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**Characterization of CD34⁺ hematopoietic cells in systemic mastocytosis:
potential role in disease dissemination.**

CD34⁺ HEMATOPOIETIC CELLS IN SYSTEMIC MASTOCYTOSIS

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ABSTRACT.

Background: Recent studies show that most systemic mastocytosis (SM) patients, including indolent SM (ISM) with (ISM_s⁺) and without skin lesions (ISM_s⁻), carry the *KIT* D816V mutation in PB leukocytes. We investigated the potential association between the degree of involvement of BM hematopoiesis by the *KIT* D816V mutation and the distribution of different maturation-associated compartments of bone marrow (BM) and peripheral blood (PB) CD34⁺ hematopoietic precursors (HPC) in ISM, and identified the specific PB cell compartments that carry this mutation.

Methods: The distribution of different maturation-associated subsets of BM and PB CD34⁺ HPC from 64 newly-diagnosed (*KIT*-mutated) ISM patients and 14 healthy controls was analyzed by flow cytometry. In 18 patients distinct FACS-purified PB cell compartments were also investigated for the *KIT* mutation.

Results: ISM patients showed higher percentages of both BM and PB MC-committed CD34⁺ HPC vs. controls, particularly among ISM cases with MC-restricted *KIT* mutation (ISM_{MC}); this was associated with progressive blockade of maturation of CD34⁺ HPC to the neutrophil lineage from ISM_{MC} to multilineage *KIT*-mutated cases (ISM_{ML}). Regarding the frequency of *KIT*-mutated cases and cell populations in PB, variable patterns were observed, the percentage of *KIT*-mutated PB CD34⁺ HPC, eosinophils, neutrophils, monocytes and T-cells increasing from ISMs_{-MC} and ISMs_{+MC} to ISM_{ML} patients.

Conclusion: The presence of the *KIT* D816V mutation in PB of ISM patients is associated with (early) involvement of circulating CD34⁺ HPC and multiple myeloid cell subpopulations, *KIT*-mutated PB CD34⁺ HPC potentially contributing to early dissemination of the disease.

Keywords: Systemic mastocytosis; mast cell; hematopoietic precursor cells; *KIT* mutation; immunophenotype.

Abbreviations:

AHNMD: associated clonal haematological non-mast cell lineage disease;

ASM: aggressive systemic mastocytosis;

ASOq: allele specific oligonucleotide quantitative;

BCP: B cell precursor;

BM: bone marrow;

BMM: bone marrow mastocytosis;

ECNM: European Competence Network on Mastocytosis;

FSC: forward light scatter;

HPC: hematopoietic precursor and stem cells;

ISM: indolent systemic mastocytosis;

ISM_{MC}: indolent systemic mastocytosis with *KIT* mutation restricted to the BM MC compartment;

ISM_{ML}: indolent systemic mastocytosis with multilineage *KIT* mutation in the bone marrow;

ISMs_{-MC}: indolent systemic mastocytosis presenting without skin lesions and *KIT* mutation restricted to bone marrow mast cells;

ISMs+_{MC}: indolent systemic mastocytosis presenting with skin lesions and *KIT* mutation restricted to bone marrow mast cells

Mab: monoclonal antibody;

MC: mast cell;

MCL: mast cell leukaemia;

PB: peripheral blood;

pDC: plasmacytoid dendritic cell;

PNA: peptide nucleic acid;

REMA: “Red Española de Mastocitosis” (Spanish Network on Mastocytosis);

sBT: serum baseline tryptase;

SSC: sideward light scatter;

SM: systemic mastocytosis;

SD: standard deviation;

2CDA: cladribine.

INTRODUCTION.

Systemic mastocytosis (SM) comprises a heterogeneous group of hematopoietic stem cell disorders characterized by an abnormal accumulation of neoplastic mast cells (MC) in the bone marrow (BM) and other tissues such as the skin. (1, 2) At presentation, SM includes both indolent (e.g. indolent SM; ISM) and aggressive forms of the disease –e.g. aggressive SM (ASM) and MC leukaemia (MCL). (2) Despite such clinical and prognostic heterogeneity, most SM patients (>90%) carry the D816V activating mutation in the stem cell factor receptor gene (*KIT*). (3-5) However, while ASM patients show involvement of multiple BM hematopoietic cell lineages by the *KIT* mutation (6-8) together with mutations in other genes (e.g. *TET2*, *ASXL1*, *SRSF2*, and *RUNX1*), (9-11) multilineage *KIT* mutation is restricted to one third of ISM cases presenting with skin lesions and a minority (<10%) of cases with ISM without skin lesions (ISMs-) or (isolated) BM mastocytosis (BMM). (3, 12) Thus, most ISM patients show a *KIT* mutation profile in the BM, restricted to MC and CD34 hematopoietic precursor and stem cells (HPC). (3) Of note, the extent of involvement of haematopoiesis by the *KIT* mutation has been proven to

be the most powerful independent variable to predict for progression of ISM to more advanced forms of the disease.(13, 14)

In parallel to the above findings, recent studies (5) (15, 16) have demonstrated that most ISM patients, including the majority of ISMs- cases show *KIT*-mutated cells in PB, even in the absence of multilineage BM involvement of haematopoiesis by the *KIT* mutation. Nevertheless, no study has investigated so far the specific compartment of circulating PB cells that carries the *KIT* mutation, particularly at the earliest disease stages.

At present it is well-established that MCs derive from BM CD34⁺ HPC.(17, 18) The majority of BM CD34⁺ precursors consist of a majority of cells already committed to different myeloid lineages -e.g., erythroid, neutrophil, monocytic, plasmacytoid dendritic cell (pDC), basophil, eosinophil and megakaryocytic- together with CD34⁺ Tdt⁺ and/or CD19⁺ B-cell precursors (BCP) and CD7⁺ T/NK precursors; consequently, only a minor percentage of all BM CD34⁺ HPC correspond to pluripotent stem cells that recirculate in PB to fill distant BM niches.(18, 19) Of note, MC-committed BM CD34⁺ HPC migrate and circulate via PB prior to their full maturation in tissues such as mucosa, skin and also BM(20). In contrast, mature MC have not been observed in PB of steady state healthy adults, and they have only been reported in a subset of SM patients, particularly among advanced SM cases (e.g. ASM and MCL).(21, 22) Consequently, circulating CD34⁺ HPC are a potential candidate cell population to carry the *KIT* mutation in PB and to account for the unexpectedly high rate of positivity for this mutation in SM; at the same time, circulating CD34⁺ HPC could lead to disease dissemination throughout the BM and skin, as observed already at diagnosis in most ISM.(23)

Here we investigated the potential association between the degree of involvement of BM haematopoiesis by the *KIT* mutation and the distribution of different maturation-associated compartments of BM and PB CD34⁺ HPC showing an immature immunophenotype compatible with neutrophil-, monocytic-, erythroid-, MC-, pDC-, basophil-, and BCP-committed precursors, in 64 ISM patients grouped according to the presence vs absence of skin lesions at diagnosis, and the pattern of BM involvement of haematopoiesis by the *KIT* D816V mutation. In a subset of

18 patients we investigated distinct (highly-purified) PB cell compartments for the presence of the *KIT* mutation.

MATERIALS AND METHODS.

Patients, controls and samples. Sixty-four ISM patients newly-diagnosed (2) at the reference centres of the Spanish Network on Mastocytosis (REMA; Mast Cell Unit, Hospital del Valle, Toledo, and; Cancer Research Centre, Salamanca, Spain), were studied. Patients were classified at diagnosis according to the pattern of involvement of distinct non-MC myeloid and lymphoid BM hematopoietic cell compartments (13) by the *KIT* mutation into 41 ISM cases with *KIT* mutation restricted to BM MC (ISM_{MC}) and 23 ISM patients presenting with multilineage *KIT* mutation (ISM_{ML}) assessed by a peptide nucleic acid (PNA)-mediated PCR-clamping method;(3) in ISM_{MC} cases, *KIT* allele burden values in whole PB were systematically <6%.(5) No patient had received cytotoxic therapy. In every case, careful skin examination was performed by highly-specialized clinicians at the REMA, skin biopsy being performed only in cases with ≥1 suspicious skin lesion(s). In parallel, paired normal BM and PB samples from 14 healthy adults (excluding individuals with past or present history of allergy) were studied. Patient (vs controls) demographics, baseline serum tryptase levels and the percentage of BM infiltration by MC at the moment of the study are shown in Table S1. Paired EDTA-anticoagulated BM and PB samples were collected in every case, after written informed consent was given by each donor according to the guidelines of the local Ethics Committee and the Declaration of Helsinki.

Detection of the *KIT* mutation. The *KIT* D816V mutation was investigated in all patients in FACS-purified (≥97% purity) BM MC, eosinophils, monocytes, neutrophils, CD34⁺ HPC and T cells -FACSaria, Becton/Dickinson Biosciences (BD), San José, CA-, using a PNA-PCR method with a sensitivity of 10⁻³;(3) in a subset of 18 patients, the *KIT* D816V mutation was also evaluated in highly-purified circulating PB CD34⁺ cells, neutrophils, eosinophils, monocytes and/or T-cells, using an allele-specific oligonucleotide quantitative PCR (ASOqPCR) approach with a greater sensitivity of <10⁻⁴, as previously described.(5, 24)

Immunophenotypic studies. Conventional diagnostic immunophenotypic BMMC studies were performed in every case at diagnosis, in parallel to the phenotypic characterization of PB CD34⁺

HPC. However, for the analysis of the levels of expression of individuals markers only cases in which exactly the same antibody clones and reagents lots were considered (n=4 healthy controls and SM cases depending on the marker between 41 and 43) as previously described in detail.(25, 26). The following combinations of monoclonal antibodies (Mab) in 6-8-color stainings were systematically used for the characterization of CD34⁺ HPC in BM and PB: i) CD9 CD45 CD36 CD105 CD34 CD19 CD38 CD71; ii) - CD45 nuclear (Nu) TdT cytoplasmic (cy) MPO CD34 CD19 CD33 – and; iii) HLA-DR CD45 CD25 CD203c CD34 CD117 CD123 - (Table S2).

Flow cytometry data acquisition was performed, as previously described.(18) in two consecutive steps immediately after sample preparation using a FACSCanto II™ instrument (BD) and the FACSDiva software (BD) for a mean of 4X10⁶ events including ≥2,000 BM and ≥100 PB CD34⁺ HPC. For data analysis, the INFINICYT™ software (Cytognos SL, Salamanca, Spain) was used. CD34⁺ HPC were identified based on their unique light scatter features and CD45^{lo/int} expression levels; for standardized gating, residual mature lymphocytes and neutrophils were used as a reference. Identification of BM and PB subsets of CD34⁺ HPC (Figure 1A and 1B) was based on the following phenotypic features: 1) B-cell precursors were identified as CD45^{lo/int} NuTdT⁺ CyMPO⁻ CD19⁺ cells with forward light scatter (FSC)^{lo} and side light scatter (SSC)^{lo} characteristics;(27) 2) neutrophil precursors were CD45^{lo/int} NuTdT⁻ CyMPO⁺ CD19⁻ cells with higher FSC and SSC features than other CD34⁺ HPC;(28) 3) nucleated erythroid precursors were identified as CD45^{lo} CD36⁺ CD105^{-/+} CD19⁻ CD71⁺ cells;(29) 4) pDC precursors displayed a HLA-DR^{hi} CD203c⁻ CD123^{int/hi} immunophenotype;(30) 5) basophil precursors were CD117^{-/+} HLA-DR^{-/+} CD203c⁺ CD123^{int/hi} CD34⁺;(31) and; 6) MC-committed CD34⁺ HPCs displayed a unique CD117^{hi} HLA-DR^{-int} CD203c⁺ phenotypic profile.(17, 18, 32) A minimum of 10 cellular events were required to establish the presence of cells within each subset of CD34⁺ HPC defined above. The remaining CD34⁺ precursors (other CD34⁺ cells) corresponded to CD34⁺ cells which did not show any of the phenotypic profiles described above.

Statistical methods. The Kruskal-Wallis and the Mann-Whitney U tests (for continuous variables) or the chi-square test (for categorical variables), were used to establish the statistical significance of differences among groups. The degree of correlation between different variables was assessed by the Spearman's rank sum test. Statistical significance was set at P values <.05 (SPSS 18.0 software, PASW Statistics for Windows, Version 18.0. Chicago, IL).

RESULTS.

Distribution of BM and PB MC in ISM. Overall, ISM patients showed an increased percentage of MC in the BM vs. healthy subjects ($p < 0.001$) (Table S1 and Figure 2). In addition, BM MC from ISM patients had significantly higher SSC values than normal BM MC ($p = 0.006$) (Figure 2C). In contrast, BM MC from ISM patients showed normal levels of expression of CD117, FcεRI and HLA-DR (Figure 2D-F). Despite the above, variable BM MC percentages and BM MC SSC, CD117 and FcεRI features were observed among the distinct subgroups of ISM patients analysed. Thus, BM MC numbers were increased in all subgroups of ISM patients vs. healthy adults ($p = 0.001$), such increase being significantly more pronounced among ISM_{ML} vs. both ISMs_{-MC} ($p = 0.003$) and ISMs_{+MC} patients ($p = 0.03$). Moreover, BM MC from ISM_{ML} patients also displayed lower CD117 ($p < 0.001$) and FcεRI levels than found in ISMs_{-MC} patients ($p = 0.03$).

The presence of extremely low levels of cells showing a MC-like phenotype (≤ 0.43 cells/ μ L) in PB was restricted to a few controls (4/14; 29%) and ISM patients (16/64; 25%) with median (range) counts of 0 cells/ μ L (0–0.43 cells/ μ L) vs. 0 cells/ μ L (0–0.31 cells/ μ L), respectively ($p > 0.05$). Interestingly, a significant correlation was observed among ISM patients between the percentage of PB MC-like cells and both a greater BM MC load ($r_s = 0.44$; $p < 0.001$) and baseline serum tryptase levels ($r_s = 0.33$; $p < 0.009$) (Table S3).

Distribution of MC-committed CD34⁺ HPC in the BM and PB of ISM patients. The percentage of BM MC-committed CD34⁺ HPCs was significantly increased vs. healthy controls among ISMs_{-MC} (Figure 3B) while they were at almost normal values among ISM_{ML} ($p > 0.05$ vs. controls) (Figure 3B). Of note, while almost every healthy subject and ISMs_{-MC} patient analysed showed MC-committed CD34⁺ HPC in PB, ISM_{ML} patients displayed significantly lower frequencies of cases with circulating CD34⁺ HPC showing a MC-committed CD117^{hi} phenotype ($p = 0.03$ and $p = 0.007$ vs. healthy subjects and ISMs_{-MC} patients, respectively) (Figure S1A). Moreover, ISMs_{-MC} patients also showed increased absolute counts of MC-committed CD34⁺ HPC in PB ($p = 0.002$ vs. healthy subjects) (Figure 4B), vs. both ISMs_{+MC} ($p = 0.006$) and ISM_{ML} cases ($p < 0.001$) who showed almost normal values (Figure 4B). Interestingly, the overall number of both BM and PB MC-committed CD34⁺ HPCs inversely correlated with serum baseline tryptase (sBT) $-r_s = -0.33$ ($p = 0.01$) and $r_s = -0.27$ ($p = 0.03$), respectively; in contrast, no

correlation was found between the percentage of BM MC and the number of neither BM nor PB MC-committed CD34⁺ HPCs (Table S3). From the clinical point of view, ISM patients who showed no MC-committed CD34⁺ HPCs in their BM, more frequently displayed organomegalies (50% vs. 6.3%, $p=0.001$), particularly hepatomegaly and/or splenomegaly (50% vs. 3.7%, $p<0.001$), together with a higher frequency of multilineage *KIT* mutation (83% vs. 31%, $p=0.01$) vs. ISM patients who showed MC-committed CD34⁺ HPC in their BM (Table S4). However, no differences were found between ISM patients with vs. without MC-committed BM CD34⁺ HPC regarding the frequency of anaphylaxis, bone lesions, skin and gastrointestinal symptoms (Table S4).

Distribution of non-MC committed subsets of BM and PB CD34⁺ HPC in ISM. Regarding other non-MC committed subsets of BM CD34⁺ HPC, ISM_{ML} patients showed lower percentages of CD34⁺ CD123^{hi} HLA-DR^{hi} pDC and CD34⁺ MPO⁺ neutrophil precursors vs. ISMs_{+MC} cases ($p=0.004$ and $p=0.01$, respectively)- (Figure 3C and 3F); such decrease was at the expenses of a greater percentage of CD34⁺ CD19⁺ B-cell precursors ($p=0.03$ vs ISMs_{-MC} and $p=0.004$ vs ISMs_{+MC}, respectively) (Figure 3D). In contrast, no significant differences were found between healthy subjects and ISM patients regarding the overall percentage of PB CD34⁺ HPC and their distinct non-MC subsets. Nevertheless, ISM patients had lower PB absolute counts of CD34⁺ MPO⁺ HPC committed to the neutrophil lineage ($p=0.003$ vs. healthy subjects) (Figure 4C), independently of the patient group ($p=0.01$ for ISMs_{-MC}, $p=0.04$ for ISMs_{+MC} and $p=0.04$ for ISM_{ML} vs. healthy controls, respectively).

A more detailed analysis showed that in addition to the greater number of PB MC-committed CD34⁺ HPCs, ISMs_{-MC} cases also had significantly ($p\leq 0.04$) increased absolute counts of total CD34⁺ HPC and other (i.e. uncommitted) CD34⁺ cells vs. all other control and patient groups (Figure 4A-D), together with lower percentages of cases showing circulating PB neutrophil lineage-committed CD34⁺ MPO⁺ HPC (vs. controls) (Figure S1B). In turn, this was translated into lower frequencies of PB CD34⁺ MPO⁺ HPC (neutrophil lineage committed precursors) among all ISM groups vs. controls ($p\leq 0.04$) (Figure S1B). In contrast, similar frequencies of controls and ISM cases showing PB CD34⁺ HPC phenotypically committed to other hematopoietic cell lineages (e.g. erythroid- pDC- and basophil-lineage committed CD34⁺ precursors) (Figure S1C - F) were found.

Presence of the *KIT* D816V mutation in different subsets of PB leucocytes and circulating CD34⁺ HPC in ISM. In 18 ISM patients (9 ISMs⁻_{MC}, 5 ISMs⁺_{MC} and 4 ISM_{ML}), we further investigated the *KIT* D816V mutation in highly-purified PB CD34⁺ HPC, eosinophils, monocytes, neutrophils and/or T-cells (Table 1). Overall, 4/9 (44%) ISMs⁻_{MC}, 5/5 (100%) ISMs⁺_{MC} and 4/4 (100%) ISM_{ML} patients tested positive for the mutation in ≥1 PB cell population: 9/43 cell populations (21%) tested positive among ISMs⁻_{MC} patients, 14/24 (58%) within ISMs⁺_{MC} and 13/14 (93%) among ISM_{ML} patients (ISMs⁻_{MC} vs. ISMs⁺_{MC}, p=0.002; ISMs⁺_{MC} vs. ISM_{ML}; p=0.02 and ISMs⁻_{MC} vs. ISM_{ML}, p<0.001), with variable patterns of positivity among different cases (Table 1). In detail, PB CD34⁺ HPC were positive for the *KIT* mutation in all (n=4/4) ISM_{ML} patients, vs. 2/9 (22%) ISMs⁻_{MC} and 3/5 (60%) ISMs⁺_{MC} cases. In turn, *KIT*-mutated T-cells were only detected among ISM_{ML} patients (n=3/3), whereas the mutation was positive in PB monocytes and/or neutrophils, from 25% and 22% ISMs⁻_{MC} patients vs. virtual all ISMs⁺_{MC} (80% and 100%, respectively) and ISM_{ML} patients (100% and 100%) (Table 1). Of note, in most cases that showed *KIT*-mutated PB eosinophils, this cell population displayed the highest allele burden from all PB cell populations investigated, including those 3/3 ISMs⁻ patients that tested positive for the *KIT* mutation in PB eosinophils (Table 1).

DISCUSSION.

Recent studies (5, 15) have demonstrated the presence of the *KIT* mutation in PB from most SM patients at diagnosis, including almost all ISMs⁺ and ASM patients, and around two-thirds of ISMs⁻ cases. Based on previous studies (33) and careful examination of the patients skin, ISM patients were subdivided here into ISMs⁻ and ISMs⁺. In the majority of ISMs⁺ and virtually all ISMs⁻ cases that carry the *KIT* D816V mutation, this mutation is restricted to MC and CD34⁺ HPC in the BM,(3) as also confirmed here. Circulating PB MC have been recurrently reported in advanced SM;(21, 34) in contrast, they have not been detected in PB of most ISM patients and healthy individuals, in line with our findings. Despite this, using a high-sensitive (sensitivity of 5X10⁻⁵), here we show for the first time the presence of extremely low numbers of cells showing a MC-like phenotype in PB of ~20% of the healthy controls and ISM patients analysed; their presence in ISM, significantly correlated with the number of BM MC and sBT.

These results support the increased frequency of MC in PB of ISM patients with higher MC-tumour load and more advanced disease, although the MC nature of the cells here identified deserves further investigations. In addition, these results also suggest that in the absence of circulating MCs in most ISM patients, PB cells other than MC would account for the presence of the *KIT* mutation in PB of most ISM cases.(35, 36) Thus, in the absence of multilineage involvement by the *KIT* mutation, CD34⁺ HPC emerge as the most probable candidate cell compartment to both carry the mutation and spread the disease via PB, throughout the BM, and other tissues (e.g. the skin). So far, the presence of the *KIT* mutation has been reported in some PB cell compartments (i.e. monocytes, T- and B-cells) (7) but not in circulating CD34⁺ HPC. Similarly, no study has analysed the potential association between the degree of involvement of BM haematopoiesis by the *KIT* mutation and the distribution of different maturation-associated compartments of BM and PB CD34⁺ HPC defined on phenotypic grounds.

Currently, it is well-established that CD34⁺ HPC preferentially localize in the BM, most of these cells being already committed to different lymphoid and myeloid lineages.(19) Despite this, immature (uncommitted) CD34⁺ HPC migrate to distant BM niches via PB, to guarantee adequate production of hematopoietic cells throughout the whole BM;(37) in parallel, MC-committed CD34⁺ HPC also migrate via the blood stream to different tissues including the skin and BM, where final maturation into functional MCs will take place.(20, 38, 39) In line with this, Dahlin *et al.* showed that MC progenitors from PB can survive, mature, and proliferate *in vitro* in the absence of stem cell factor (SCF) and *KIT* signalling.(40)

As expected, our results showed that ISM patients had increased percentages of BM CD34⁺ HPCs showing a CD117^{hi} HLA-DR-/int CD203c+ phenotype (vs. healthy controls), particularly among ISMs_{-MC} and ISMs_{+MC} patients, but not ISM_{ML} cases. Of note, a similar profile was also observed in PB, where ISMs_{-MC} patients, but not ISMs_{+MC} and ISMs_{+ML} cases, showed increased counts of MC-committed CD34⁺ HPCs. Overall, these results resemble those of Georgin-Lavialle *et al.*, who reported an increased rate of detectable circulating MC precursors in PB in SM vs. both healthy donors and cutaneous mastocytosis (CM). In the study of Georgin-Lavialle *et al.*, a greater frequency of cases showing circulating MC precursors was found among ASM and SM-AHNMD patients vs. ISM, while here a clearly distinct pattern was observed with lower numbers of MC-committed CD34⁺ HPCs in ISM_{ML} vs. ISM_{MC} patients. Such

apparent discrepancy might be due to the fact that Georgin-Lavialle *et al.*,(41) investigated a population of circulating CD34⁺ CD117⁺ MC precursors and not CD34⁺ CD117^{hi} (HLA-DR^{int} CD203c⁺) cells showing a MC-committed phenotype as done here; such methodological differences might lead to the detection of more mature CD34⁺ MC precursors (or even MC) among ASM, MCL and ASM-AHNMD patients by Georgin-Lavialle.(41) In turn, Dahlin *et al.*(42) have recently described a rare population of Lin⁻ CD34^{hi} CD117^{int/hi} FcεRI⁺ MC progenitors in PB of healthy adults and patients with well-controlled asthma which are both numerically (≈ 0.001% vs. 0.001%) (40) and phenotypically fully coincident with the MC-committed CD34⁺ HPC population here identified in healthy controls and ISM patients (data not shown), including low FcεRI⁺ expression assessed here only in a subset of cases.(21, 34, 43)

Of note, while the frequency of cases with circulating MC-like cells did not vary significantly among the different subgroups of ISM, the number of PB CD34⁺ CD117^{hi} HPC showing a MC-committed phenotype decreased from ISMs⁻ to both ISMs⁺MC and ISMs⁺ML cases. Although this could reflect a decreased ability to produce MC among ISM_{ML} vs. ISMs⁻ cases, it might also be due to a greater migration rate of MC-committed CD34⁺ HPC out of the blood to e.g. the skin, BM and other tissues, with decreased overall PB numbers. In line with this later hypothesis, here we observed both i) a direct correlation between sBT and the percentage of BM MC and more immature MC phenotypes (decreased SSC, FcεRI and CD117) which are typical features of ISM cases with greater tumour mass and multilineage involvement of BM haematopoiesis by the *KIT* mutation (14) and ii) an inverse correlation between sBT and the number of both BM and PB MC-committed CD34⁺ HPC. Altogether, these results might point out a preferential commitment of CD34⁺ HPC towards the MC lineage both in PB and BM at the earliest disease stages (i.e. ISMs⁻), followed by an increased migration of MC-committed CD34⁺ precursors to tissues where tumour-MC accumulate in ISMs⁺. Alternatively, it might also reflect an early blockade of maturation of CD34⁺ HPC towards the MC lineage, once the *KIT*-mutation involves hematopoietic cell lineages other than MC, with infiltration of tissues other than BM, leading also to a greater frequency of hepatomegaly and/or splenomegaly, as reported here. In contrast, other signs and symptoms of the disease (e.g. pruritus, abdominal pain/cramping, bone lesions and anaphylaxis) did not correlate with the absence of BM MC-committed CD34⁺ HPC, in line with previous findings showing that a higher D816V *KIT* mutation

allele burden, despite being associated with more advanced disease, did not correlate with these clinical manifestations of ISM.(44) In contrast to our findings, the frequency of CD34⁺ PB MC precursors identified by Dahlin *et al.* (40) was similar between controls and SM patients. However, this apparent discrepancy might be due to the subtype of SM patients analyzed by Dahlin *et al.* (40) as they might correspond to the most common ISMs⁺ variant of SM which also showed here a percentage of MC-committed CD34⁺ HPC similar to healthy controls.

Overall, ISM_{ML} patients also showed lower percentages of BM CD34⁺ HPC committed to both the neutrophil and pDC lineages, at the expense of a greater CD34⁺ B-cell precursor counts vs. ISM_{MC} cases. Similarly, ISM_{ML} patients displayed lower PB absolute counts of neutrophil-committed CD34⁺ HPC vs. healthy subjects, a feature shared by other ISM_{MC} cases. In contrast, ISMs_{MC} patients had significantly higher numbers of PB CD34⁺ HPC and uncommitted CD34⁺ cells vs. controls and other ISM patients. These results suggest that among circulating CD34⁺ HPC, the presence of the *KIT* mutation could initially favor their maturation towards cell lineages other than the neutrophil lineage, particularly to the MC lineage, while at more advanced disease stages, increased migration of CD34⁺ MC precursors outside PB and/or progressive blockade of maturation to distinct myeloid lineages might occur, similarly to what has been reported for other myeloid malignancies.(18, 45, 46)

In order to test this hypothesis, we investigated the presence of the *KIT* mutation in distinct compartments of PB cells, including CD34⁺ HPC, in a subset of patients. As expected, all ISMs_{MC} and ISM_{ML} patients had ≥1 PB cell population that tested positive for the mutation, while this only occurred in around half of all ISMs_{MC} cases investigated, in line with previous findings in whole PB leukocytes.(5) Moreover, progressively higher numbers of *KIT*-mutated cell populations with increasingly higher allele burden were detected from ISMs_{MC} to ISMs_{MC} and ISM_{ML} patients. Thus, PB CD34⁺ HPC from all ISM_{ML} patients were positive for the *KIT* mutation, while only a fraction of cases with the *KIT* mutation restricted to BM MC carried the *KIT* mutation in PB circulating CD34⁺ HPC; similarly, while ISM_{ML} cases systematically showed *KIT* mutated T-cells in PB, these systematically had wild-type *KIT* among ISM_{MC} patients. In parallel, the frequency of *KIT*-mutated PB monocytes and neutrophils progressively increased from ISMs_{MC} and ISMs_{MC} to ISM_{ML} and, while almost every cell compartment was mutated among ISM_{ML} cases, a more heterogeneous mutational profile was observed in ISMs_{MC} and ISMs_{MC}

patients. Unexpectedly however, a fraction of ISM_{MC} (i.e. MC-restricted *KIT* mutation in BM) showed *KIT*-mutated PB neutrophils and monocytes. This apparently discrepancy between the PB and BM results is probably due to the distinct sensitivity of the conventional routine diagnostic PNA PCR method used to analyze BM cells and establish multilineage involvement of hematopoiesis by the D816V *KIT* mutation (10^{-3}) vs that of the more sensitivity ASOqPCR assay used for the evaluation of the purified PB monocytes and neutrophils (10^{-4}).⁽⁵⁾ Most interestingly, among the distinct mutated cell compartments, eosinophils were those usually showing the highest allele burden, particularly among ISMs- cases. Altogether, these results suggest that circulating PB CD34⁺ cells could be responsible for the dissemination of SM throughout the BM and to other tissues (i.e. the skin) at the same time they might contribute, together with other hematopoietic cell compartments, to the high rate of positivity for the *KIT* D816V mutation in the PB of ISM patients, particularly among ISMs- cases. Despite this, normal residual wild-type *KIT* haematopoiesis still appears to predominate in ISM as a rather low *KIT* allele burden was detected among circulating PB CD34⁺ HPC from most patients; the reason for the discrepancies observed in a fraction of ISM patients, regarding multilineage involvement in PB vs BM, deserves further investigations.

Authorship Contributions: A. M. and C. T. performed experiments, analysed the data, interpreted the results, made the figures and wrote the paper; M. J-A. performed *KIT* mutation experiments and analysed/interpreted the results; N. D. participated in the organization of the samples and performed *KIT* mutation experiments; AC. G-M. analysed data, interpreted results and critically reviewed the paper; I. A-T. and A. M. recruited the patients and controls, followed the patients and critically reviewed the paper; L. S-M. collected the samples and critically reviewed the paper; C. C. and JI. M-G. contributed with technical support; A. H. and JI. S-G. critically reviewed the paper; L. E. supervised the study, performed clinical follow-up of the patients and critically reviewed the paper; A. O. designed the research, supervised the study and wrote the paper.

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Table 1. Distribution of the *KIT* mutation in highly-purified circulating CD34⁺ HPC and other mature subsets of PB leucocytes.

Diagnostic subtype	BM <i>KIT</i> mutation profile	N of CD34 ⁺ HPC other cells analyzed*	PB CELL POPULATION					
			CD34 ⁺ HPCs	Eosinophils	Monocytes	Neutrophils	T-cells	Total
ISM _s -	MC	2,150/15,000	-	-	NT	-	NT	-
ISM _s -	MC	1,950/15,000	-	-	-	-	-	-
ISM _s -	MC	2,600/15,000	-	-	-	-	-	-
ISM _s -	MC	1,300/15,000	-	-	-	-	-	-
ISM _s -	MC	561/15,000	-	-	-	-	-	-
ISM _s -	MC	6,000/15,000	-	-	+ (0.26%)	-	-	+
ISM _s -	MC	3,600/15,000	-	+ (4.5 %)	-	-	-	+
ISM _s -	MC	652/15,000	+ (0.3%)	+ (10%)	-	+ (0.2%)	-	+
ISM _s -	MC	4,500/15,000	+ (2.4%)	+ (17.1%)	+ (0.29%)	+ (0.75%)	-	+
Total <i>KIT</i>+ ISM_s-_{MC} cases			2/9 (22%)	3/9 (33%)	2/8 (25%)	2/9 (22%)	0/9 (0%)	4/9 (44%)
ISM _s +	MC	2,400/15,000	-	-	-	+ (0.23%)	-	+
ISM _s +	MC	3,000/15,000	-	+ (1.8%)	+ (2.4%)	+ (0.7%)	-	+
ISM _s +	MC	900/15,000	+ (0.03%)	NT	+ (1%)	+ (0.69%)	-	+
ISM _s +	MC	1,000/15,000	+ (0.02%)	+ (4%)	+ (2.36%)	+(0.50%)	-	+
ISM _s +	MC	1,300/15,000	+ (0.20%)	-	+ (1.01%)	+ (0.24%)	-	+
Total <i>KIT</i>+ ISM_s+-_{MC} cases			3/5 (60%)	2/4 (50%)	4/5 (80%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
ISM _s +	ML	360/15,000	+ (4%)	NT	+ (0.9%)	+(0.37%)	+ (1.8%)	+
ISM _s +	ML	1,200/15,000	+ (1%)	NT	+ (8.1%)	+(0.66%)	+ (0.18%)	+
ISM _s +	ML	2,200/15,000	+ (14%)	-	NT	+ (4.1%)	+ (0.63%)	+
ISM _s +	ML	3,150/15,000	+ (9%)	NT	+ (56%)	NT	NT	+
Total <i>KIT</i>+ ISM_s+-_{ML} cases			4/4 (100%)	0/1 (0%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	4/4 (100%)
Total cases			9/18 (50%)	5/14 (36%)	9/16 (56%)	10/17 (59%)	3/16 (19%)	13/18 (72%)

Results expressed as absence (-) vs. presence (+) of *KIT* mutation in each cell population analyzed and percentage of positive cells (i.e. *KIT* allele burden). *The number of PB eosinophils, monocytes, neutrophils and T-cells analyzed per patient was always of 15×10^3 for each population. PB, peripheral blood; BM, bone marrow; ISMs-, indolent systemic mastocytosis without skin lesion; ISMs+, indolent systemic mastocytosis with skin lesion; MC, mast cells; NQ, not quantifiable; NT, not tested.

FIGURE LEGENDS.

Figure 1. Bivariate flow cytometry dot plot histograms illustrating the most relevant phenotypic profiles used for the identification of different subsets of bone marrow (BM; panel A) and peripheral blood (PB; panel B) $CD34^+$ hematopoietic progenitor and precursor cells (HPC) from a representative ISM patient.

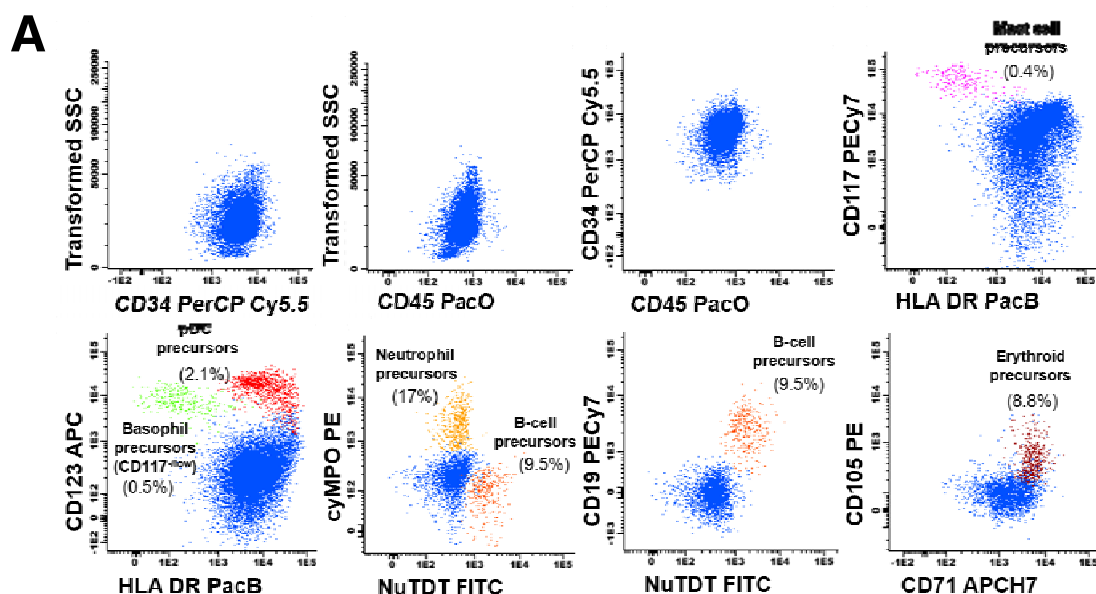
Figure 2. Percentage of bone marrow (BM) (panel A) and peripheral blood (PB) mast cells (MC) (B) and their most relevant phenotypic features in the BM (panels C, D, E and F) from adult indolent systemic mastocytosis (ISM) patients grouped according to the pattern of involvement of BM haematopoiesis by the *KIT* mutation and the presence vs absence of skin lesions (vs healthy controls). Notched-boxes extend from the 25th to the 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles, respectively. P-value <.05 * vs controls, Δ vs ISMs-_{MC} cases and *f* vs ISMs+_{MC} patients. ISMs-_{MC}, indolent systemic mastocytosis presenting without skin lesions and *KIT* mutation restricted to BM mast cells; ISMs+_{MC}, indolent systemic mastocytosis presenting with skin lesions and *KIT* mutation restricted to BM mast cells; ISM_{ML}, indolent systemic mastocytosis with multilineage *KIT* mutation.

Figure 3. Distribution of total $CD34^+$ HPC and $CD34^+$ mast cell (A and B), plasmacytoid dendritic cell (pDC) (C), B-cell (D), basophil (E), neutrophil (F), erythroid (G) and other $CD34^+$ HPC (H) in the bone marrow (BM) of adult indolent systemic mastocytosis (ISM) patients grouped according to the pattern of involvement of BM haematopoiesis by the *KIT* mutation and the presence vs absence of skin lesions (vs healthy controls). Notched-boxes extend from the 25th to the 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles, respectively. P-value <.05 * vs controls, Δ vs ISMs-_{MC} cases and *f* vs ISMs+_{MC} patients. ISMs-_{MC}, indolent systemic mastocytosis presenting without skin lesions and *KIT* mutation restricted to BM mast cells; ISMs+_{MC}, indolent systemic mastocytosis presenting with skin lesions and *KIT* mutation restricted to BM mast cells; ISM_{ML}, indolent systemic mastocytosis with multilineage *KIT* mutation.

Figure 4. PB absolute count of total CD34⁺ hematopoietic stem and precursor cells (HPC) (A) and CD34⁺ mast cell-(B), neutrophil-(C) and other (D) HPC subsets in adult indolent systemic mastocytosis (ISM) patients grouped according to the pattern of involvement of BM haematopoiesis by the *KIT* mutation and the presence vs. absence of skin lesions (vs healthy controls). Notched-boxes extend from the 25th to the 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles, respectively. P-value <.05 * vs controls and Δ vs ISMs^{-MC} cases. ISMs^{-MC}, indolent systemic mastocytosis presenting without skin lesions and *KIT* mutation restricted to BM mast cells; ISMs^{+MC}, indolent systemic mastocytosis presenting with skin lesions and *KIT* mutation restricted to BM mast cells; ISM_{ML}, indolent systemic mastocytosis with multilineage *KIT* mutation.

Figure S1. Frequency of cases showing CD34⁺ mast cell-(A), neutrophil-(B), erythroid-(C), basophil-(D), B-cell (E) and plasmacytoid dendritic cell-(pDC) (F) committed precursors in peripheral blood among adult indolent systemic mastocytosis (ISM) patients grouped according to the pattern of involvement of BM haematopoiesis by the *KIT* mutation and the presence vs absence of skin lesions (vs healthy controls). P <.05 * vs controls and Δ vs ISMs^{-MC} cases. ISMs^{-MC}, indolent systemic mastocytosis presenting without skin lesions and *KIT* mutation restricted to BM mast cells; ISMs^{+MC}, indolent systemic mastocytosis presenting with skin lesions and *KIT* mutation restricted to BM mast cells; ISM_{ML}, indolent systemic mastocytosis with multilineage *KIT* mutation.

Figure 1.



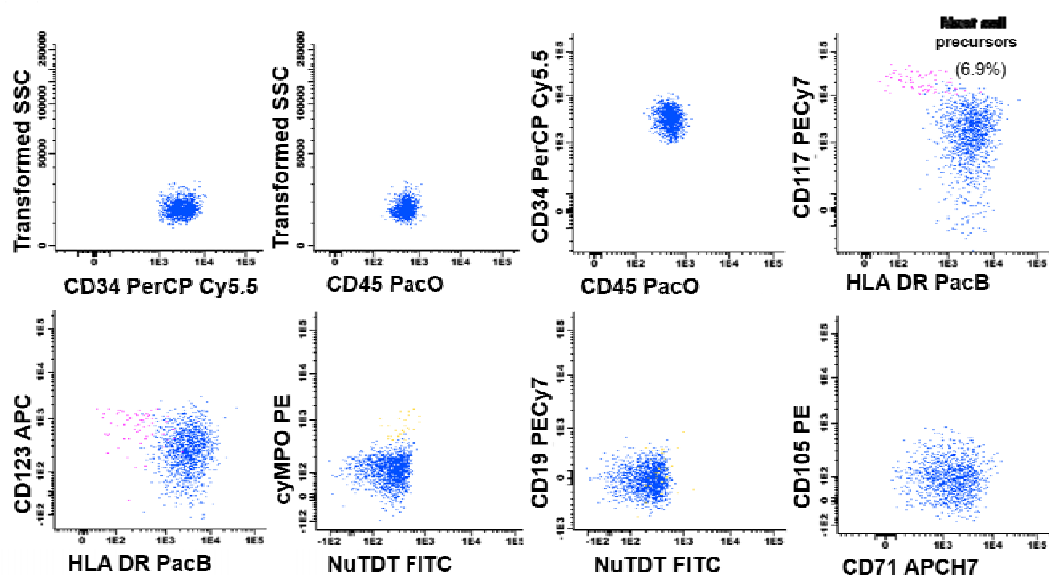
B

Figure 2.

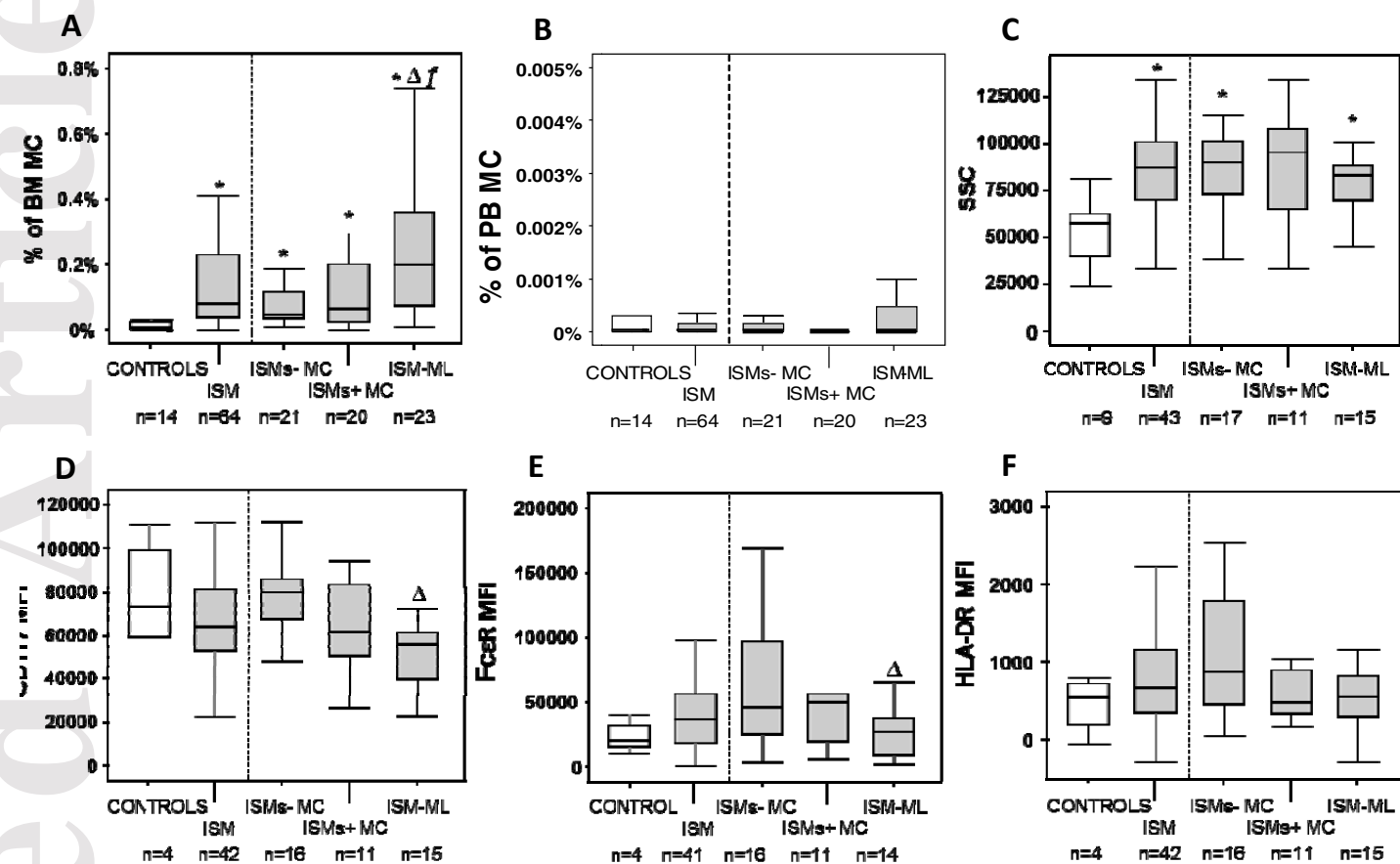


Figure 3.

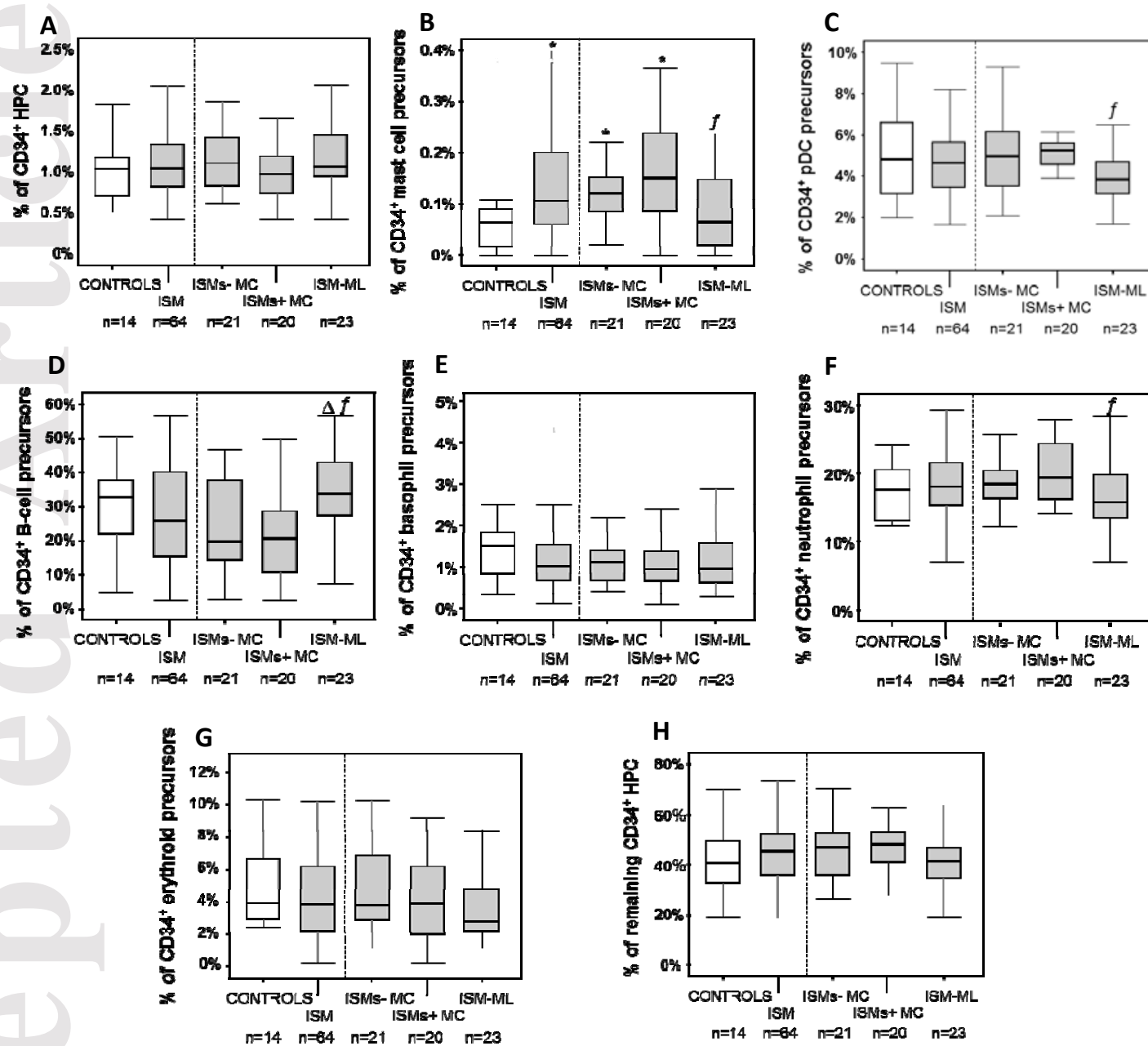
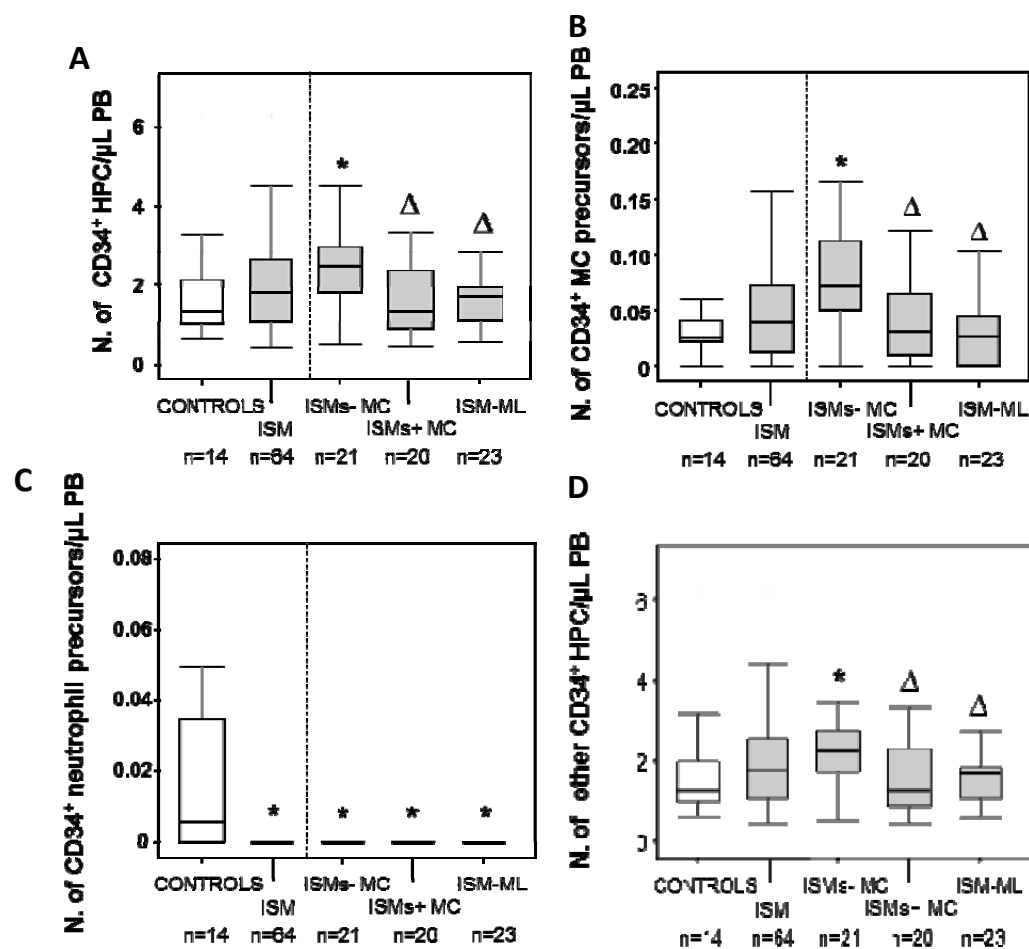


Figure 4.



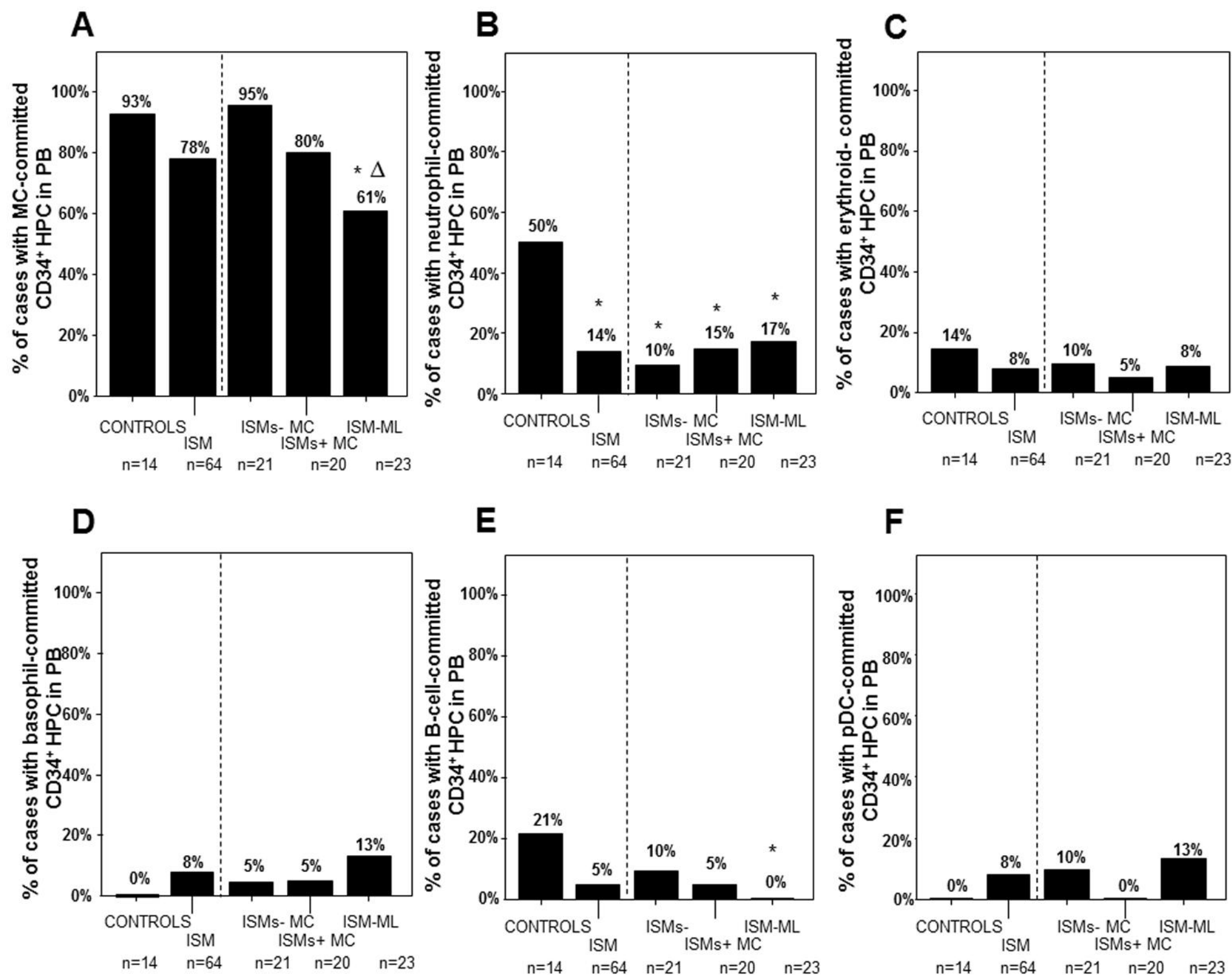


Table S1. Demographics, serum tryptase levels and percentage of BM MC in controls (n=14) vs adult indolent systemic mastocytosis patients (n=64) distributed according to the presence vs absence of skin lesions and the pattern of involvement of BM hematopoiesis by the *KIT* mutation.

Variable	Controls (n=14)	ISM (n=64)	P value	ISMs ⁻ _{MC} (n=21)	ISMs ⁺ _{MC} (n=20)	ISM _{ML} (n=23)	P value
Age (y) ¹	52±17	47±11	NS	46±9	43±10	50±12	NS
Sex (M/F)	5/9 (36%/64%)	29/35 (45%/55%)	NS	13/8 (62%/38%)	6/14 (30%/70%)	10/13 (44%/56%)	NS
Serum tryptase (ng/ml) ¹	10.0±5.0	54.1 ± 61.9	<0.001	28.1± 15.9	31.2±24.5	98±84.2	<0.001*,**
% BMMC ¹ by FCM	0.01%±0.01%	0.16%± 0.22%	<0.001	0.08%±0.07%	0.13%±0.15%	0.3%± 0.3%	0.03*,**

Results expressed as number of cases (percentage) or as¹mean ± one standard deviation. SM, systemic mastocytosis; ISMs⁻_{MC}, indolent systemic mastocytosis presenting without skin lesions and *KIT* mutation restricted to bone marrow (BM) MC; ISMs⁺_{MC}, indolent systemic mastocytosis presenting with skin lesions and *KIT* mutation restricted to bone marrow (BM) MC; ISM_{ML}, indolent systemic mastocytosis with multilineage *KIT* mutation; y, years; M, male; F, female; BM, bone marrow; MC, mast cell; FCM, flow cytometry; NS, statistically not significantly different (p>0.05).*, ISMs⁻_{MC} vs ISM_{ML}, and; **, ISMs⁺_{MC} vs ISM_{ML}.

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Table S2. List of monoclonal antibodies used for the immunophenotypic analysis of CD34⁺ HPC in both BM and PB samples from systemic mastocytosis patients and control subjects.

Antibody conjugate	Clone	Source
CD9-PacB	MEM-61	Exbio Antibodies ^a
CD19-PECy7	J3-119	Beckman Coulter ^b
CD25-FITC	2A3	BD Biosciences ^c
CD33-APC	P67.6	BD Biosciences ^c
CD34-PerCPCy5.5	8G12	BD Biosciences ^c
CD36-FITC	CLB-IVC7	Sanquin ^d
CD38-APC	HB-7	BD Biosciences ^c
CD45-PacO	HI30	Invitrogen ^e
CD71-APCH7	M-A712	BD Biosciences ^c
CD105-PE	1G2	Beckman Coulter ^b
CD117-PECy7	104D2D1	Beckman Coulter ^b
CD123-APC	AC145	Miltenyi Biotec ^f
CD203c-PE	97A6	Immunotech ^g
FcεRI-FITC	Polyclonal	Invitrogen ^e
HLA-DR-PacB	L243	Biolegend (Taper) ^h
MPO-PE	MPO-7	Dako ⁱ
TdT-FITC	HT-6	Dako ⁱ

Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; PacB, pacific blue; PE, phycoerythrin; PerCPCy5.5, peridinin chlorophyll protein-cyanine 5.5; PECy7, phycoerythrin-cyanine 7; PacO, pacific orange ^aExbio Antibodies (Vestec, Czech Republic); ^bBeckman Coulter (Miami, FL, USA); ^cBD Biosciences (San Jose, CA, USA); ^dSanquin (Amsterdam, The Netherlands); ^eInvitrogen (Carlsbad, CA, USA); ^fMiltenyi Biotec (Cologne, Germany); ^gImmunotech (Marseille, France); ^hBiolegend (San Diego, CA, USA); ⁱDako (Glostrup, Denmark).

Table S3. Indolent systemic mastocytosis: correlation between both the percentage of mast cells (MC) in the bone marrow (BM) and baseline serum tryptase levels and the percentage of peripheral blood (PB) MC and CD34⁺ BM and PB MC precursors.

	%BM MC		Serum tryptase (ng/ml)	
	r _s	P-value	r _s	P-value
% PB MC	0.44	<0.001	0.33	0.009
% BM CD34 ⁺ MC precursors	-0.17	NS	-0.33	0.01
% PB CD34 ⁺ mast cell precursors	-0.13	NS	-0.27	0.03

r_s, Spearman correlation coefficient; BM, bone marrow; MC, mast cell; PB, peripheral blood; NS, statistically not significantly different (p>0.05).

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Table S4. Indolent systemic mastocytosis: relationship between the presence vs absence of bone marrow mast cell-committed CD34⁺ precursors and the clinical features of the disease.

Disease features	ISM		P value
	Presence of CD34 ⁺ BM MC precursors (n=58)	Absence of CD34 ⁺ BM MC precursors (n=6)	
Anaphylaxis	38%	50%	NS
Organomegalies	6.3%	50%	0.001
Lymphadenopathies	6.3%	0%	NS
Hepatomegaly and/or splenomegaly	3.7%	50%	<0.001
Moderate/severe skin symptoms*	22%	0%	NS
Moderate/severe GI symptoms**	21%	17%	NS
Bone lesions***	14%	0%	NS
% cases with PB MC	3.5%	17%	NS
Multilineage <i>KIT</i> mutation	31%	83%	0.01

Results expressed as percentage of cases. ISM, indolent systemic mastocytosis; BM, bone marrow; PB, peripheral blood; MC, mast cells. NS, statistically not significantly different (p>0.05). *Pruritus; **Abdominal pain/cramping; ***Osteoporosis and/or osteopenia were not considered bone lesions.

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