ICCS e-Newsletter
CSI Case

Laura Sánchez-Muñoz, Jose Mario T. Morgado, Cristina Teodosio

Instituto de Estudios de Mastocitosis and
Centro de Investigación del Cáncer
Spanish Network on Mastocytosis
Spain
Case Summary
History and physical examination

- A 63-year-old male suffering from **skin lesions** 5 years ago (beginning in the back, chest and arms) that turn red with temperature changes. Biopsy: cutaneous mastocytosis.

- Occasional **flushing** without trigger. No abdominal symptoms, no anaphylactic episodes.

- Image probes: abdominal ultrasonography, normal, no organomegalies; bone densitometry: **osteoporosis**.

- Increased and maintained basal **serum tryptase**: >24ng/ml (normal <11.5ng/ml).
## Peripheral blood cell counts

<table>
<thead>
<tr>
<th>CBC</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WBC</strong>: 4.2 x 10^9/l</td>
<td>(4.5 – 11) *</td>
</tr>
<tr>
<td><strong>RBC</strong>: 4.66 x 10^{12}/l</td>
<td>(4.5 – 5.5)</td>
</tr>
<tr>
<td><strong>Hgb</strong>: 15.9 g/dl</td>
<td>(13.0 – 16.5)</td>
</tr>
<tr>
<td><strong>Hct</strong>: 45.7%</td>
<td>(40.0 – 54.0)</td>
</tr>
<tr>
<td><strong>MCV</strong>: 98.3fl</td>
<td>(80.0 – 99.0)</td>
</tr>
<tr>
<td><strong>MCH</strong>: 34.2pg</td>
<td>(27.0 – 31.0) *</td>
</tr>
<tr>
<td><strong>MCHC</strong>: 34.8g/dl</td>
<td>(33 – 37)</td>
</tr>
<tr>
<td><strong>RDW</strong>: 13.6%</td>
<td>(11.5 – 14.5)</td>
</tr>
<tr>
<td><strong>Plts</strong>: 204 x 10^9/l</td>
<td>(120 – 400)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CBC differential</th>
<th>Normal</th>
</tr>
</thead>
</table>

# Biochemical parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptase</td>
<td>24</td>
<td>5.5 – 13.5 mg/l</td>
</tr>
<tr>
<td>B2 microglobulin (mg/dl)</td>
<td>1.97</td>
<td>0.80 - 2.20 mcg/ml</td>
</tr>
<tr>
<td>LDH (IU/l)</td>
<td>243</td>
<td>230 - 530 mU/mL</td>
</tr>
<tr>
<td>Total IgE</td>
<td>5.33kU/l</td>
<td></td>
</tr>
</tbody>
</table>
Work-up and evaluation

• Bone marrow (BM) biopsy and aspiration in EDTA was performed under the suspicion of Systemic Mastocytosis (SM).

• Flow cytometric immunophenotyping was performed on this sample.

• Data acquisition was performed in a FACSCanto II, with FACS Diva (BDB); data analysis was done with the INFINICYT software (Cytognos SL).
## List of FCM tubes

### Acquisition in a FACSCanto II with FACS Diva:
Pac Blue / Pac Orange / FITC / PE / PerCP-Cy5.5/ PECy7 / APC / APCH7

<table>
<thead>
<tr>
<th>Tube 1</th>
<th>Control/ CD45/Control/Control /CD34/ CD117/ Control /Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 2</td>
<td>CD2 / CD45/ IgE / CD25 /CD34/ CD117/ CD203c /Control</td>
</tr>
<tr>
<td>Tube 3</td>
<td>HLA-DR/ CD45/ CD35 / CD59 /CD34/ CD117/ CD123 /Control</td>
</tr>
<tr>
<td>Tube 4</td>
<td>HLA-DR/ CD45/ CD63 / CD32 /CD34/ CD117/ CD123 /Control</td>
</tr>
</tbody>
</table>

| Tube 5          | HLA-DR / CD45/cyControl / cyControl / CD34/CD117/cyControl/Control |
| Tube 6          | HLA-DR/ CD45/cyTryptase/cyChymase/CD34/CD117/ cyCPA / Control    |
Recommended Analysis Strategy
**Tube 1: Analyze the presence of Mast Cells**

<table>
<thead>
<tr>
<th></th>
<th>Pac.B</th>
<th>Pac.O</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PECY7</th>
<th>APC</th>
<th>APCH7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tube 1</strong></td>
<td>Control</td>
<td>CD45</td>
<td>Control</td>
<td>Control</td>
<td>CD34</td>
<td>CD117</td>
<td>Control</td>
<td>Control</td>
</tr>
</tbody>
</table>

Control, unstained negative control.

At least two mAb to accurately identify MC are necessary.
Critical Parameter: a double-step data acquisition procedure is mandatory

1) in a first step, acquire and store $2-5 \times 10^4$ events, corresponding to the whole sample cellularity;
2) draw a live gate including CD117+ events

3) in a second step, a minimum of $10^2$–$10^3$ events falling in a CD117++ electronic live gate should be stored;
4) refine selection of Mast cells: CD117++, CD45+, CD34-

At least two mAb to accurately identify MC are necessary
Conclusion

Presence of 0.1% Mast cells can be identified on the bone marrow aspiration.

Are these mast cells immunophenotypically normal or aberrant?
**Tube 2: Analyze the presence of aberrant Mast Cells**

<table>
<thead>
<tr>
<th></th>
<th>Pac.B</th>
<th>Pac.O</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PECY7</th>
<th>APC</th>
<th>APCH7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tube 2</strong></td>
<td>CD2</td>
<td><strong>CD45</strong></td>
<td>IgE*</td>
<td><strong>CD25</strong></td>
<td>CD34</td>
<td>CD117</td>
<td>CD203c</td>
<td>CD69</td>
</tr>
</tbody>
</table>

*Indirectly observed by anti-IgE staining.

CD25 should be included to accurately distinguish normal vs. aberrant MC. Other markers (CD2, CD203c, CD69) are optional to define the complete immunophenotypical pattern of MC.
Tube 2: Analyze the presence of aberrant Mast Cells

Acquire only CD117+++ events

Identify Mast cells: CD117+++, CD45+, CD34-
characterize the immunophenotype of Mast cells: CD203c, CD25, CD2, IgER

Tube 2: Analyze the presence of aberrant Mast Cells (MC)

Once identified...
Always compare MC autofluorescence with the expression of mAb to correctly characterize MC immunophenotype.

Expression of mAb on BM MC

○ = Isotype control place
Interpretation of the results

• Percentage of bone marrow mast cells: 0.1%.

• Immunophenotype of BM mast cells: CD117++, CD45+, CD34-, Rlge+, CD25++, CD2-/+dim, CD203c++.

• Apart from mast cell population, the overall cellularity should be analyzed, to rule out the presence of myeloid displastic signs or even other hematological non-MC lineage disease.

PROBABLE DIAGNOSIS: SYSTEMIC MASTOCYTOSIS
• Differential diagnosis: presence of $CD25^{\text{bright+}}$ BM MC can only be found in a subset of Hypereosinophilic Syndromes (HES), characterized by the $FIP1L1$-$PDGRFA$ fusion, morphologically abnormal MC expressing CD25 and lacking on $KIT$ mutation.

• In our case, although no compact BM MC aggregates were detected (WHO major criterion), three minor WHO diagnostic criteria were detected:
  - morphologically atypical BM MC
  - CD25$^{\text{bright}}$ BM MC
  - D816V $KIT$ mutation on BM MC

FINAL DIAGNOSIS: SYSTEMIC MASTOCYTOSIS
Mastocytosis are “Rare Diseases” characterized by the presence of clonal MC in tissues.

- Organs most frequently affected are: skin, BM, bone, liver, spleen, lymph nodes, gastrointestinal tract.
- Symptoms and signs are related to the tissue MC burden, to the release of MC mediators or both.
- Codon 816 KIT somatic mutations present in almost all SM patients (except for WDSM and some MCL).
Diagnostic algorithm in suspected Mastocytosis

Complete clinical work-up. Allergologic work-up. Abdominal ultrasonography, DEXA scan. + Peripheral blood count, routine biochemistry, serum cholesterol, LDH, B₂-microglobulin, tryptase.

Bone marrow biopsy and aspirate

Morphology
Histology and Immunohistochemistry
FC Immunophenotype
Molecular biology in purified cell lineages

Integral diagnosis & Classification
**Cutaneous Mastocytosis:**
Typical skin lesions = typical clinical signs + positive histology with infiltrates of MC.

**Systemic Mastocytosis (SM):**

**Major criteria:**
1) Multifocal, dense infiltrates of mast cells (≥ de 15 MC in aggregates) detected in sections of bone marrow and/or other extracutaneous organ(s), by tryptase immunohistochemistry or other special stains.

**Minor criteria:**
1) >25% of MC in the infiltrates in BM or other organs are spindle-shaped or have atypical morphology.
2) Detection of c-kit point mutation at codon 816 in bone marrow, blood or other extracutaneous organ.
3) CD25/CD2 bright+ MCs in bone marrow, blood or other extracutaneous organs.
4) Serum total tryptase persistently >20 ng/ml (unless there is an associated clonal myeloid disorder, in which case this parameter is not valid).
Bone marrow biopsy: Histopathology

Tryptase staining (x500) of a BM biopsy specimen from Indolent SM without dense compact MC aggregates.

Small non-compact MC aggregate containing less than 15 MC and thus, not diagnostic for SM. It should be noted that there is a mixture of both round and spindle shaped MC.

This picture suggests (but not confirms) a diagnosis of SM.

Tryptase staining (x500) of a BM biopsy specimen from Indolent SM with dense compact MC aggregates.

Perivascular MC aggregate containing more than 15 mast cells and thus, fulfilling the WHO major criterion for SM.
CD25 staining (x500) of a BM biopsy specimen from Indolent SM.

Utility of the immunostaining of CD25 in SM.

Mast cell aggregates composed by round shaped MC can be seen. The expression of CD25 confirms the diagnosis of SM.

Tryptase staining (x100) of a BM biopsy specimen from SM.

Co-existence of diffuse MC infiltration (arrow) and hypercellular hematopoiesis (open arrow). This histological pattern strongly suggests the diagnosis of Aggressive SM.
Toluidine blue staining of a BM smear from SM (x400).

This image illustrates the value of toluidine blue in the identification of MC in BM smears. MC metachromatic granules are clearly shown, while other nucleated BM cells are stained in blue pale color. Mast cell aggregates are clearly seen as well as an admixture of round and spindle shaped MC.
Bone marrow smears: MC morphology

May-Grünwald Giemsa staining of a BM smear from SM (x600).

Abnormal MC (arrows) together with an admixture of eosinophils, lymphocytes and plasma cells.

Toluidine blue staining of a BM smear from SM (x600).

Atypical mast cells with metachromatic granules are shown.

Atypical MC may include not only spindle-shaped MC but also round or polygonal MC with eccentric oval nucleus, elongated cytoplasmic extensions, hypogranulated cytoplasm with focal granule accumulation with or without granule fusions phenomena.
FCM diagnosis of Mastocytosis

**Critical Parameters**

1. BM aspiration should be performed **firmly and quickly** to obtain a sufficient number of BM fragments.

2. Always perform BM aspiration and biopsy when suspect a Systemic Mastocytosis.

3. Heparinized or EDTA anticoagulated BM aspirate should be processed within the first 24h after collection.

4. Only **fresh samples** with high cell viability should be used for the enumeration of mast cells.

5. Assessment of the **basal MC autofluorescence** levels should always be performed, use either an unstained tube or appropriate fluorochrome-matched isotype controls.
FCM diagnosis of Mastocytosis

6. Staining of a BM smear containing BM particles with 1% Toluidine blue (ph: 0.5), to get an overall impression on the MC content of the sample, prior to immunophenotyping. If the BM MC load is very low, it is advisable to perform duplicates or triplicates of the staining tubes.
Panel of Monoclonal Antibodies (mAb)
• Depends on the flow cytometer facilities and the specific mAb reagents available.
• Recommended: a short screening panel for mastocytosis and a larger panel to be applied when aberrant MC are detected.
• For identification of the MC population a double staining with CD117 and CD45 is desirable.

Four color combinations recommended for immunophenotypic analysis of BM MC.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Step 1: screening of mastocytosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>Control</td>
<td>CD45</td>
<td>CD117</td>
</tr>
<tr>
<td>2</td>
<td>IgE*</td>
<td>CD25</td>
<td>CD45</td>
<td>CD117</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Step 2: further MC characterization panel</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CD35</td>
<td>CD59</td>
<td>CD45</td>
<td>CD117</td>
</tr>
<tr>
<td>2</td>
<td>CD63</td>
<td>CD32</td>
<td>CD45</td>
<td>CD117</td>
</tr>
<tr>
<td>3</td>
<td>cy Control</td>
<td>cy Control</td>
<td>CD45</td>
<td>CD117</td>
</tr>
<tr>
<td>4</td>
<td>cy Total Tryptase</td>
<td>cy Chymase</td>
<td>CD45</td>
<td>CD117</td>
</tr>
</tbody>
</table>

Control: unstained negative control; CPA: Carboxypeptidase A3; cytoplasmic markers are preceded by "cy", otherwise we refer to membrane markers.
*Indirectly observed by anti-IgE staining.
# FCM diagnosis of Mastocytosis

## Panel of Monoclonal Antibodies (mAb)

Eight color combinations recommended for immunophenotypic analysis of BM MC.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Pacific Blue</th>
<th>Pacific Orange</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PECY7</th>
<th>APC</th>
<th>APCH7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>CD45</td>
<td>Control</td>
<td>Control</td>
<td>CD34</td>
<td>CD117</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>CD2</td>
<td>CD45</td>
<td>IgE*</td>
<td>CD25</td>
<td>CD34</td>
<td>CD117</td>
<td>CD203c</td>
<td>CD69</td>
</tr>
</tbody>
</table>

**Step 1: screening of mastocytosis**

<table>
<thead>
<tr>
<th>Tube #</th>
<th>HLA-DR</th>
<th>CD45</th>
<th>CD35</th>
<th>CD59</th>
<th>CD34</th>
<th>CD117</th>
<th>CD123</th>
<th>CD25</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HLA-DR</td>
<td>CD45</td>
<td>CD35</td>
<td>CD59</td>
<td>CD34</td>
<td>CD117</td>
<td>CD123</td>
<td>CD25 ~</td>
</tr>
<tr>
<td>2</td>
<td>HLA-DR</td>
<td>CD45</td>
<td>CD63</td>
<td>CD32</td>
<td>CD34</td>
<td>CD117</td>
<td>CD123</td>
<td>CD25 ~</td>
</tr>
<tr>
<td>3</td>
<td>HLA-DR</td>
<td>CD45</td>
<td>cy Control</td>
<td>cy Control</td>
<td>CD34</td>
<td>CD117</td>
<td>cy Control</td>
<td>CD25 ~</td>
</tr>
<tr>
<td>4</td>
<td>HLA-DR</td>
<td>CD45</td>
<td>cy Total Tryptase</td>
<td>cy Chymase</td>
<td>CD34</td>
<td>CD117</td>
<td>cy CPA</td>
<td>CD25 ~</td>
</tr>
</tbody>
</table>

Control: unstained negative control; CPA: Carboxypeptidase A3; cytoplasmic markers are preceded by "cy", otherwise we refer to membrane markers.

*Indirectly observed by anti-IgE staining.

~To be included when a double MC population (CD25+ and CD25- MC) is observed with the screening panel.

Carboxypeptidase A3 antibody was a kind gift from AF Walls (Southampton, UK); Anti-Tryptase antibody was kindly provided by LB Schwartz (Richmond, VA, USA).
Further characterization of Mast Cells immunophenotype

- To completely characterize the immunophenotype of MC, it is necessary to analyze the expression of different proteins related to activation, liberation and complement regulatory proteins.

- Also, the information given by the protease content as tryptase, carboxipeptidase and chymase is of great value, although not all of this mAb are commercially available.

- Although this panel is not necessary for the diagnosis of Systemic Mastocytosis, it gives information regarding the profile of BM MC in order to subclassify the patients into the different subtype of the disease.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Pac.B</th>
<th>Pac.O</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PECY7</th>
<th>APC</th>
<th>APCH7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1:</td>
<td>HLA-DR</td>
<td>CD45</td>
<td>CD35</td>
<td>CD59</td>
<td>CD34</td>
<td>CD117</td>
<td>CD123</td>
<td>CD25*</td>
</tr>
<tr>
<td>Tube 2:</td>
<td>HLA-DR</td>
<td>CD45</td>
<td>CD63</td>
<td>CD32</td>
<td>CD34</td>
<td>CD117</td>
<td>CD123</td>
<td>CD25*</td>
</tr>
<tr>
<td>Tube 3:</td>
<td>HLA-DR</td>
<td>CD45</td>
<td>cy Control</td>
<td>cy Control</td>
<td>CD34</td>
<td>CD117</td>
<td>cy Control</td>
<td>CD25*</td>
</tr>
<tr>
<td>Tube 4:</td>
<td>HLA-DR</td>
<td>CD45</td>
<td>cy Total Tryptase</td>
<td>cy Chymase</td>
<td>CD34</td>
<td>CD117</td>
<td>cy CPA</td>
<td>CD25*</td>
</tr>
</tbody>
</table>

Control: unstained negative control; CPA: Carboxypeptidase A3; cytoplasmic markers are preceded by "cy", otherwise we refer to membrane markers.
~To be included when a double MC population (CD25+ and CD25- MC) is observed with the screening panel.
Different immunophenotypical patterns of BM MC from Mastocytosis

1. **Activated mature immunophenotype profile:**

   Aberrant expression of CD25 and CD2 and over-expression of activation and de-granulation markers.

   Detected in **good prognosis** categories: ISM.

2. **Resting mature profile:**

   No aberrant CD2 or CD25 expression. Normal expression of activation or de-granulation markers. Higher content of proteases and high SSC characteristics.

   Typical from **Well-differentiated SM** subtype.

3. **Immature profile:**

   Aberrant CD25 expression in the absence of CD2. Decreased CD117 and IgER expression, and increased positivity for CD123, HLA-DQ, and HLA-DR together with abnormally low levels of cytoplasmic tryptase and carboxipeptidase in association with decreased light scatter features.

   Found in **poor prognosis** categories: ASM, MCL
Different immunophenotypical patterns of BM MC from Mastocytosis

Indolent Systemic Mastocytosis (SM)  Well-differentiated SM  Aggressive SM

FCM utility at early stages of the disease

- Patients with proven skin mastocytosis or patients lacking skin lesions referred because of anaphylaxis / severe MC –mediator related symptoms.
- Normal or slightly increased serum tryptase levels.
- Absence of dense compact MC aggregates in bone marrow biopsy.

FCM can be the only objective diagnostic parameter. In many cases two populations of MCs (normal and aberrant) are detected by FCM. (see an example in next slide)
FCM utility at early stages of the disease

1. Acquisition

- 50mil events

2. MC Identification

- Aberrant MC
- Normal MC

3. MC immunophenotypic characterization

- Aberrant MC
- Normal MC
FCM analysis of peripheral blood

Presence of peripheral blood circulating MCs correlates with aggressive forms of the disease.
Molecular biology in purified cell lineages

- Cell separation by using FACS Aria sorter → **PURITY > 95%**

D816V KIT mutation determination in each population separately
Prognosis of Mastocytosis: molecular biology

Modified from: Escribano et al., JACI, 2009; Updated April 2012.

- KIT mutation restricted to MC population
- Multilineal (myeloid and lymphoid) KIT mutation

**Probability of disease progression (n=354)**

<table>
<thead>
<tr>
<th>Years</th>
<th>10 years</th>
<th>20 years</th>
<th>30 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC Mutation</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Multilineal Mutation</td>
<td>19%±7%</td>
<td>36%±10%</td>
<td>52%±12%</td>
</tr>
</tbody>
</table>
FCM diagnosis of Mastocytosis. **Summary.**

- When a SM is suspected, it is mandatory to refer the patient to a **Reference Center** to complete the diagnosis and follow-up of the disease.
- Detection of **CD25-bright** expression on BM MC by FCM is diagnostic of Systemic Mastocytosis.
- Characterize the complete BM MC immunophenotype to subclassify the patients into the **different categories** of the disease.
- Establish the mutational pattern on purified BM cell lineages to analyze the presence of **KIT mutation** in each population and determinate the **risk of progression** at the time of diagnosis.
FCM diagnosis of Mastocytosis. References.


