($n=133$) according to the newly proposed algorithm for the diagnosis and classification of mastocytosis (Figure 1)

Subtype of mastocytosis	*No. of cases (%)*	*Skin lesions*	*Anaphylaxis*	*CD25^{++} bone marrow mast cells*	*Clonal bone marrow mast cells*[a]	*Compact bone marrow mast cell aggregates*	*Morphologically atypical bone marrow mast cells*
Cutaneous mastocytosis	9/133 (7%)	9/9	1/9	0/9	0/9	0/9	0/9
Well-differentiated systemic mastocytosis	9/133 (7%)	9/9	1/9	0/9	7/9	8/9	0/9
Indolent systemic mastocytosis with skin lesions	82/133 (61%)	82/82	14/82	82/82	79/82	64/82	76/82
Indolent systemic mastocytosis without skin lesions	17/133 (13%)	0/17	17/17	17/17	15/17	12/17	16/17
Systemic mastocytosis associated to other hematological non-mast cell lineage disease	6/133 (4.5%)	3/6	1/6	6/6	6/6	6/6	5/6
Aggressive systemic mastocytosis	8/133 (6%)	6/8	3/8	8/8	8/8	8/8	8/8
Mast cell leukemia	2/133 (1.5%)	0/2	0/2	2/2	2/2	2/2	2/2
Total	133	109/133 (82%)	37/133 (27.8%)	115/133 (86.5%)	117/133 (94.4%)	100/133 (75.2%)	107/133 (80%)

Results are expressed as number of positive cases from all cases analyzed and percentage between brackets, or just as number of positive cases from all cases analyzed.
[a]Clonal bone marrow mast cells: including all *KIT* mutations other than D816V or a clonal result in a HUMARA test in females.

Table 6 Distribution of mastocytosis patients according to β_2-microglobulin and LDH serum levels

Subtype of mastocytosis[a]	*Increased serum β_2-microglobulin*	*Decreased serum LDH*	*Increased serum β_2-microglobulin and decreased serum LDH*
Cutaneous mastocytosis	0/9	0/9	0/9
Well-differentiated systemic mastocytosis	0/9	2/9	0/9
Indolent systemic mastocytosis (with skin lesions)	8/82	15/82	2/82
Indolent systemic mastocytosis (without skin lesions)	1/17	2/17	0/17
Systemic mastocytosis associated to other hematological non-mast cell disease	2/6	1/6	0/6
Aggressive systemic mastocytosis	7/8	7/8	7/8
Mast cell leukemia	2/2	2/2	2/2

LDH: lactic acid dehydrogenase.
Results are expressed as number of positive cases from all cases analyzed and percentage between brackets.
[a]Cases were classified according to the algorithm proposed in Figure 1.

lesions, $P=0.51$) (Table 5). A more detailed analysis of the clinical symptoms of both groups of patients showed a higher frequency of both mast cell mediators release-associated symptoms in between the acute episodes and *KIT* mutation restricted to mast cells, among indolent systemic mastocytosis without skin lesions *vs* indolent systemic mastocytosis with skin lesions (56 *vs* 24% and 82 *vs* 76%, $P=0.003$ and $P=0.071$, respectively) (Table 5).

Regarding aggressive systemic mastocytosis, 8 of 11 patients, classified as such by the WHO criteria, had aggressive disease with C-findings in the absence of pathologic fractures. Most interestingly, all but one of these eight aggressive systemic mastocytosis patients, showed increased β_2-microglobulin in association with decreased LDH serum levels, both parameters being found in only a minority of all indolent systemic mastocytosis, with skin lesions and other indolent forms of the disease (2/105; 2%) (Table 6). Conversely, the other three aggressive systemic mastocytosis cases as per the WHO, had stable disease and they were placed in this category only because of the presence of skeletal lesions in the absence of other clinical findings of aggressiveness (C-findings); in these three patients, vertebral osteoporotic fractures appeared 18, 8, and 5 years after the onset of aggressive systemic mastocytosis after a follow-up of 39, 34, and 15 years from disease onset, respectively. Among these three cases, disease progression was found in one of them, with *KIT* mutation involving mast cells and both myeloid and lymphoid lineages, 38 years after the onset. Noteworthy, all the three cases showed normal β_2-microglobulin and LDH serum levels.

Proposed Algorithm for Refined Diagnosis and Classification of Mastocytosis

Based on the above findings, a new algorithm for refined diagnosis and classification of mastocytosis was developed (Figure 1). Through this algorithm, patients without mastocytosis could be easily identified because of the systematic absence of both: (1) skin lesions and (2) *KIT* mutation or clonal bone marrow mast cells, if at least, (3) one of the following additional criteria is present at diagnosis: (1) absence of anaphylaxis; (2) normal bone marrow mast cell immunophenotype; (3) absence of compact bone marrow mast cell aggregates; or (4) cytomorphologically normal bone marrow mast cells (Figure 1). Among mastocytosis, cutaneous mastocytosis is defined by the absence of (1) *KIT* mutation/clonal bone marrow mast cells and (2) compact mast cell aggregates in the bone marrow, with (3) a normal bone marrow mast cell immunophenotype (Figure 1). In turn, systemic mastocytosis cases systematically had $CD25^{bright+}$ bone marrow mast cells and/or compact bone marrow mast cell aggregates in the bone marrow biopsy (Figure 1).

Among systemic mastocytosis, well-differentiated systemic mastocytosis could be identified and discriminated from other disease subtypes because of coexisting skin lesions—usually nodular or plaque form (data not shown), compact bone marrow aggregates, and a mature bone marrow mast cell immunophenotype, typically in the absence of the *KIT* D816V mutation (Figure 1). In turn, indolent systemic mastocytosis without skin lesions, patients were characterized by the absence of mastocytosis in the skin in association with severe systemic mast cell mediator-related symptoms (eg anaphylaxis) (mast cell-activation syndrome) and low mast cell burden (<10% clonal bone marrow mast cells by flow cytometry and <30% bone marrow mast cell infiltration by histopathology) (Figure 1). Mast cell leukemia displayed a high bone marrow mast cell burden (>10% of marrow mast cell by flow cytometry and/or >30% bone marrow mast cell by histopathology) usually in the absence of mastocytosis in the skin (Figure 1; Table 5). Finally, coexistence of $CD25^{bright+}$ and compact bone marrow mast cell aggregates identified indolent systemic mastocytosis with skin lesions and aggressive systemic mastocytosis patients, in addition to systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease cases. Distinction between indolent systemic mastocytosis

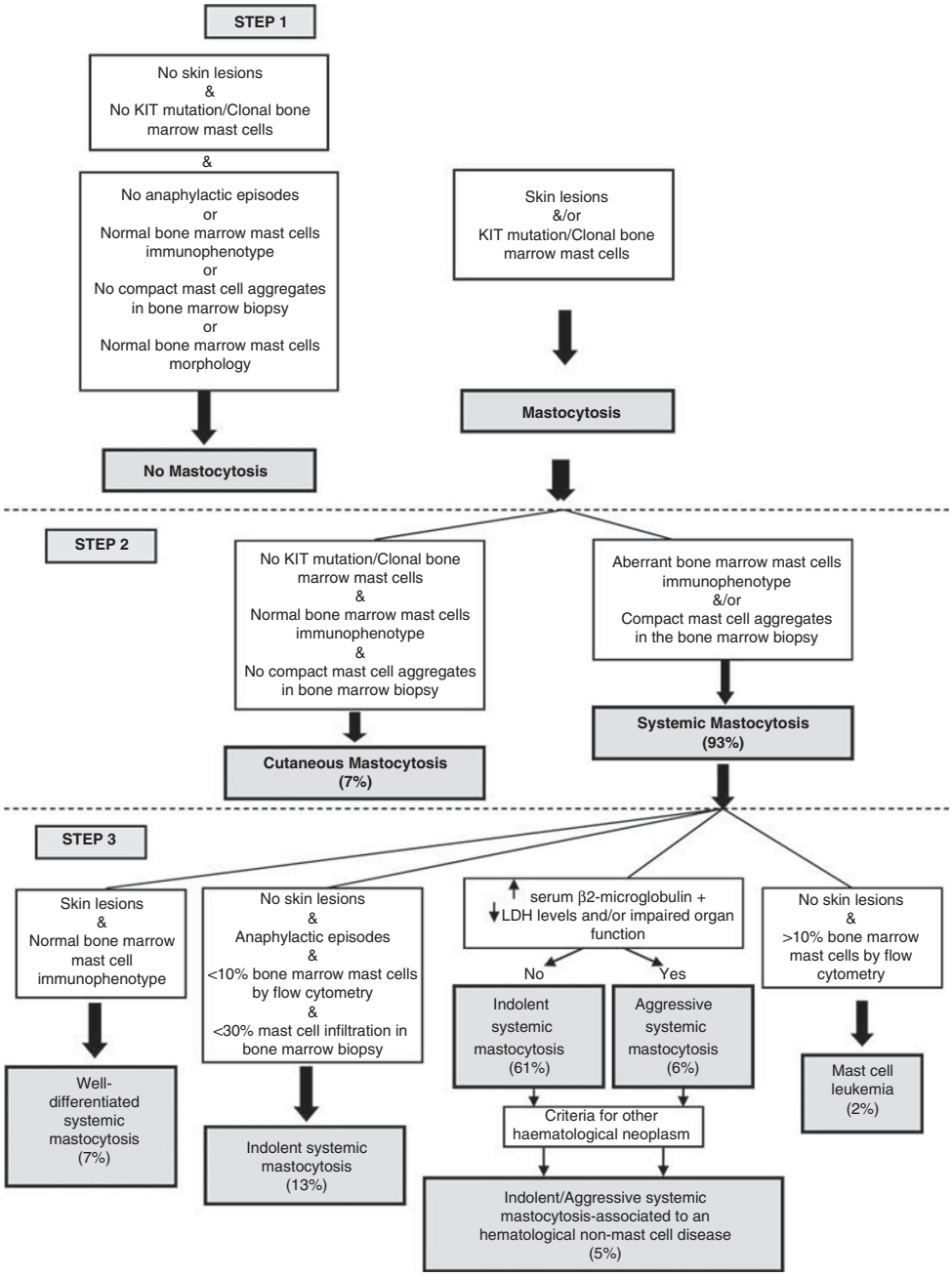

Figure 1 Proposed REMA algorithm for the diagnosis and classification of different subtypes of mastocytosis. The specific percentage of mastocytosis patients ($n = 133$) included in each category is shown between brackets.

and aggressive systemic mastocytosis required further WHO criteria such as C-findings (ie bone marrow organ dysfunction with cytopenias, organomegaly with organ failure, and severe malabsorption with hypoalbuminemia and weight loss, and large sized osteolysis with local mast cell infiltration), typically associated with increased β_2-microglobulin and decreased LDH serum levels in the later patients (ie aggressive systemic mastocytosis) (Table 6). In turn, specific diagnosis of systemic mastocytosis associated with other clonal hematological non-mast cell lineage disease cases was defined based on cytomorphological, histopathological, immunophenotypical, and molecular findings related to the associated with other clonal hematological non-mast cell lineage disease (Figure 1; Table 5).

Prospective Evaluation of the Newly Proposed REMA Algorithm (Validation Group)

In order to explore the utility of the proposed algorithm, we prospectively applied it in an *out-of-sample* mode, to another group of 117 cases with

Table 7 Distribution of mastocytosis patients according to the WHO classification and the new proposed algorithm and refined reclassification

Subtype of mastocytosis	*Testing series* (n = *133*)		*Validation series* (n = *117*)[a]	
	WHO classification no. of cases (%)	*Proposed reclassification no. of cases (%)*	*WHO classification no. of cases (%)*	*Proposed reclassification no. of cases (%)*
Cutaneous mastocytosis	20 (15%)	9 (7%)	1 (1%)	1 (1%)
Indolent systemic mastocytosis	93 (70%)	99 (74%)	60 (51%)	66 (56%)
Indolent systemic mastocytosis with skin lesions		82 (61%)		45 (38%)
Indolent systemic mastocytosis without skin lesions		17 (13%)		21 (18%)
Well-differentiated systemic mastocytosis	NI	9 (7%)	NI	1 (1%)
Systemic mastocytosis associated to other hematological non-mast cell lineage disease	6 (5%)	6 (4.5%)	1 (1%)	1 (1%)
Aggressive systemic mastocytosis	11 (8%)	8 (6%)	3 (3%)	0
Mast cell leukemia	2 (1%)	2 (1.5%)	0	0
Unclassifiable cases	1 (1%)	0	4 (3%)	0

NI: not included; WHO: World Health Organization.
Results are displayed as number of cases and percentage between brackets.
[a]Forty-eight cases suspected of having mastocytosis were not diagnosed of mastocytosis with both the REMA and WHO criteria.

suspected diagnosis of mastocytosis who were consecutively referred to the Instituto de Mastocitosis from October 2007 to December 2009. Based on this strategy, 117/117 cases (100%) with mastocytosis and clonal mast cell disorders compatible with mastocytosis were correctly classified (Table 7). Compared with the WHO criteria, differences were as follows (1) 4/117 (3%) patients were unclassifiable by the WHO as they showed only 1 or 2 minor criteria with no major criterion in the absence of mastocytosis in the skin; (2) 3/117 indolent systemic mastocytosis cases according to the REMA's criteria who were classified as aggressive systemic mastocytosis by the WHO, only because they had skeletal lesions but no other 'C'-findings.

Discussion

During the past 15 years, major advances have been achieved in both the diagnosis and classification of mastocytosis[2–4,12,34] with very detailed studies about the cytological,[6] immunohistochemical,[5] immunophenotypic,[25,33] and molecular features of mast cells in mastocytosis. Undoubtedly, the WHO criteria represented a major step forward in both the diagnosis and classification of mastocytosis, contributing to the distinction between good- (eg cutaneous mastocytosis and indolent systemic mastocytosis) *vs* poor-prognosis subgroups (eg aggressive systemic mastocytosis, mast cell leukemia). However, since the initial proposal, only one study based on 59 patients[19] has prospectively evaluated the WHO criteria. Interestingly, preliminary data from this study indicate that based on the WHO criteria, some patients suffering from a true systemic mastocytosis cannot be classified as such. In line with these findings, it has been suggested by consensus groups that specific subtypes of mastocytosis may not fulfill the WHO diagnostic criteria, particularly among well-differentiated systemic mastocytosis and indolent systemic mastocytosis without skin lesions cases.[2] Additional data also indicate that prediction of outcome among indolent systemic mastocytosis, as well as the distinction between both cutaneous mastocytosis *vs* systemic mastocytosis and indolent systemic mastocytosis *vs* aggressive systemic mastocytosis, and between stable *vs* progressive disease could also benefit from the introduction of further objective criteria.[12,18] Altogether these observations point out the need for additional refined criteria.[19]

In this study, we retrospectively applied the current WHO criteria to the diagnosis and classification of a group of 133 cases suspected of mastocytosis and a heterogeneous group of 855 controls. Overall, no false-positive cases were found with only one case that could not be classified. However, despite this high diagnostic efficiency, 15 cases could have been more precisely classified. Most of these 15 cases corresponded to well-differentiated systemic mastocytosis and patients with an overall picture similar to indolent systemic mastocytosis, who were classified as either cutaneous mastocytosis or aggressive systemic mastocytosis.

Regarding the individual WHO criterion, presence of dense multifocal bone marrow mast cell infiltrates was highly specific of mastocytosis, since it was not observed in any of the control cases; however, around one fourth of all good-prognosis patients lacked on the major criterion for systemic mastocytosis at the same time they showed normal or slightly increased (<20 ng/ml) serum baseline tryptase; these findings point out the need for more sensitive diagnostic criteria (eg other minor WHO criteria), in line with previous observations.[19] In around 80% of our systemic mastocytosis cases, an abnormal bone marrow mast cell morphology was

found, typically including an admixture of spindle-shaped mast cell and either round or polygonal mast cells. In turn, spindle-shaped fully granulated mast cell in the absence of other aberrant changes were found in 4% of all bone marrow control samples, mainly among myelodisplastic syndrome. Altogether, these findings support the utility of careful morphological analysis of bone marrow smears for the detection of abnormal bone marrow mast cells in mastocytosis. However, it should be noted that false-negative results were relatively frequently observed, particularly in well-differentiated systemic mastocytosis where mast cells were round, usually with a clearly increased size, and they were fully granulated with frequent degranulation phenomena making them morphologically rather similar to normal bone marrow mast cells.

Since in the early stages of the disease, indolent systemic mastocytosis patients usually display very low mast cell burden in the absence of bone marrow mast cells aggregates, demonstration of the presence of either clonal or phenotypically aberrant mast cells in the bone marrow and/or other tissues by highly sensitive methods becomes essential. In line with other reports, the presence of $CD25^{bright+}$ bone marrow mast cells appeared to be a highly specific diagnostic criteria being found in most systemic mastocytosis patients,[19,25,35–39] but in only a few control bone marrow samples, all of which corresponded to FIP1L1/PDGFRA + chronic eosinophilic leukemia and myelodisplastic syndrome, in line with previous observations;[10] altogether these findings support the usage of CD25 expression on bone marrow mast cells as a surrogate marker for mast cells clonality. Noteworthy, the presence of clonal mast cells assessed through *KIT* mutation or a clonal HUMARA test (in bone marrow mast cells from women lacking *KIT* mutations) in highly purified mast cells was found in virtually every case (94%), confirming previous observations;[18,27] this indicates that *KIT* mutation in bone marrow mast cells may have a higher diagnostic value than other WHO criteria.

Conversely, serum tryptase >20 ng/ml was the less sensitive WHO diagnostic criteria, since it was only found in $\approx$70% of our cases; in addition, serum tryptase >20 ng/ml was also identified among systemic secondary or idiopathic (non-clonal) mast cell-activation syndrome patients, supporting its lower specificity *vs* other WHO criteria.

Based on these findings, we propose a new objective algorithm for refined diagnosis and classification of mastocytosis, which includes the WHO criteria with the exception of serum tryptase, plus other objective clinical (eg systemic mast cell-activation symptoms and mastocytosis in the skin) and laboratory data (eg β_2-microglobulin and LDH serum levels); this new diagnostic algorithm may be easily applied in the clinical setting to patients suspected of having mastocytosis. Once this new algorithm was designed, it was prospectively evaluated in an independent series of patients, confirming its improved efficiency over the WHO criteria; this was particularly true for the distinction between cutaneous mastocytosis and systemic mastocytosis, specially among well-differentiated systemic mastocytosis and to a lower extent also for indolent systemic mastocytosis without skin lesions.

Once the diagnosis of systemic mastocytosis has been established, further subclassification into specific disease entities is mandatory for adequate prognostic stratification and patient management. Also here, the proposed new algorithm could have an improved efficiency, particularly as regards the combined usage of additional objective criteria (eg serum β_2-microglobulin and LDH levels in addition to 'C'-findings) for the distinction between indolent systemic mastocytosis and aggressive systemic mastocytosis.

If only mastocytosis in the skin is present, diagnosis of cutaneous mastocytosis could be made, while presence of bone marrow mast cell aggregates or $CD25^{bright+}$ or mutated/clonal bone marrow mast cell, in addition to mastocytosis in the skin, supports the diagnosis of systemic mastocytosis. Among the later patients, well-differentiated systemic mastocytosis showed unique features: coexistence of skin involvement and compact bone marrow mast cell aggregates with round shape and larger $CD25^{-}$ mast cell; in addition, *KIT* D816V mutation[13] or other *KIT* mutations[14] were found in only a few well-differentiated systemic mastocytosis cases. Whenever well-differentiated systemic mastocytosis do not show bone marrow mast cell aggregates, failure of the current WHO criteria (but not the new algorithm) would most likely occur. Conversely, indolent systemic mastocytosis without skin lesions and mast cell leukemia were identified because of lack of mastocytosis in the skin in association with anaphylaxis and a relatively low (indolent systemic mastocytosis without skin lesions) or high (mast cell leukemia) mast cell burden. Finally, coexistence of $CD25^{bright+}$ mast cell and compact bone marrow mast cell aggregates, in addition to mastocytosis in the skin and/or the *KIT* D816V mutation, identified a heterogeneous group of patients including indolent systemic mastocytosis with skin lesions, aggressive systemic mastocytosis, and systemic mastocytosis—associated with other clonal hematological non-mast cell lineage disease. Among them, systemic mastocytosis—associated hematological non-mast cell lineage disease—was easily identifiable on the basis of clinical, cytomorphological, histological, immunophenotypical, and molecular features of the associated with other clonal hematological non-mast cell lineage disease. Conversely, the distinction between indolent systemic mastocytosis and aggressive systemic mastocytosis required additional and more objective parameters on top of 'C'-findings. In this regard, combined assessment of serum

β_2-microglobulin and LDH appeared to be particularly useful since, with both parameters, some clinically stable aggressive systemic mastocytosis cases as per the WHO, would be reclassified as indolent systemic mastocytosis. Interestingly, these later cases showed severe osteoporosis with pathological fractures as the only C-finding; in line with our previous observations,[18] neither severe osteoporosis nor pathological fractures secondary to osteoporosis should be included as a 'C'-finding, except in cases with demonstrated mast cell infiltration in lesional areas. If this holds true, these 3/11 cases who had aggressive systemic mastocytosis according to the WHO, would be reclassified as indolent systemic mastocytosis by the new algorithm here proposed with strong implications in patients' prognosis and life expectancy. Noteworthy, none of the other aggressive systemic mastocytosis patients showed pathological fractures.

In summary, here we confirm the robustness of the WHO criteria for the diagnosis and classification of mastocytosis but provide evidence for the need of additional refinement of the WHO system with the potential introduction of the proposed new parameters and algorithm, which are particularly useful for the diagnosis of well-differentiated systemic mastocytosis, patients at early stages of the disease (eg indolent systemic mastocytosis without skin lesions) and discrimination between indolent systemic mastocytosis and aggressive systemic mastocytosis.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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