It’s an EAACI Research Fellowship: 3 months from 15th September to 15th December in Belgium (University Antwerp 2610 – Campus drie Eike – Department Allergology and Reumatology)

In particular, the frequency follow the specific programme: 3 times a week (Monday, Tuesday and Wednesday in the morning and afternoon work in a Lab for learning many protocols; 3 times a week (Wednesday in the evening, Thursday and Friday morning and evening in the clinical hospital with the patients).

The purpose and the principal object was:

**Can behaviour analysis of individual mast cells and basophils lift the veil of anaphylaxis in mastocytosis?**

Mastocytosis is a complex multisystem disorder that is characterized by a pathologic accumulation of neoplastic mast cells in one or different organs. This clonal mast cell expansion is oftentimes associated with point mutations, especially the DB16V mutation. These mutations result in gain-of-function of their surface receptor c-Kit (CD117) for stem cell growth factor (SCF). The clinical presentation of mastocytosis is extremely heterogeneous. Clinics vary from a generally benign limited skin disease (cutaneous mastocytosis, CM) to a systemic disease (systemic mastocytosis, SM) with distinct grades of aggressiveness, viz. indolent systemic mastocytosis, smouldering systemic mastocytosis, aggressive systemic mastocytosis and mast cell leukaemia. Anaphylaxis, potentially life-threatening and fatal, is one of the cardinal clinical features of indolent systemic mastocytosis and affects over 50% of the patients, particularly those without skin involvement. In these patients, occurrence of anaphylaxis is unpredictable and can result from both IgE-dependent and IgE-independent triggering of mast cells and basophils. Remarkably, the total mast cell burden, hitherto “best” reflected by the level of serum tryptase, does not predict the risk nor clinical outcome of anaphylaxis. Moreover, in patients with systemic mastocytosis and a concomitant IgE-mediated wasp/honeybee venom allergy, an inverse relationship has been demonstrated between the risk of sting-induced anaphylaxis and serum tryptase. Furthermore, in patients with aggressive systemic mastocytosis and mast cell leukaemia who have higher mast cell burden, anaphylaxis is an exceptional clinical presentation. Taken together, these data indicate that the burden of neoplastic mast cells is not critical for anaphylaxis in systemic mastocytosis.

The overarching aim of this research project is to gather more fundamental insights in the functional behaviour of mast cell and basophil in systemic mastocytosis patients with or without anaphylaxis. For this purpose, the project was subdivided in 3 objectives:

1. **Objective 1**: is there a different expression of activating and inhibitory receptors between resting mast cells and basophils from patients with and without anaphylaxis? (Figure 1A).
Objective 1: Is there a different expression of activatory and/or inhibitory receptors?

Objective 2: Do mast cells and basophils from patients with or without anaphylaxis respond differently to IgE-dependent and IgE-independent stimuli? (Fig. 1B).

Objective 3: Can we identify an imbalance between the activating and inhibitory pathways that might account for distinctive mast cell and basophil functions in phenotypically different patients? (Fig. 1C).
Figure 1B: Study of cultured mast cell and basophils activation in patients and controls. **Fig 1C.** Balance of activating and inhibitory pathways in cultured mast cell and basophils in patients and controls.

With respect to the objectives we focused on the study of mast cells rather than basophils, first of all because for the study of basophils it is not necessary to set up a culture and then because the study of basophils provided for a simpler learning.

In fact about my work in LAB in these months:

**LABORATORY**

I learned many protocols and to verify my preparation it was possible to compare the results of my experiments with those of my tutor.

Protocols have this purposes:

1) **How to make a culture of mast cells? How to use peripheral blood and bone marrow samples**

Most of the protocols practiced necessitated the use of mast cells in culture; therefore it was fundamental to learn how to make a culture of mast cells both from peripheral blood and from bone marrow. Mononuclear cells were isolated with a density separation, out of these mononuclear cells the CD34+ progenitor cells were enriched using the EasySep Human CD34 selection kit according to the manufacturer’s instructions. Also the viability of the cells were examined with a 7AAD viability straining.

After isolation the CD34+ cells were cultured in a serum free methylcellulose medium supplemented with IL-3 and SCF for 14 days. After 14 days the cells where cultured in IMDM medium supplemented only with SCF for another two more weeks.
Figure 2a – 2b: in the days 0,3,7,14 and 21 it is necessary to control the growth and the check for contamination.

Figure 3a-b-c: Isolated CD34+ cells after magnetic separation.

2) Turning cytometry data into results

I was learning to use Flow cytometry with FACSCanto II and Fascalibur and the new program with my device “FCS Express 6 Cytometry” for analyze the data with the graphs and the plot plots obtained with the execution of the experiments.

3) Typing the human cultured mast cells

Mature mast cells were defined as CD117+ and CD203c+. Immunophenotyping contains staining of the activating receptors (MGRPRX2 and the high affinity IgE-receptor) and inhibitory receptors (CD300a and CD32B). CD63 is a molecular marker of degranulated human mast cells. CD25 is very important for the diagnosis of SM. In fact aberrant expression of CD2 and/or CD25 by bone marrow, peripheral blood or other extracutaneous tissue mast cells is currently used as a minor World Health Organization diagnostic criterion for systemic mastocytosis.
Figure 5a-f: (4a) cultured human MC express MRGPRX2 on their cell membrane, (4b) all MC express CD300a and FceRI (4c). (4d) Expression of CD32 and (4e) CD25 (4f) in the last on the right MC in rest don’t express CD63.

Figure 5a - 5b
**4) ACTIVATION** with the culture: studying the activation mast cells, with focus on: do you have MRGPRX2 in your membrane of mast cells? Are the mast cells activable with substance P? Looking at degranulation (CD63 upregulation) and increase in intracellular calcium.

Cross-linking of the high affinity receptor for IgE antibodies (IgE Rec I) and/or engagement of MGRPX2-G protein-coupled receptors results in Ca\(^{++}\) influx leading to a cascade of phosphorylations of intracellular signaling molecules such as Syk and Lyn. Subsequently the signal converges to mitogen-activated protein kinases (MAPKs/JAK, etc.). Finally, the process might culminate in degranulation with release of histamine and other mediators through the fusion of intracellular granules with the cell membrane and leads to the up-regulation of CD203c, CD11b/CD18 complex, and mainly CD63 as shown in Figures 1 A-B.

Our hypothesis was: the balance between activating and inhibitory pathways in response to IgE-dependent and IgE-independent stimuli in mast cells determines the risk of anaphylaxis in patients with SM. The purpose was to compare the effect of IgE-dependent and IgE-independent stimuli on bone marrow (BM) and peripheral blood (PB) derived cultured mast cells. Cells were stimulated with anti-IgE or with IgE-independent triggers such as substance P or other molecules (fig. 1 C). Then various read outs like Ca\(^{++}\) influx, phosphorylation of intracellular signalling molecules and surface molecules (e.g. CD63)
Figure 6 d-e-f: intracellular calcium measurements after activation with buffer (6a), with Substance P (6b), with anti-FcεRI (6c). It can be noted that there is a greater activation in the graph of the Sub P (6b) respect to the Buffer (6a); below CD63 measurements after activation with buffer (6d), with Substance P (6e), with anti-FcεRI (6f).

It was very useful as it allowed me to correct any errors in methodology in the laboratory and with the use of electronic devices for the analysis of the dot plots. Here are some interesting results in “Activation’s mast cells experiments”:

Figure 7a-b-c
Figure 7a-b-c: Comparison of my results with the one's of my mentor
Shows the expression of CD63 after 3 or 20 minutes in some samples of PB and BM, as can be seen the results are comparable, we can observe almost the same results. Finally, in Figure 8c: which represents the RATIO of Substance P (measurement experiment of calcium influx after mast cellular activation) values for 6 patients whose bone marrow blood and other peripheral blood (dashed line) were analyzed, we can see how the results between my experiments and those of my tutor are compared. The values are almost similar.

In this graph above (figure 8) we can observe how an expression of MRGX2 is almost always accompanied by an increased expression of CD63 (both after 3 minutes from activation and after 20 minutes) with $R^2 (0.9339)$ for CD63 20 min.
5) Without culture typing of bone marrow: which characteristics have mast cells that are found in the bone marrow in patients with suspected mastocytosis?

This protocol is very useful, as it allows the characterization of mast cells using a bone marrow sample without the use of cells in culture, characterization in particular of: CD25, MRGPRX2, FcεRI and CD300a. The working solutions of the experiment were as follows: RBC Lysis Buffer, PBS BSA 0.5% and Parapormaldehyde 0.1%. After lysis of the RBC the cells, after washing and centrifugation twice, added PBS BSA 0.5 % and after centrifugation were strained with the marker described above, after last centrifugation added Parapormaldehyde 0.1%, finally analysed with flow cytometry.

Fig 9a-h: Immunophenotyping of full bone marrow of patient with suspected mastocytosis in my experiments. After isolated positive CD117 cells, can observe how there is a positive expression of the following parameters taken into consideration (MRGPRX2, CD300a, FCERI, CD25); the positivity of CD25 in particular allows us to diagnose systemic mastocytosis (fig. 9e).
CLINICAL HOSPITAL

I learned many protocols:

- A) Management of patients with hypertriptasemia and Mastocytosis with different symptoms and the patients with anaphylaxis (for example idiopathic anaphylaxis or for imenoptera bee and wesp)
- B) Management of patients with Indolent form, Smouldering or Aggressive Mastocytosis, use of drugs such as Midostaurin, Masatinib or Avapritinib
- C) Management of patients with allergy to hymenoptera venom, from the anamnesis, to the diagnosis and use of specific immunotherapy
- D) Management of allergy to penicillins and other beta-lactams (for exemple cross-reactivity with Cephalosporin): correct anamnesis for to decide the follow-up and then choose which tests to do and choose the precise order (SPT, IDT and challenge oral or endovenous/intramuscolar test)
- E) Management of allergy to another antibiotics (for exemple fluorochinolonics and macrolids) or also another drugs: NSAID’s, corticosteroids, anestetics, heparines, contrast media and biologicals with skin test and provocatie tests
- F) Management of desensitization of many medicaments, in particular chemioterapics like carboplatinum
- G) Management of patients with food allergy or allergic rhinitis
- H) Management of patients with Chronic Spontaneous Urticaria and subcutaneous Xolair therapy (Omalizumab)
- I) Management of patients with different diseases: Systemic lupus erythematosus, autoinflammatory diseases, hypereosinophilic syndromes, vasculitis, sarcoidosis and many others.

The approximately number of the patients observed is 150 patients

My personal reflection and how I can improve for the future:

First and foremost, it is a great thing to have learned that there are many methods for typing mast cells. There is still much to know, but there are some protocols that I could continue to use once I have returned to my country of origin. In particular the protocol for the bone marrow typing (without use of culture) to be able to see if there is a different characterization of mast cells in patients with suspected mastocytosis and not, but especially in patients with mastocytosis with or without anaphylaxis.

In the first place certainly it was very useful for me to observe and learn the management of patients with mastocytosis, both the indolent and the more severe and aggressive forms. In Sardinia (Italy) there is the possibility to continue to follow a large number of patients over time, they will need a careful and scrupulous follow-up, including the collaboration of colleagues hematologists, dermatologists, geneticists and all those who intend to work in team with us.

The allergy to hymenoptera venom, although still underestimated from an epidemiological point of view, represents an important cause of morbidity and mortality worldwide, and also in Italy. The prevention of future allergic reactions in patients who have developed a systemic reaction is achieved through proper management of the patient in an emergency and subsequent allergological counseling for diagnosis, prescription of auto-injectable adrenaline and, where indicated, specific immunotherapy with hymenoptera (venom immunotherapy, VIT). It’s therefore fundamental for me to have learned the fundamental bases for the management of this type of patient (also considering the strong correlation between Mastocytosis and allergy to hymenoptera venom).

Hypersensitivity to drugs has increased in the twenty-first century due to an explosion in new and targeted medications to address disease through personalized and precision medicine. Drugs are among the main triggers of anaphylaxis, but identification of the culprit drug is frequently difficult. I understand that to confirm diagnosis of the causative agent, medical records and clinical history are
fundamental. The management of the patient with drug allergy will be very useful in my clinical activity as it will allow me to have a correct approach to this complex and still little known problem. Finally the **management of all the other diseases not mentioned** has certainly been a great help for all that includes seriously doing allergology in this vast and still little known field.

Thank you to Didier Ebo, Vito Sabato, Jessy Elst, Chris Bridts et all (a special thanks to all because they listened to me patiently and throughout this period every day they gave me all their knowledge). Thank you to another people of staff of Clinical Hospital UZA and LAB (Building eight) - CAMPUS DRIE EIKEN

**References and bibliography**

- 3 - Matito A, Curr Allergy Asthma Rep 2014; 14:450;
- 4 - van Anrooij B, J Allergy Clin Immunol 2013; 132:125-30
- 5 - Cop N, Decuyper II,Faber MA, Sabato V,Bridts CH, Hagendorens MM, De Winter BY,De Clerck LS, and Ebo DG; Cytometry Part B (Clinical Cytometry) 00B:00–00 (2016)