Research Fellowship Report

Project tittle: Bone involvement in systemic mastocytosis: a study on the utility of clinical characteristics, analytical markers and radiological changes on the prevention, diagnosis and prognosis of bone pathology in patients with mastocytosis.

Fellow: Tiago Azenha Rama

Type of fellowship: Short Term Research Fellowship

Duration: 3 months (September – November)

Home Institution: Centro Hospitalar Universitário São João/Faculdade de Medicina da Universidade do Porto, Porto, Portugal.

Host Institution: Instituto de Estudios de Mastocitosis, Hospital Virgen del Valle, Toledo, Spain.

Introduction

The term systemic mastocytosis (SM) refers to a heterogeneous group of rare diseases, characterized by the presence of clonal mast cells (MC) in one or more tissues, such as the skin, the gastrointestinal tract and the bone marrow, among others. Bone is one of the most frequently affected tissues in SM and both extremes of disease (osteoporosis and osteosclerosis) may be found.

Bone metabolism relies on osteoblastic and osteoclastic functions, the endpoints for formation and resorption mechanisms, respectively. The receptor activator of nuclear factor kappa-B ligand (RANKL) is one of the main inducers for osteoclastogenesis, its effects being blocked by osteoprotegerin (OPG). MC may play an important role on such mechanisms, as they release proinflammatory cytokines such as TNF-α, IL-1, and IL-6 that stimulate osteoclastogenesis, and interferon (IFN)-γ, or interleukin (IL)-13 that seem to have inhibitory effects. The role of other cytokines released by MC, such as regulated upon activation normal, T-cell expressed and secreted (RANTES), osteopontin (OPN) and oncostatin-M (OSM), is less clear.

This research fellowship had two purposes. First, to quantify MC mediators potentially related to bone regeneration and resorption, in order to identify profiles that could correlate with the presence of osteoporosis and osteosclerosis. Second, to provide the fellow with clinical expertise in the field of mast cell disorders.

Tasks performed during the stay at CLMast

Laboratorial work

In order to achieve the aforementioned objective, a cross-sectional study was designed with 120 adult SM patients. Demographical, clinical and analytical data were collected for each patient, from medical records. The fellow was assigned the task of choosing multiplex kits to be acquired. In order to do so, he personally contacted several companies.

Previously frozen stored plasma samples were analyzed using Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad), using a customized multiplex procartaplex kit containing IFN-γ, IL-1β, IL-6, IL-13, OSM, OPG, RANK), TNF-α (Thermo Fisher Scientific, Waltham, MA, USA), and two uniplex kits, each containing OPN and RANTES (Thermo Fisher Scientific, Waltham, MA, USA).

All included patients were diagnosed according to the WHO classification. These were assigned to three different groups according to bone disease status: 1) absence of bone disease, 2) osteoporosis and severe osteopenia and 3) diffuse osteosclerosis. Rules defined by the Ethics and Clinical Trials committee of Hospital Virgen del Valle were followed.

Patient selection

120 adult patients with SM diagnosed and followed-up at the Instituto de Estudios de Mastocitosis de Castilla-La Mancha (CLMast), were randomly selected, by the fellow. More than 400 medical records were reviewed. Inclusion criteria comprised having systemic mastocytosis, bone remodelling markers and stored plasma measured at the date of blood collection and a bone densitometry, performed at most 1 year before, or 1 year after blood sample collection. Exclusion criteria included having mild to moderate osteopenia and well differentiated systemic mastocytosis.

Laboratorial procedure

All samples were previously thawed at room temperature, vortexed for 30 seconds and centrifuged at 10000 x g, for 10 minutes, following transference into sterile tap-top tubes. Meanwhile, the wash buffer concentrate (10X) was brought to room temperature and vortexed for 15 seconds. A 1x wash buffer solution was prepared mixing 20 mL of the wash buffer concentrate (10x) with 180 mL deionized water. The same procedure was performed with the universal assay buffer (10x). Simplex bead vials were vortexed for 30 seconds. For simplex kits (RANTES and OPG) the simplex beads were previously prepared, by adding 100 µL of each Simplex bead vial (50X) to a wash Buffer (1X) to a final volume of 5 mL. Detection antibody mixture was then prepared adding 60 µL of each detection antibody concentrate to detection antibody diluent to a total of 3 mL. Next, standard antigen vials were centrifuged at 2,000 × g for 10 seconds. A total of 50 µL of universal assay buffer was added into each standard vial, which were then vortexed for 10 seconds and centrifuged at 2,000 × g for 10 seconds. All vials were incubated on ice for 10 minutes. The contents of each vial were pooled into one of the vials and filled up with sample type specific buffer to a total volume of 250 µL, which was vortexed for 10 seconds. Serial 4-fold dilutions of the reconstituted standards were prepared, using the PCR 8-tube strips, by transferring 50 µL of the content of the vial into the next (1 to 2, 2 to 3 and so forth), already containing 150 µL of universal assay buffer and mixing through pipetting up and down for a total of 10 times. Tube 8 served as a blank, being left with 200 µL. These PCR 8-tube strips were kept on ice until needed.

Plates with 98 wells were used. The first two rows were used for standards/blank, using the aforementioned PCR 8-tube. The remaining wells were assigned to samples. Each sample was placed in duplicate in adjacent columns. Previously diluted (simplex kits) and undiluted (multiplex kits) 50 µL of magnetic bead solution were added to each well, using a multichannel pipette. The plate was secured into a hand-held magnetic plate washer, for 2 minutes to allow the beads to accumulate. Maintaining the magnetic plate the supernatant liquid was removed through quick inversion into a sink and blotting the inverted assembly onto several layers of paper towels to remove any residual solution. The magnetic beads were washed placing 150 µL of wash Buffer into each well, allowing the beads to accumulate on the bottom of each well for 30 seconds to allow. The aforementioned removal of supernatant was repeated. The washing procedure was repeated once.

Universal assay buffer (25 µL) was added to each well. Afterwards, the standards, blanks and wells (25 µL) were placed into each well, the plate was sealed and left to incubate on a plate shaker, at 500 rpm, for 60–120 minutes, at room temperature, followed by another washing procedure done twice. Detection antibody mixture (25 µL) was added to each well, the plate was sealed and left to incubate for 30 minutes at 500 rpm, followed by another washing procedure done twice. Streptavidin-R-phycoerythrin (SAPE) solution (50 µL) was added to each well, the plate was sealed and left to incubate for 30 minutes at 500 rpm, followed by another washing procedure done twice. Finally, the reading buffer (120 µL) was added to each well, the plate was sealed and left to incubate for 5 minutes at 500 rpm, followed by reading on the MagPIX instrument.

Results

As shown in figure 1, compared with normal bone patients, those with osteoporosis had significantly higher levels of bone resorption cytokines - IFN-γ ($p=0.05$), IL-1β ($p=0.05$) and IL-6 ($p=0.05$). There was also a trend for increased OSM ($p=0.078$) and TNF-α ($p=0.074$).

Those with osteosclerosis had significantly lower levels of bone resorption cytokines - IFN-γ ($p=0.03$) and RANKL ($p=0.04$) - and higher levels of bone formation cytokines - OPN ($p<0.01$) and RANTES ($p=0.01$). There was also a trend for lower IL-13 ($p=0.072$) and IL-6 ($p=0.062$).

Conclusions

This study confirms a proinflammatory/osteoclastogenic cytokine profile in osteoporosis and provides novelty describing not only increased expression of bone formation cytokines in SM patients with osteosclerosis, but also decreased expression of proinflammatory/osteoclastogenic cytokines.

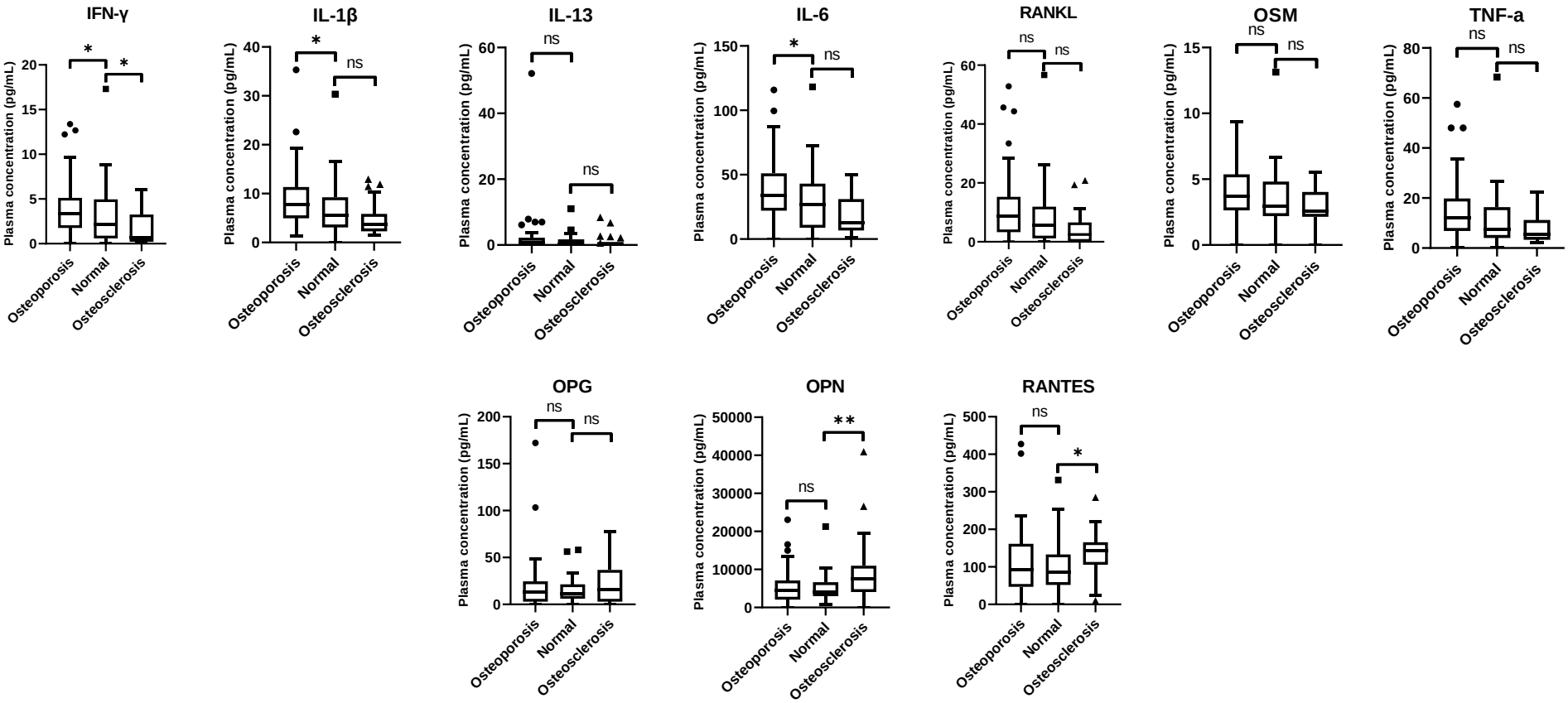

Figure 1. Comparison between cytokine levels for osteoporosis vs normal bone and normal bone vs osteosclerosis, in systemic mastocytosis patients. IFN,Interferon; IL, interleukin; OPG, osteoprotegerin; OPN, Osteopontin; OSM, oncostatin M; RANTES, regulated upon activation normal, T-cell expressed and secreted; RANKL, receptor activator of nuclear factor-κB ligand; TNF, tumor necrosis factor.

Clinical Work

During the 3 months' rotation, the fellow was also given the opportunity to acquire medical knowledge through the practice of medicine with mastocytosis and MC activation syndromes.

During the first week, the fellow was introduced onto daily routines: medical consultations, peripheral blood collection, bone marrow (BM) aspirate and biopsy, flow citometry, BM smear preparation for citology and citology observation. The fellow was assigned the task of filling out laboratory requests (blood work, BM aspirates, BM and skin biopsies), for all patients consulted during the rotation. In order to do so, he reviewed the medical records of more than 300 patients. During the first week, he also joined Prof. Almudena Matito, or Prof. Ivan Alvarez-Twoze, observing their consults, and was taught on how to perform BM aspirates and biopsies.

Starting at the second week, the fellow was asked to perform medical consults for new patients, consulting with Prof. Almudena Matito, or Prof. Ivan Alvarez-Twoze, after each one. The fellow consulted a total of 59 patients and observed a total of 32 consults performed by the aforementioned specialists.

The fellow also performed 5 spirometries before and after a bronchodilator, 14 skin biopsies and 32 BM aspirates/biopsies having observed the BM smears for all of them.

The task of filling patient diagnosis in CLMast databases was also assigned to the fellow. In order to fulfil this task, the fellow had to read more than 200 medical records.

Personal reflection

The fellow would like to separate personal gains resulting from the scholarship into 4 different dominions: acquisition of medical knowledge on mast cell diseases, development of laboratorial work competences, cultural enrichment and further development of previously attained competencies on the Castilian language.

Acknowledgements

First of all, the fellow would like to thank EAACI for the opportunity to learn and work on an excellence centre, something that would had been impossible had the fellow not won the fellowship and Centro Hospitalar Universitário de São João an especially the Immunoallergology department for supporting and endorsing this fellowship.

The fellow would like to gratefully acknowledge Prof. Almudena Matito for sharing her knowledge and critical views on mast cell diseases, Prof. Ivan Alvarez-Twose for his invaluable clinical teachings, Dra. Inés Torrado for her teachings on allergology and companionship, Prof. Laura Sanchez-Muñoz for her invaluable help on acquiring multiplex kits and performing the assays, to Prof. Ana Henriques for her precious teachings on all the laboratory work and on performing the assays. Finally the fellow would like to gratefully acknowledge nurse Rosa for her assistance during bone marrow aspirates/biopsies and skin biopsies, and lab technicians Mrs. Rosa Gálan and Mr. Juan Carlos Fernandez for their help on finding and acquiring frozen samples.