

## **EAACI FELLOWSHIP AWARD 2017 – FINAL REPORT**

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Host supervisor: **Christiane Hilger, PhD**

Duration: 01/04/2017 – 30/06/2017

Location: Department of Infection and Immunity, Luxembourg Institute of Health,

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Title of the project: **“IN-DEPTH PROTEOMIC ANALYSIS OF PROPOLIS ALLERGENS USING COMBINATORIAL PEPTIDE LIGAND LIBRARIES (CPLL)”**.

### **1. INTRODUCTION**

#### **1.1- ALLERGY TO PROPOLIS**

Propolis (bee glue) is the resinous substance that bees collect from living plants for the construction and protection of their nests. Since antiquity, the health promoting benefits of propolis as a food ingredient have long been recognized. It has antibacterial, antifungal, and antiviral properties and a wide range of other beneficial biological activities <sup>1</sup>. Although propolis is now broadly available as a dietary supplement worldwide, it has the potential to cause substantial allergic reactions when ingested or externally used by patients with a history of allergy. Several case reports of allergic reactions have been reported and European studies are reporting a sensitization rate of 1.2-6.6% <sup>2</sup>. A multicentre study in Finland evaluated in an interval of 5 years the sensitization to propolis among 3885 and 5130 adults, respectively. This study noted the almost threefold increase in the prevalence of sensitization to propolis from 0.5 to 1.4 % <sup>3</sup>. In a Polish study on atopic children with eczema, propolis was found to occupy rank 2 in children (16.5% sensitization) and rank 4 in adolescents (5.4%) <sup>4</sup>. Allergic reactions to propolis may manifest as contact cheilitis, contact stomatitis, perioral eczema, labial edema, oral pain, peeling of lips, and dyspnea. Despite the known allergenic potency of propolis extracts, the presence and molecular origins of IgE-mediated hypersensitivity to this beehive product have not yet been investigated.

## 1.2 - IDENTIFICATION OF TRACE ALLERGENS USING CPPL METHODOLOGY

The analysis of propolis proteins appeared to be difficult due to the inherent characteristics of its matrix: very low protein content (less than 1%) and high amounts of interfering compounds such as lipids, phenolics and pigments. Over the last decade, the development of proteomics tools led to the discovery of trace allergens as well as allergenic proteins present in lower concentrations in large varieties of natural sources. In the present work, improved protein extraction, enrichment and 2D-DIGE separation combined with the use of combinatorial peptide ligand libraries (CPPL) enrichment <sup>5</sup> of very low concentration proteins were used in order to provide an in-depth proteomic analysis of propolis allergenic content.

## 2. MAJOR OBJECTIVES

- The identification of IgE-binding proteins in propolis extracts
- The prediction of the groups of allergic patients susceptible to exhibit allergic reactions after ingestion or external exposure to propolis allergens,
- The comparison of the allergenic patterns of propolis samples originating from different climatic zones.

## 3. METHODS

### 3.1- PATIENTS SERA

Sera of polysensitized allergic patients were selected according to their symptoms, positive skin prick test results and positive ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) with serum-specific IgE  $\geq 0.71$  kIU/l (CAP class  $\geq 2$ ). Blood samples from allergic individuals living in Luxembourg and Tehran were drawn at the time of the visit at the Immunology-Allergy department, Centre Hospitalier de Luxembourg and at the Immunology, Asthma and Allergy Research Institute of Tehran, respectively. Patients' sera were separated by centrifugation and stored at  $-20^{\circ}\text{C}$  until use. For each analysis, the serum from a healthy individual with a normal total immunoglobulin E (IgE) concentration has been selected as control.

### 3.2- PROPOLIS EXTRACTS

Propolis samples were collected from 4 geographically distinct areas in Iran (Alborz, Gilan and Tabriz) and Belgium (Witry). Different propolis protein extracts (in PBS and different detergents) have been evaluated by 1D and 2D SDS-PAGE analyses for protein quantity and quality. A first evaluation of IgE-reactivity has been done by 1D-IgE immunoblotting using sera of pollen-allergic patients living in Luxembourg and Iran (Figure 1). Propolis extracts with representative patterns have been further analyzed and compared using multiplex-fluorescence labelling (NH DyeAGNOSTICS, Germany) and CPLL methodology.

For sample preparation prior to the CPLL treatment, two times 5 g of Iranian propolis samples for I5 and I3 (total 10 g) and four times 5 g (20 g) of the Belgian propolis were cryo-grinded and suspended in 15 mL of 10 mM phosphate buffer pH 7.06 containing 3 M urea and 0.2% NP-40 (PUN buffer) and Tris buffered saline pH 8 containing 10 mM Tris, 150 mM NaCl and 1% Triton X 100 (TBS buffer), respectively. After a few minutes shaking to wet the propolis particles, the suspension was shaken overnight at room temperature and the supernatant was separated by centrifugation. All supernatants were collected and then pooled together thus forming the PUN extract for Iranian propolis samples and TBS extract for the Belgian propolis sample. In order to eliminate pigments and other polymeric materials, PUN extracts were precipitated using cold acetone containing 0.07% 2-ME. These mixtures were stored at -20 °C overnight and then the supernatants eliminated by centrifugation. By adding 200 µl of cold acetone and vortexing, the remaining pellets were washed and the supernatants eliminated by centrifugation during 15 min at 5300 rpm at 4 °C. Pellets from the Iranian (I5 and I3) and Belgian propolis extracts were then dissolved in 3 mL of PUN and TBS buffer, respectively. The obtained PUN and TBS solutions were then dialyzed against 3 M urea and TBS solution containing protease inhibitors (Roche). All extracts were kept at -20 °C before use for CPLL treatment. The same protocol has been used for propolis EtOH extracts.

### 3.3- CPLL TREATMENT

Protein capture by CPLL was performed on EtOH, TBS and PUN propolis extracts using the ProteoMiner™ Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer instructions. All steps were performed at room temperature. Incubation and washes on the rotary shaker were all conducted at 10 rpm. Briefly, 20 µL of ProteoMiner beads were washed three times with 200 µL

of wash buffer containing 150 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4. After elimination of buffer excess by centrifugation, 400 µL of optimized PUN protein extract was loaded. The mixture is gently rotated for 2 hours at room temperature. Beads were separated by centrifugation and the supernatant (flowthrough) was collected. The flowthrough of each extract is collected and stored at -20 °C for further analyses. Afterwards, CPLL beads were washed (3 times) with the addition of 200 µL of wash buffer and rotated from end-to-end over a 5 min period. After all wash buffer has been removed by centrifugation, bead pellet was washed with 200 µL deionized water and rotated end-to-end for 1 min. The mixture is centrifuged at 1,000 x g for 30–60 sec to remove water and the supernatant discarded. The remaining pellet is incubated in 20 µL of rehydrated elution reagent (8 M urea, 2% CHAPS, 5% acetic acid) and lightly vortexed for 5 sec. The mixture is then incubated at room temperature, lightly vortexed several times over a period of 15 min. The eluate was collected by centrifugation at 1,000 x g for 30–60 sec. This step was repeated two more times and collected elutions were pooled and kept at -20 °C until further analyses.

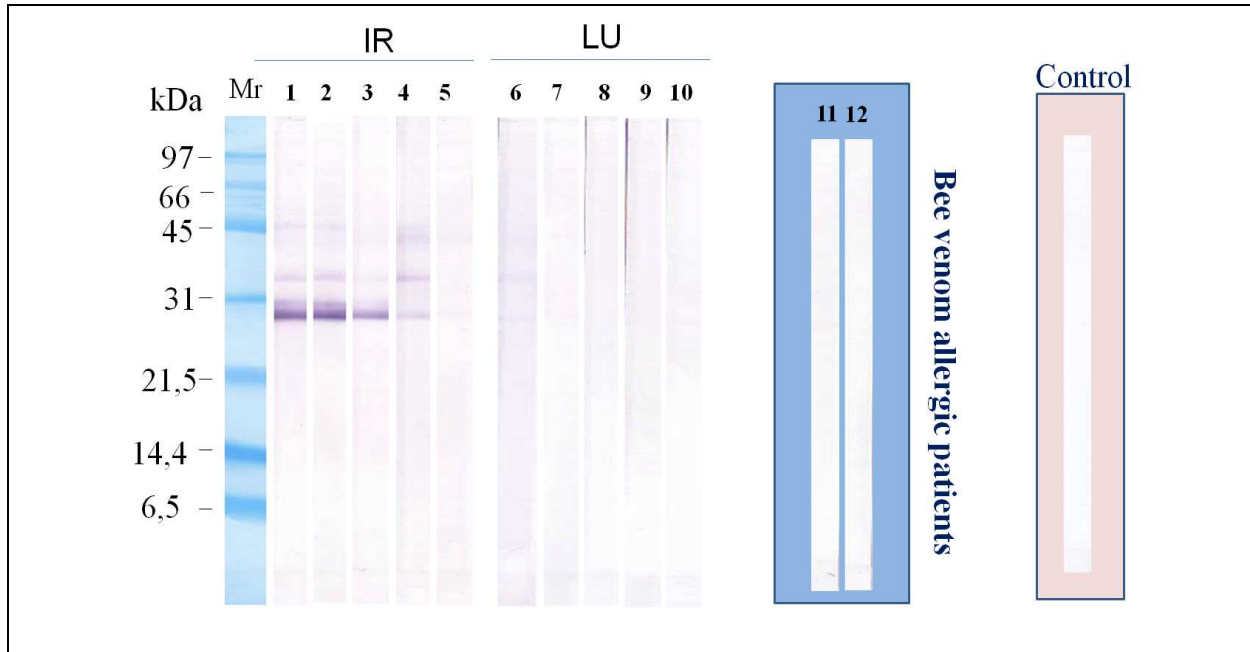
### 3.4. 2D-DIGE EXPERIMENTS

Prior to protein labeling using the Refraction 2D labelling kit (NH DyeAGNOSTICS, Halle, Germany), lyophilized CyDye including “Gdye 100 sp” and “Gdye 300 12G” were solubilized in DMF to obtain a stock solution of 1 mM. To limit any effects of photobleaching on the fluors, all subsequent steps are performed in the dark. The dye-to-protein ratio was around 400 pmol of dye for 50 µg of protein for each extract. It is recommended to label the pooled internal standard with the Cy2 dye and then to perform a dye swap with each of the sample types using Cy3 and Cy5. The labeling reaction is performed on ice for 30 min, and stopped by the addition of lysine to quench any unreacted dye (for 10 min on ice). After CyDye labeling, the three samples were separated on the same 2D gel and then imaged for the resulting fluorescence associated with each CyDye. For 2D separations, the labeled samples were mixed appropriately for loading onto the first dimension IPG strips by in-gel rehydration (50 µg protein for each labeled extract per gel). The samples were added to the adequate volume of loading buffer in order to achieve the following final concentrations: 7 M urea, 2 M thiourea and 4% CHAPS. The IPG strip (non-linear pH 3-10, Bio-Rad, Hercules, USA) was incubated overnight in the loading buffer mixture and then applied to the first dimension using an IEF cell device. After focusing, the IPG strip was subjected to a

two-step equilibration procedure with SDS buffer prior to the second dimension separation on an horizontal flatbed electrophoresis apparatus (HPE™ BlueHorizon™ Flatbed System, Serva, Heidelberg, Germany). After 2D separation, the gels are directly imaged with a laser scanner (Typhoon 9400, GE Healthcare) enable to excite the three dyes independently and having the necessary band pass filters to avoid cross talk.

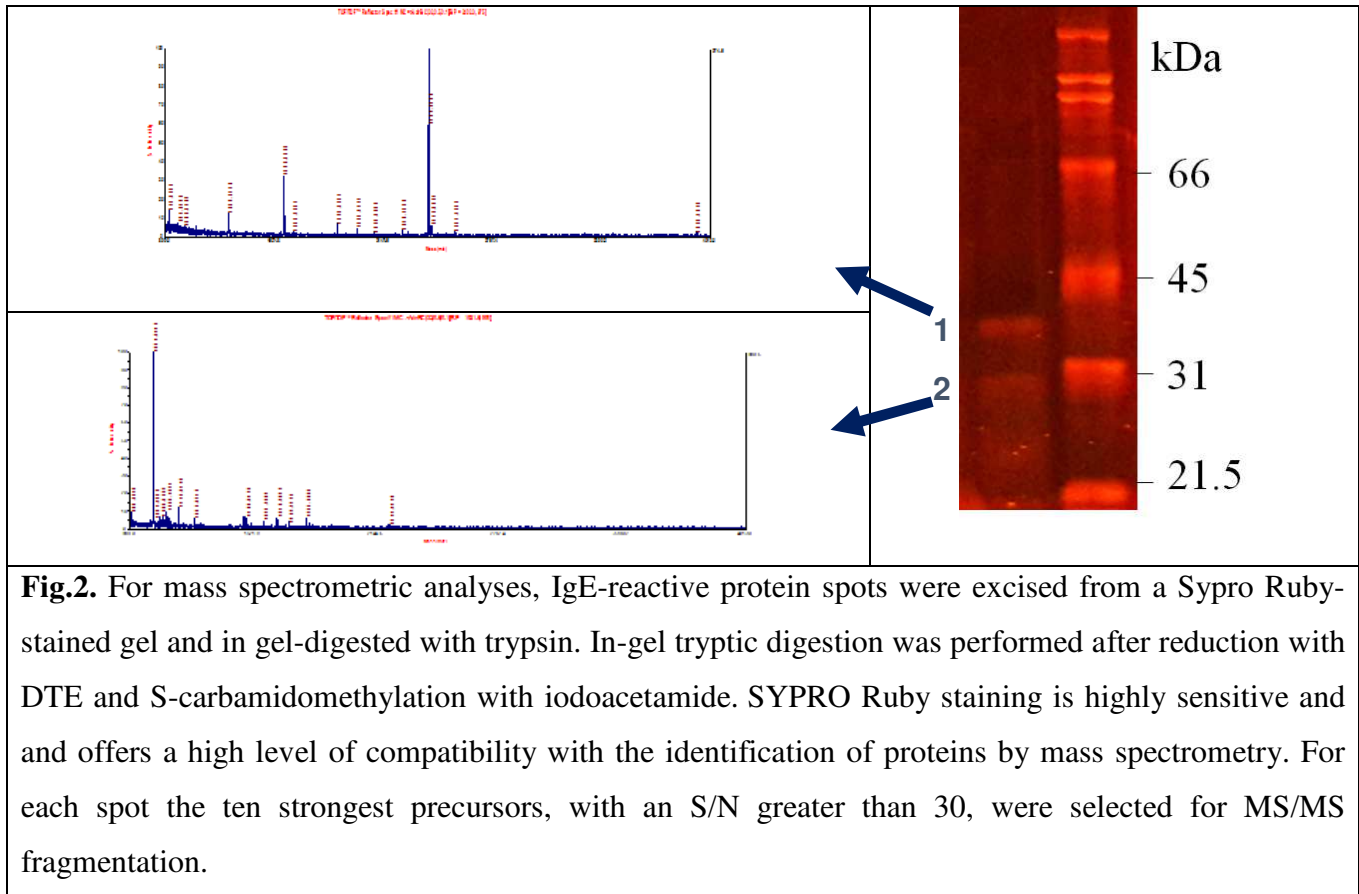
## 4. RESULTS

### 4.1. 1-DE (SDS-PAGE) AND SPECIFIC IGE SCREENING



**Fig. 1.** SDS-PAGE 15% and IgE immunoblotting on propolis extracts probed with sera of pollen allergic patients living in Iran (IR) and Luxembourg (LU). Mr represents the molecular weight marker; lanes 1–10, screening of 10 patients sera by Western blotting for specific IgE antibodies to Iranian propolis extract (from Tabriz). The strips were displayed according to IgE binding patterns. Iranian patients showed well-marked IgE reactivity to two proteins of about 30 and 36 kDa. No specific IgE reactivity was observed with sera from patients suffering from bee venom allergy and in the control strip.

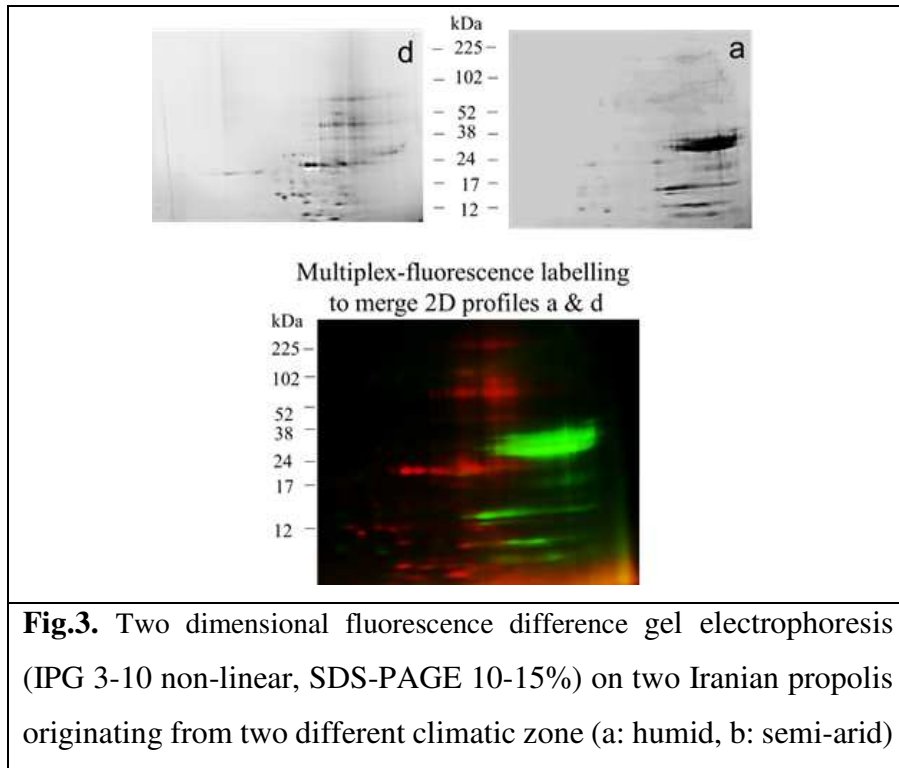
## 4.2. MS/MS ANALYSIS ON IGE REACTIVE PROTEINS



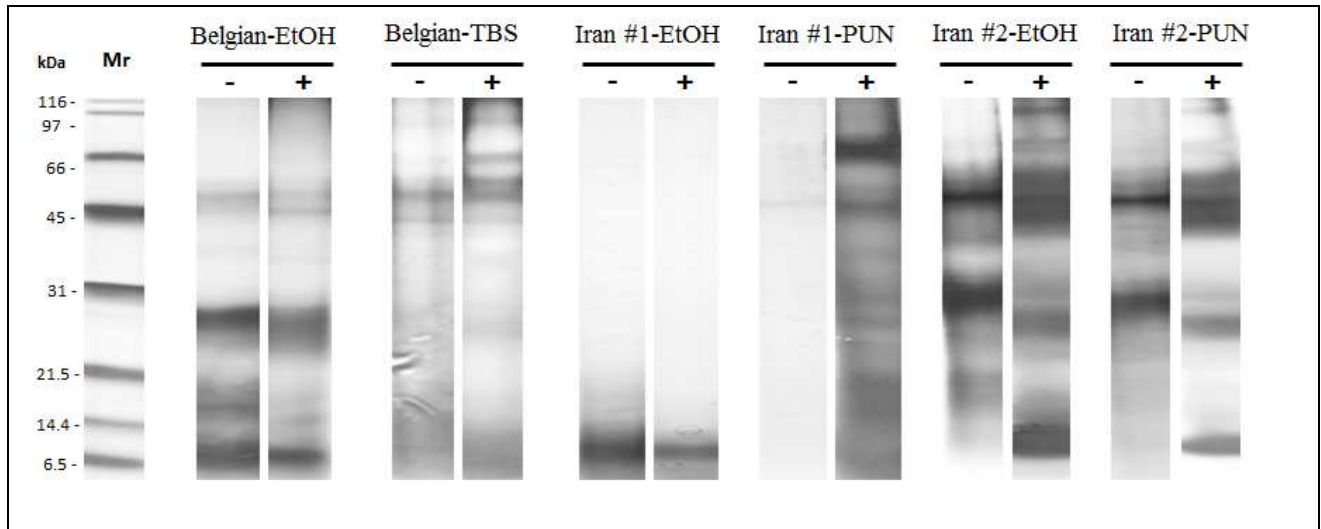
MS/MS based MASCOT search using NCBI database were statistically significant and identified the IgE reactive protein spots with at least two peptides and relevant MASCOT scores. At least two proteins reported as cross-reactive allergens in literature have been identified from propolis samples.

## 4.3. 2D-DIGE EXPERIMENTS

As depicted by the Figure 3, 2D-DIGE analyses point out significant differences between the protein profiles of Iranian propolis collected from two geographically distinct areas.



#### 4.4. CPLL TREATMENT OF DIFFERENT PROPOLIS EXTRACTS



**Fig.4.** SDS-PAGE protein profiles of 6 different propolis extracts before (-) and after (+) protein enrichment and CPLL treatment. Iranian propolis extracts #1 and #2 correspond to propolis samples collected from Tabriz and Alborz provinces (Iran), respectively. The Belgian propolis originated from Witry (Northern Belgium).

In order to explore the proteome of different propolis samples in view of studying the repertoire of propolis allergens, two analytical approaches were combined. The first approach consisted in setting up a protocol to optimally extract proteins from propolis tissues which were poorly soluble in conventional extraction buffers. The use of surfactants was efficient to qualitatively and quantitatively increase the number of solubilized proteins in Iranian propolis extracts originating from Tabriz (Iran #1-PUN). In contrast, conventional ethanolic extraction of propolis samples collected from Witry (Belgian-EtOH) and Alborz (Iran #2-EtOH) led to richer protein containing extracts when compared to TBS and PUN extracts, respectively. The second approach using the ProteoMiner bead technology was then applied to different propolis extracts in order to unravel under-represented proteins. As depicted in Fig.4., protein concentration followed by a CPLL treatment allowed the detection of several non-redundant proteins in Belgian-TBS, Iran #1-PUN, Iran #2-PUN and Iran #2-EtOH propolis extracts.

## **5. CONCLUSION AND PERSPECTIVES**

The extended proteomic knowledge on major protein families triggering IgE-mediated allergies constitutes a good research basis to identify novel allergenic molecules from novel allergenic sources as well as to predict potential cross-reactivity among various allergenic species.

Our preliminary results showed that propolis extracts comprise potential cross-reactive IgE reactive proteins able to sensitize pollen allergic patients. Besides, our 2D-DIGE experiments mirrored significant differences in protein content among propolis samples originating from different Iranian regions. In-depth proteomic experiments using CPLL are still in progress to further determine the nature of propolis putative allergens and to evaluate the risks of IgE cross-reactivity with other allergenic sources. To the best of our knowledge, this work represents the pioneer proteomic investigation on propolis extracts and provides useful insights for further studies on the allergens of this widely used dietary supplement.

## **6. OTHER RESEARCH ACTIVITIES**

-Participation in the weekly meeting of the “Molecular and Translational Allergology” research group, headed by Dr. Christiane Hilger and Dr. Annette Kühn.

-Participation in seminars of the Department of Infection and Immunity of the Luxembourg Institute of Health, headed and animated by Professor Markus Ollert.



-Participation in the Annual EAACI congress held in Helsinki, June 17-21, 2017 with a poster presentation awarded by a poster prize.

## **7. ACKNOWLEDGEMENT**

First of all, I would like to thank the European Academy of Allergy and Clinical Immunology (EAACI) for giving me the opportunity to collaborate with a world-renowned institution, on the cutting edge of innovation and technology in the field of molecular allergy. I would like to express my special appreciation and thanks to my host supervisor Dr. Christiane Hilger, for encouraging and mentoring this research project. This work would not have been possible without her valuable support and constructive supervision. I would also like to thank Professor Markus Ollert for inviting me to the “Department of Infection and Immunity of the Luxembourg Institute of Health” and for opening the doors to this collaborative project. I would especially like to thank Stéphanie Kler for her precious technical support and for setting up ideal experimental conditions for this project. Her experience on honeybee products as an amateur beekeeper has been greatly valuable and strengthened the relevance of this study. It was also a pleasure to work with Dominique Revets providing me constructive advises in the field of proteomics. I learned a lot from our passionate discussions. Finally, I would like to thank all the members of the “Molecular and Translational Allergology” research group for welcoming me in a warm and considerate atmosphere. These three months allowed me to experiment novel laboratory methodologies in a highly international scientific environment and were very rewarding. I hope that this fellowship will pave the way for further collaboration with this brilliant research group.

## **8. REFERENCES**

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