Final Report

Medium-term EAACI Research Fellowship 2018

Research Title: House dust mite molecular sensitization profile - A 20 years' assessment of *Dermatophagoides pteronyssinus* allergic patients concerning clinical data and specific IgE to the major allergens Der p 1, Der p 2 and Der p 23

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This report describes my research project, activities and achievements over the period of my fellowship (1st May 2018 – 31st October 2018) at the Department of Pathophysiology and Allergy Research, Medical University of Vienna, Austria.

Background and Rationale:

Allergic diseases are steadily rising and affecting a larger number of individuals. House dust mite (HDM) allergy affects approximately 2% of the world's population, thus being a cause of major healthcare and economic burden.

The progress in molecular biology over the last years has allowed the identification and characterization of single allergens in detail at a molecular level. Component-resolved-diagnostics (CRD) offer the possibility of higher diagnostic precision and allow for better management of patients. Little is known about longitudinal patterns of the development and changes of IgE to distinct allergen components. Recently CRD approaches demonstrated that IgE responses to timothy grass and dust mites during childhood could increase in molecular complexity over time through "molecular spreading", in which sensitization to a primary allergen precedes an increase in the number of recognized molecules. Monomolecular sensitization might expand to polymolecular recognition and this phenomenon seems to correlate with clinical symptoms.

Nevertheless, there is not yet available a broader view of the evolution of IgE molecular responses throughout a long period of time in groups of patients with different atopic diseases, ages, type and level of sensitizations in real life, without any bias usually associated to clinical trials.

In most studies, IgE antibody reactivity has been analyzed using singleplex assays with a limited number of allergen extracts that cannot distinguish genuine sensitization from cross-reactivity. The use of species specific components is extremely necessary in places like tropics where co-exposure to non-crossreacting mites genera is common.

Also, allergic patients are characterized by the production of specific IgE antibodies against allergens, whereas allergen-specific IgG antibodies occur in both allergic and nonallergic individuals. Allergen-specific IgG antibodies play a role in allergen-specific clinical tolerance, either occurring naturally or induced by allergen-specific immunotherapy (AIT). For certain allergens, IgE and IgG responses differ and allergen-specific IgG antibodies may be associated with exposure to the respective allergen.

The microarray multiplex technology allows measurement of allergen specific IgE and IgG antibody responses to many allergen components. This allows an unprecedented precision of mapping the sensitization profiles down to the level of disease-causing allergen molecules. The MeDALL allergen-chip is based on microarray technology for diagnosis and monitoring of IgE and IgG reactivity profiles towards a wide-ranging variety of allergens. The chip contains a panel of 13 components from *Dermatophagoides pteronyssinus* (Der p) - Der p 1, Der p 2, Der p 4, Der p 5, Der p 7, Der p 10, Der p 11, Der p 14, Der p 15, Der p 18, Der p 21, and Der p 23 and clone 16-encoded allergen.

Study goals and objectives

The main objective was to assess a 20-year-longitudinal evolution of IgE and IgG responses to Der p molecular allergens in HDM sensitized patients who had suffered or are still suffering from allergic symptoms and to establish a relationship with progression of the disease over time.

As secondary objectives we aimed to characterize the HDM molecular sensitization profile in a population of patients with allergic rhinitis and/or allergic asthma from 20 years ago and to analyse clinical symptoms recorded on patients' files. We also wanted to assess if patterns of IgE and IgG responses to distinct allergen components might be associated with different allergic diseases and different severities of each one of them. We wondered if the molecular sensitization profile might be a reasonable tool to monitor and possibly predict the course of disease and we also sought to consider the effect of AIT on specific IgE or specific IgG levels.

Methods

The study population was selected from a group of patients followed in the Department of Allergy and Clinical Immunology of the Centro Hospitalar de Setúbal, E.P.E. – São Bernardo Hospital with a history of allergy and sensitization to HDM Der p assessed approximately 20 years ago by specific IgE to total extract (ImmunoCAP, Thermofisher Scientific).

Regarding the recruitment eligibility criteria, we selected males and females aged between 18 and 80 years, being patients who started to be followed in the Department of Allergy and Clinical Immunology since March 1994 until June 2000 with clinical manifestations of allergy and documented sensitization to HDM Der p assessed by specific IgE to Der p total extract

(ImmunoCAP). All patients with these characteristics necessarily had to sign the informed consent form for inclusion in the study.

Data sources

- Clinical follow-up and sera collection: Patients were contacted by phone to come to a hospital visit during Winter season, where an assessment of the clinical history of the disease progression over time was performed, after signing the informed consent form. The patient was asked about data concerning how the disease progressed until the present time or if there were any development of new symptoms, changes in geographic residence and present symptoms and treatment medication. A blood sample analysis was collected to be evaluated with MeDALL-chip.
- Medical records data and stored sera bank: Data from patients' files was also collected, from the time when the first blood sample was taken approximately 20 years ago, concerning symptoms and treatment medication in previous Fall/Winter seasons and the date when the allergic symptoms started. Also, the 20-year-old stored blood samples of each patient were analysed by MeDALL-chip.
- Definition of variables: Clinical outcomes were defined from medical records and follow-up visit, with a gravity score, concerning asthma and rhinitis symptoms and the treatment medication used, according to Global Strategy for Asthma Management and Prevention (GINA 2018 update) and Allergic Rhinitis and its Impact on Asthma (ARIA guidelines 2016 revision), respectively.

Ethics approval was asked and obtained from the Ethics Committee of the Centro Hospitalar de Setúbal E.P.E. – São Bernardo Hospital and from the National Commission of Data Protection. An informed consent form was obtained from all the research participants by the principal investigator during the unique visit to the hospital for research purposes.

Clinical files selection, patients' recruitment, clinical data assessment, collection of new blood samples and recovery of stored blood samples took place in the Department of Allergy and Clinical Immunology, namely in the Immunology and Molecular Biology Laboratory of the Centro Hospitalar de Setúbal, E.P.E. – São Bernardo Hospital, Setúbal, Portugal. The analysis of the blood samples (stored from 20 years ago and from the present) was performed with microarray technology using MeDALL, in the Center for Pathophysiology, Infectology and Immunology, Dept. of Pathophysiology and Allergy Research / Division of Immunopathology, Medical University of Vienna, Austria.

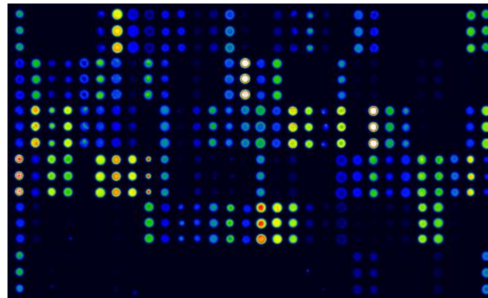
Allergen-specific IgE and IgG measurements

For analysis of the samples, a customized allergen chip based on ISAC technology (Thermo Fisher Scientific, Uppsala, Sweden) that was developed in the FP7-funded European Union project MeDALL (MeDALL-chip) was used at the Medical University of Vienna (EK1641/ 2014) (Lupinek et al., 2014). All sera were analyzed anonymously. Aliquots of 30 µl serum were incubated on the microarray and after 120 min of incubation at room temperature, slides were washed, and fluorochrome-labeled IgE specific detection antibody (Thermo Fisher) was added and incubated for 30 min at room temperature.

Figure 1. Chips into washing buffer, on magnetic stirrer / **Figure 2.** Each chip comprises 6 arrays containing serum or sample diluent/calibrator.

For detection of IgE, sera were applied undiluted, for detection of IgG, samples were diluted 1:50 in sample diluent (Phadia AB). For calibration and detection of background signals a calibrator serum and sample diluent (Phadia AB), respectively, were included in each run of serum analysis. Chips were then washed, dried and analyzed using a Laser Scan Confocal microarray reader. The results were evaluated using Phadia Microarray Image Analysis (MIA) software and reported in ISAC Standardized Units for IgE and IgG detection (ISU) by using a standard calibration curve. The cut off was 0.3 ISU-E for IgE and 0.1 ISU-E for IgG, according to instructions from the manufacturer.

Figure 3. Detection of allergen-specific IgE and IgG by the MeDALL allergen-chip. Each of the allergen molecules on the chip was spotted in triplicates (groups of three dots aligned vertically), signal strength is visualized in false colours with increasing fluorescence intensities from blue to red/white.



The MeDALL-chip comprises 176 allergen components including aero- and food allergen components which have recently been described and validated (Lupinek et al., 2014).

Results

In this study, the number of selected patients was 55, with 54,5% being females, with a mean age of 37,4 ± 14, minimum of 20 and maximum of 78 years old. Mean age of the patients when the first sera were collected was 18,4 years old, approximately 20 years ago. The assessed demographic characteristics of participants included presence of current and past symptoms of atopic dermatitis, allergic rhinitis and allergic asthma. Also, the periodicity and severity of symptoms were assessed accordingly to data present in past clinical files and information collected with current clinical history to the three atopic diseases. Regarding asthma symptoms,

patients were asked about symptom control and use of relief medication. Patients were also questioned about their follow-up with an allergy specialist, family history of atopic diseases, presence of food or other airborne allergies and changes in geographic residency. Regarding use of AIT, participants were inquired about having received it and the composition, period and symptom improvement were analyzed in the past clinical files.

The clinical results together with the data obtained by multiplex assay MeDALL-chip for IgE and IgG detection are under conjoint analysis with special focus on HDM components. For each patient a comparison between current and 20-years-ago sera should allow better understanding the evolution of sensitization patterns across time.

Figure 4. Overview of the HDM molecules contained on the MeDALL allergen-chip, results being displayed for IgE results in pairs of “o” (old) and “n” (new) sera from each patient.

Discussion

Microarrayed allergens are increasingly used to determine the molecular profile of IgE sensitization in populations. However, to our knowledge, the present study is the first real-life study comparing IgE and IgG responses to a large panel of allergens throughout a period of 20 years in a group of well-characterized HDM allergic patients with different atopic diseases and distinct severities. The main strength of the project was the possibility to compare the results between patients who had HDM AIT with the ones who hadn´t.

However, some problems were anticipated as the rate of patients accepting inclusion in this research study being low, regarding the 20-year-gap, especially if they no longer had allergic symptoms. Having this in consideration, the decision of measuring IgG specific antibodies was made, being the research course adapted from the original plan.

Regarding the eventual impact in future research, results of the present study will answer the long-sought question of how IgE and IgG reactivity profiles in adult allergic patients may change or remain constant during life-time, being valuable also for better understanding when allergen-specific interventions such as avoidance or AIT should be undertaken or not.

Extended results and discussion will be subject of a near future scientific paper.

Other activities

- Expression, purification and characterization of recombinant olive, *Parietaria* and peanut allergens

In this fellowship I had the possibility to learn and assist in expression, purification and characterization of novel recombinant olive and *Parietaria* pollen allergens as well as peanut allergens. The main purpose was to study certain unknown features of those such as allergenic activity and possible association with allergic disease phenotypes. For this, the allergens were expressed as recombinant proteins in *Escherichia coli (E. coli)* and/or baculovirus infected insect cells. They were purified, biochemically characterized and after that, used to study IgE recognition frequency and allergenic activity in basophil activation experiments using sera of clinically well-characterized pollen-allergic and peanut-allergic patients.

Recombinant proteins can be manufactured in bacteria, yeast, insect cells and mammalian cells. The most widespread expression system is *E. coli* with the advantage that it is relatively cheap, easy to handle and the proteins are produced in large amounts. Peanut profiling, Ara h 5 was expressed in *E. coli*.

However, this system has the disadvantage that certain proteins are found in the form of inclusion bodies and need a refolding step, which can be critical for the functionality of the allergen. Few proteins cannot be properly expressed in *E coli* because they require correct disulfide bond formation and/or posttranslational modifications and therefore need to be expressed in eukaryotic cells. For this, expression in insect cells is used. The advantage of insect cells for allergen production is that the most of the proteins are well folded and therefore are easier to purify. Nevertheless, insect cells and mammalian cells are more complicated to handle than bacteria or yeast, and the manufacturing costs are also higher. We used baculovirus infected insect cells for the expression of Ole e 10 and Ole e 11 proteins.

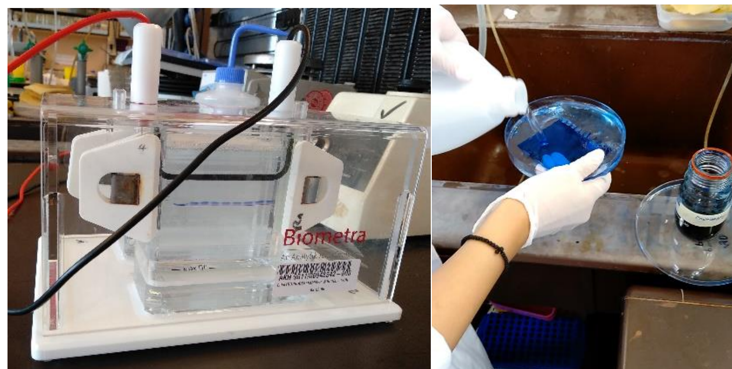
Figure 5. Blue/white selective LB agar plates containing antibiotics for the selection of appropriate DH10Bac™ transformants containing insert with bacmid DNA (white colonies) / **Figure 6 and 7.** Agarose gel electrophoresis

The constructs containing the desired DNA sequence were transformed into DH10Bac^{tm} *E. coli* cells to generate high molecular weight recombinant bacmid DNA. Baculovirus infected insect cells were used for the expression of the recombinant proteins.
Optimal expression of recombinant proteins in various expression systems including *E. coli* can be easily achieved when the vectors and host cells are carefully chosen, and the growth conditions are properly controlled. Culture conditions and the induction of expression have profound effects on the way the recombinant protein is produced and directly influence the strategies for protein purification. All proteins were purified by Ni^{2+}-affinity chromatography. For this procedure urea or imidazole containing buffers were used.

Figure 8. Purification of recombinant protein on Ni-NTA resin in a column.

After the purification step, I also had the possibility to perform SDS-PAGE to confirm the presence and purity of the protein previously expressed.

Figure 9. SDS-PAGE/ **Figure 10.** Coomassie blue staining



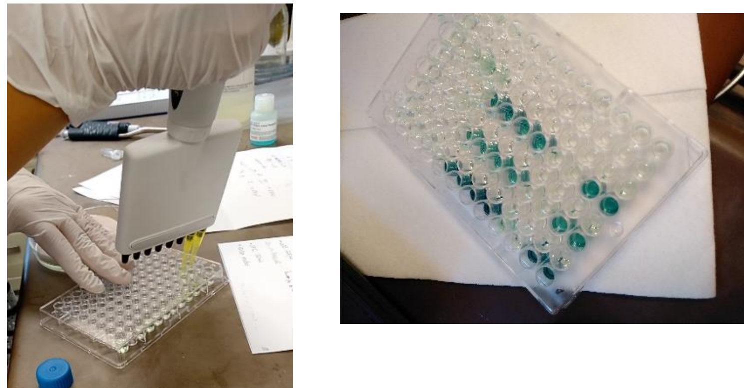
- ELISA

The enzyme-linked immunosorbent assay (ELISA) technique is the gold standard for quantitation of soluble proteins and provides rapid and consistent results that are easy to analyze.

We next analyzed the immunological properties of the produced recombinant proteins or peptides (Ole e 1 expressed in insect cells and Par j 2-derived synthetic peptides expressed in *E. coli*) by ELISA to determine their IgE reactivity.

ELISA plates were coated with allergen and left overnight. After that the plates were washed, blocked and incubated with the sera from allergic patients and negative controls. Bound IgE antibodies were detected with anti-human IgE antibodies. Optical density values (OD) were measured on ELISA reader.

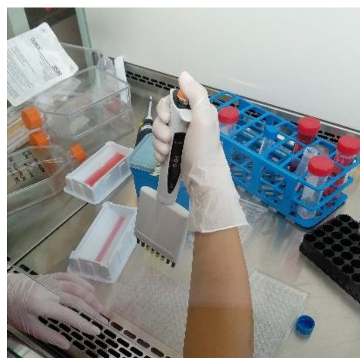
Figure 11 and 12. Direct ELISA



- RBL assay

Rat basophilic leukemia (RBL) cell-lines transfected with human FceRI have been developed. These cells can be cultured permanently, allowing for more flexibility and independence from the availability of human basophils, which are nonfunctional in 8–20% of subjects, and improved standardization. The human FceRI allows these cells to bind IgE from the sera of allergic subjects and subsequently to be activated in an allergen specific manner. Specific IgE sera screening studies are usually nonfunctional immunochemical methods which cannot measure the biological activity of proteins. Therefore, we used an assay with RBL cells transfected with human FceRI for analyzing the activity of olive allergen-derived synthetic peptides.

Figure 13. RBL assay



- Analysis of clinical relevance of molecular monosensitization in HDM allergy

AIT is an effective therapeutic approach in HDM sensitized individuals with allergic respiratory disease. However, in comparison with AIT for grass or birch pollen, AIT for HDM shows significantly lower therapeutic success. This reduced efficacy might be explained by variable amounts or even absence of some of the main HDM allergens (Der p 1, Der p 2, Der p 23) in the different commercial extracts. Recently Der p 23, a peritrophin-like protein, was identified as a new major Der p allergen. Its recent commercial availability for diagnostics allowed us to identify a new group of patients sensitized only to Der p 23 and not to other major HDM allergens (Der p 1 and Der p 2). Therefore, we raised the question about the IgE-reactivity profile, regarding other Der p components, in this type of patients. For this purpose, we sought to analyze with MeDALL chip a group of HDM-allergic patients with IgE to Der p 23 but not to Der p 1 or Der p 2.

Patients were selected from the database of the Department of Allergy and Immunology of the Hospital de São Bernardo, Centro Hospitalar de Setúbal E.P.E., in Portugal. The study was approved by the local ethics committee and the national data protection commission. The database was scanned for the patients with perennial allergy to HDM who were referred to our institution between 2010 and 2018. The sera were transferred to the Department of Pathophysiology and Allergy Research, Medical University of Vienna, Austria, for MeDALL chip analysis.

Regarding the results, we found that almost all of the patients that met the inclusion criteria mentioned above were only sensitized to Der p 23, whereas no sensitization to other HDM allergens present on the chip (Der p 1, 2, 4, 5, 7, 10, 11, 14, 15, 18, 21 and clone 16) was detected.

Patient nº	**MeDAAL Chip** (ISU; positive>0,3)
4	HDM sensitizations **Positive to:** Der p 23: 0,65 **Negative to**: Der f 1, Der f 2, Der p 1, Der p 2, Der p 4, Der p 5, Der p 7, Der p 10, Der p 11, Der p 14, Der p 15, Der p 18, Der p 21 and Lep d 2
	Other sensitizations: none

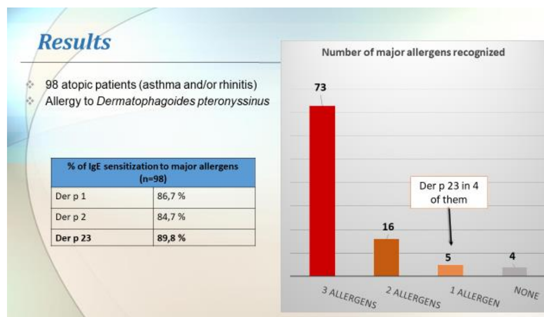
Although Der p 23 is only present in low amounts in house dust and also in HDM extracts, it is capable of inducing high IgE titers and our data have clearly shown that this allergen has clinical relevance even in patients who are negative to all other HDM components. This group of Der p 23-monosensitized HDM allergic patients will hardly benefit from SIT, in view of the probably low concentration of this component in the vaccine extracts.

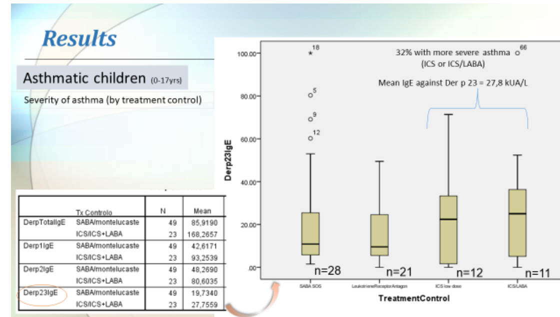
The results of this data were submitted for publication in Practitioner's Corner - Journal of Investigational Allergology and Clinical Immunology. The data was also presented in the Portuguese National Conference of Allergy and Clinical Immunology Departments (RISUS /VoAI 2018) and won the 1st place in *ex eaquo*.

- Study presentation at the World Allergy School - XII World Congress on Asthma, COPD and Immunopathology

During the Fellowship period, I submitted for presentation at World Allergy School – XII Word Congress on Asthma, COPD and Immunopathology a study previously done by my research group in Portugal about Der p 1, Der p 2, Der p 23, House Dust Mite Major Allergens Sensitization Profile in a Portuguese Population.

Concerning this subject, we analyze sera of 98 pediatric and adult HDM-allergic patients, living in different areas, with positive IgE to Der p total extract and then tested for Der p 1, Der p 2 and Der p 23 reactivity. Our study showed a high prevalence of sensitization to Der p 23, also found in German and American studies, but relatively low IgE titers, similarly to other European countries, Canada and Japan. Cases of Der p 23 monosensitization were found, also described in other cohorts. Asthma was related to more frequent recognition of all major allergens (significant for Der p 1 and 2) when comparing to rhinitis. Asthma severity in children seemed associated to higher IgE to total Der p and Der p 2. Regarding mean IgE values to Der p 23, paediatric patients with every day medication for asthma symptoms (ICS or ICS/LABA) presented with higher levels of IgE to Der p 23.





This oral presentation was awarded with the 2nd place among applications submitted.

- Study presentation at the European Academy of Allergy and Clinical Immunology 2018 Congress

I had the opportunity to participate in the EAACI 2018 Congress, which was in Munich, Germany during my Fellowship, also with work submitted and presented in two poster sessions:

  - Improving efficacy of beta lactams allergy diagnosis
  - A very unusual expression of anaphylaxis to almonds – a case of allergy to oil body allergens

- Participation at Seminars of Department of Pathophysiology, Medical University of Vienna

During the 6-month period of the Fellowship I had the opportunity to present at 3 lectures/seminars in the Department of Pathophysiology at the Medical University of Vienna, regarding different subjects in allergy field:

  - Analysis of B cell repertoires in the GI tract in peanut allergy and in early childhood development (Scott Boyd, MD. PhD., Stanford University, USA)
  - Asthma from early childhood to adolescence – from endotype discovery to stratified treatments and prevention (Adnan Custovic, MD. PhD., Imperial College London, UK)
  - Allergy to milk proteins (Léon Knippels, MSc, PhD., Utrecht, Netherlands)

I attended also at the 9th Retreat of the Center for Pathophysiology, Infectiology and Immunology of the Medical University of Vienna, where I had the opportunity to learn about the current projects in the lab.

For the future

The Fellowship has provided a successful research experience which couldn´t be more useful for a young scientist and medical doctor as me. Besides, much fruitful collaboration between the host and home institution were enabled. Combining the strengths of my home institution (the collection of sera and clinical information of allergic patients over a period of more than 20 years) and the worldwide outstanding reputation in the field of molecular allergology of the Center for Pathophysiology Infectiology and Immunology of the Medical University of Vienna, some future projects had already started. For example, peanut-allergic patients and *Parietaria* and olive pollen-allergic patients were selected regarding IgE to total extract and IgE to major allergens and sera was sent to the Center and stored for future analysis.

Acknowledgments

It was a great honor and a pleasure to develop this research project in such an outstanding working group, at the Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology of the Medical University of Vienna. This amazing and unforgettable experience would not have been possible without the support of many people.

Therefore, I really like to express my huge gratitude for this incredible opportunity to my host supervisor Professor Rudolf Valenta. I would also want to profoundly thank to my mentor in the laboratory and the person who taught me all the methodologies, Dr. Yulia Dorofeeva, who became also a good friend. I appreciate very much the support and guidance given by all the members in Professor Rudolf Valenta's group, especially by Renata Kiss and Dr. Christian Lupinek.

Acknowledgments also to my home institution, particularly to Head of Department and my PhD supervisor Professor Filipe Inácio, a visionary researcher without whom this project could not become real, to Dr. Elza Tomaz, my co-Home supervisor, and to Dr. Ana Paula Pires who has led the investigation in our laboratory. A special thanks also to Professor Luís Taborda Barata, my PhD co-supervisor.

I want to express my enormous gratefulness to EAACI for providing me the chance to work in a foreign laboratory and broad my horizons, contributing for my professional and personal development. I am fully excited to continue the scientific collaboration with further research projects in the future.

I plan to present the generated results at the annual EAACI Congress 2019, which will take place in my home country. I also pretend to publish the data in one or more scientific papers as well as include the information in the thesis for acquiring the PhD degree.

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