EAACI Fellowship – Final report 2018

Awardee: Moulin Charlotte, PhD Student

Study of class-switch history of mucosal B cells in the asthma lung

Fellowship: Medium-term Research Fellowship 6-month period (10th June 2018 to 10th December 2018)

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Host Supervisors: Dr. Louisa K. James, Dr. Hamish King

Host Institution: The Blizard Institute, Barts and the London Medical School, Queen Mary University of London, London, United-Kingdom

Introduction

Asthma is a chronic respiratory disease, affecting 5-10% of the world population. It is an inflammatory disorder of the airways where adaptive and innate immune cells act together with epithelial cells and cause airway hyperresponsiveness, overproduction of mucus and airway narrowing[1]. Two third of asthma patients present specific-IgE against common aeroallergen(s) detectable in blood, presumably reflecting local IgE production in the lung. This local IgE production can be also detected in the absence of serum specific IgE in some non-atopic patients[2]. In contrast, healthy subjects, who tolerate common aeroallergens, demonstrate regulatory responses and increased production of IgA[3], as also observed in allergic patients who underwent allergen-specific immunotherapy (*Pilette JI 2007*). The regulation of IgA production in (allergic) asthma remains however elusive.

As antibodies undergo affinity maturation, they can undergo class switch recombination (CSR). This involves isotype switching according to the heavy chain gene segment order (IgM>IgD>IgG3>IgG1>IgA1>IgG2>IgG4>**IgE>IgA2**). Based on pilot data from our laboratory showing decreased IgA1 and IgA2 mRNA levels in bronchial biopsies from patients with asthma, we hypothesize that CSR to IgA is dysregulated in asthma, in particular the theoretical sequencial switch from IgE to IgA2.

Due to the little and variable quantity of material from patients (endobronchial biopsies may give from 500 to 10,000 ng of RNA), and the difficulty to address the hypothesis as to whether a class switch from IgE to IgA2 does exist, we decided to use *Antibody Repertoire (high-troughput) sequencing*, allowing from low RNA quantity to identify with high accuracy Ig isotypes, including discrimination between IgA1 and IgA2 and lineage tracing to identify putative IgA2 clones originating from IgE.

The aim of this 6-month research project was to address a fundamental question related to CSR in the human lung. The switch of antibody genes from IgE to IgA2 that we hypothesize has not previously been described. Using antibody repertoire sequencing to discriminate isotypes subset has, up to now, never been applied to lung biopsies from asthma patients.

Working hypothesis

We hypothesise that the patterns of local CSR differ between asthma and healthy subjects, favouring IgE over IgA and that this underlies the impaired synthesis of IgA from lung B cells in asthma.

Project Time-line

Month 1: Learning process

To start the project, I received in the Host institute training in RNA extraction, cDNA library production, and qPCR using mock samples supplied by the host lab, such as frozen tonsil cells and fresh nasal polyps. This allowed me to learn molecular biology skills and to test the reproducibility of different methods.

Bioinformatics data from the host institution were used to learn how to process and treat the data with pRESTO software (bioinformatic methods were learnt from the first month to the last month, in parallel with ongoing biological sample manipulation). This education was essential to obtain skills for data analysis, allowing me to return to my home institute with sufficient knowledge to manage the raw data.

Month 2: cDNA library production and protocol optimization
The cDNA library produced during the training was presenting two peaks: one at the expected size, a second (minor) from a higher size. This phenomenon was already observed by the host institute and I tried to optimize the protocol, first on tonsil cells and nasal polyps, to reduce this unexpected library part, by testing different amounts of starting RNA, number of PCR cycles and PCR primer concentrations in order to establish the optimal parameters (allowing to reduce this second part altough not removing it completely).

Month 3-4: cDNA library production on project's samples
Compared to previous samples, our bronchial biopsy samples were of different origin and quality (small sized endobronchial biopsies, with variable RNA quality and number of cells of interest). The optimal selected parameters for cDNA library production were not sufficient to optimally solve the cDNA library production issue in all biopsies (broadly due to poor RNA quality). A first batch of sample was analyzed by deep sequencing on Illumina miSeq by the Genome Center from the Blizard Institute containing a library with this double peak profile, to evaluate its influence. It was found that the double peak profile impacted the deep sequencing data, particularly leading to a reduction of sequences percentage passing the FastQC. Following this observation I used the technique of E-gel size selection on the concerned sample to obtain unique peak library. A second batch of sample was sequenced to ensure that this processus of size selection improve the FastQC results.

Month 5: Second run of cDNA library production from another sample series
Due to difficulties in obtaining good cDNA libraries from our 24 samples (only 9/24 were successful), we reactivated our collaboration with the team from the University of Marseille (Pr Chanez P. and Dr. Gras D.), who provided additional biopsies from asthma and control subjects. These biopsies were submitted to RNA extraction, cDNA libraries production, and size selection on a part of the sample to allow a total of 29 samples processed and analysed (Tab 1. from both asthma and control subjects).

	Control (n = 8)	**Mild Asthma (n = 7)**	**Severe Asthma (n = 14)**
Gender			
M	4	4	6
F	4	3	8
Age (mean)	55	50	51
Atopy	40 %*	43 %	60 %
Rhinosinusitis	0 %	71 %	87 %
Treatment			
ICS	0	71 %	100 %
LAβ2	0	71 %	100 %
SAβ2	0	28 %	100 %
OCS	0	0 %	62 %

Tab 1. Data table from patient included in the final antibody repertoire sequencing analysis
*3 patients with missing data (atopy status)

Month 6: Final deep sequencing and data analysis
My last month was spent analyzing the bioinformatics data generated by the NGS, with the help of the Dr. Hamish King working on antibody repertoire sequencing (REPseq) data analysis at the Blizard Intitute with Dr. Louisa K. James. This analysis of 29 bronchialbiopsy samples was started at the host institute and it is still ongoing at home institute, owing to my training for such analysis. At the end of this fellowship I was also invited by Prof. Hannah Gould to present my data in her lab at the King's College London.

Preliminary Results

Percentage of unique sequences and mutation frequencies

Preliminary analysis of the deep sequencing data allowed us to identify the percentage of all different antibody isotypes in the airway from asthma patients compared to controls. We also analysed the antibody mutation frequencies of these sequences, reflecting antibody maturation affinity of the B-cell response.

Contrary to our hypothesis, we found a trend towards increased IgA1 in severe asthma compared to controls (becoming statistically significant, p=0.02 when excluding one control with chronic cough) and no significant change in IgA2. IgE was detected in half of severe asthma patients (7/14) and in one mild asthma patient (1/7). No local IgE was detected in control subjects (Fig.1). We also analyzed the percentage of immunoglobulins in IgE+ and IgE- severe asthma patients to see wether IgE expression impacted the other antibody (sub)classes (Fig.2). In IgE+ severe asthma patients, we detected increased synthesis of IgA1 and IgA2 as well as IgD, suggesting a "poly-isotypic" response in those patients, whereas IgG1 was decreased (Fig. 2).

Regarding the mutation frequencies, no significant differences were observed between the different isotypes between asthma and controls except for IgG4 (Fig.3), which increased in severe asthma patients.
Our hypothesis was that an IgE-IgA2 class switch can arise in B cells from the healthy mucosa, and could be impaired in asthma. As no IgE was detected locally in controls, we studied IgA2 mutation frequencies in severe asthma (IgE+ *versus* IgE-) based on the following idea: as a sequential switch would be associated with an antibodies with higher affinities (and higher mutation rate), a higher IgA2 mutation rate in IgE+ severe asthma would be in favor of our hypothesis. However, the IgA2 mutation frequencies were not significantly different between the two groups of this limited series of patients (Fig.4).

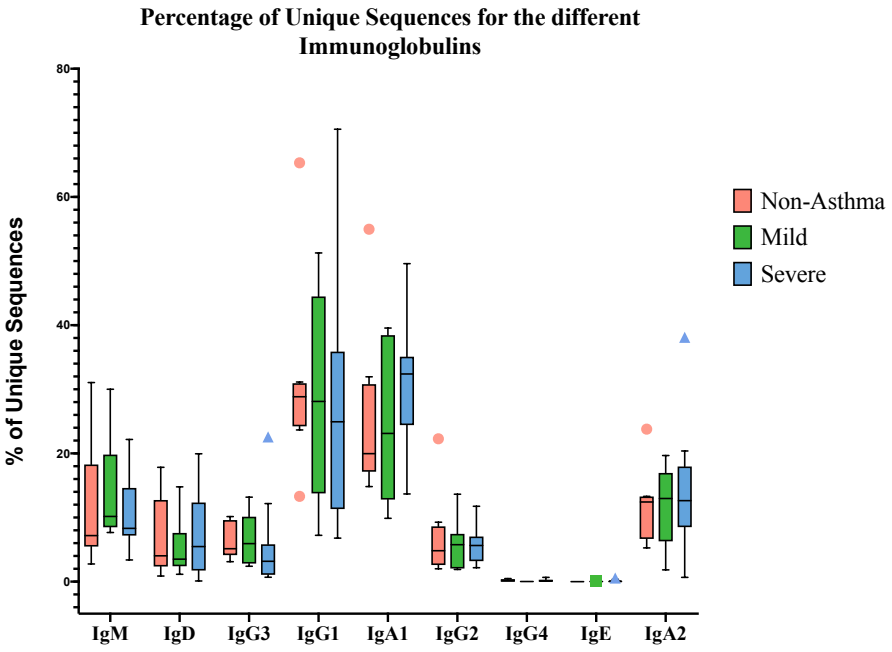


FIGURE 1. Boxplot with median of grouped unique sequences percentage in controls, mild and severe asthma subjects (controls n=8, mild asthma n=7 and severe asthma n=14). Sequences were pre-treated with pRESTO 0.5.10 with 3 sequences per UMI as threshold to build the consensus sequences.

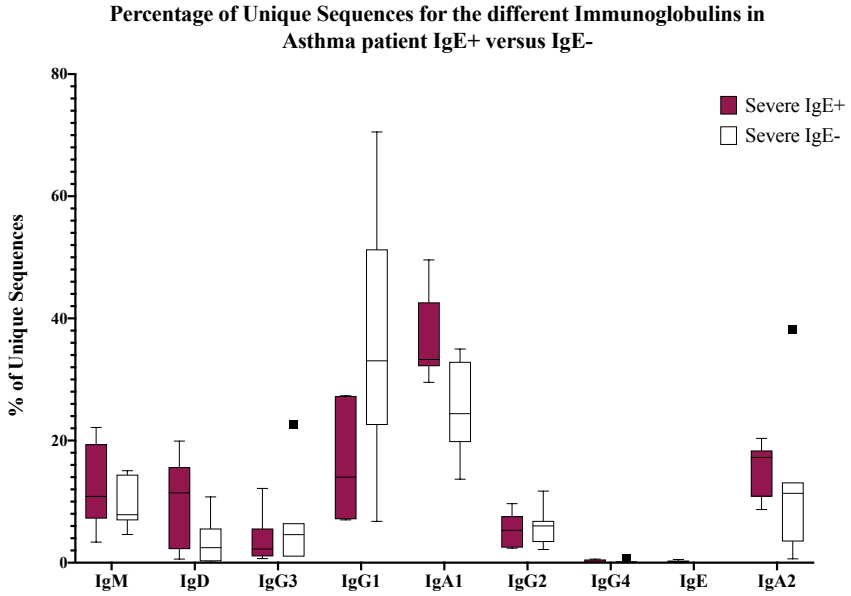


FIGURE 2. Boxplot with median of grouped unique sequences percentage in severe asthma patients displaying or not IgE switching (IgE+ severe asthma n=7, IgE- severe asthma n=7). Sequences were pre-treated with pRESTO 0.5.10 with 3 sequences per UMI as threshold to build the consensus sequences.

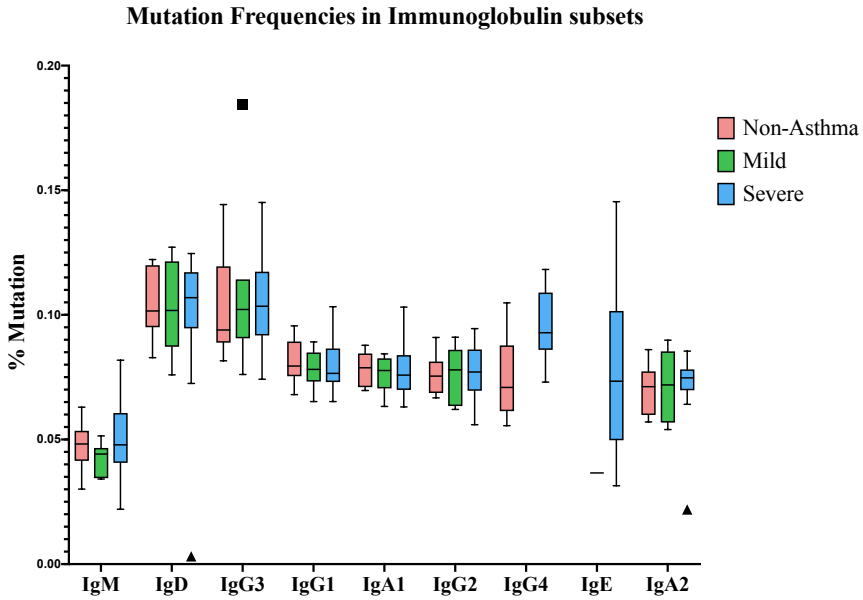


FIGURE 3. Boxplot with median of grouped mutation frequencies
(controls n=8, mild asthma n=7 and severe asthma n=14)
The mutation frequencies were obtained with the function "observedMutations" from Shazam.

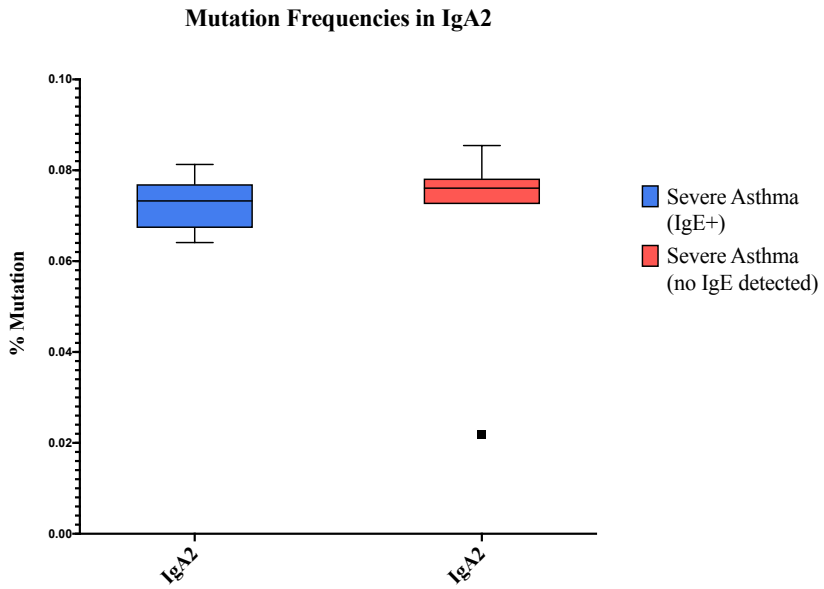
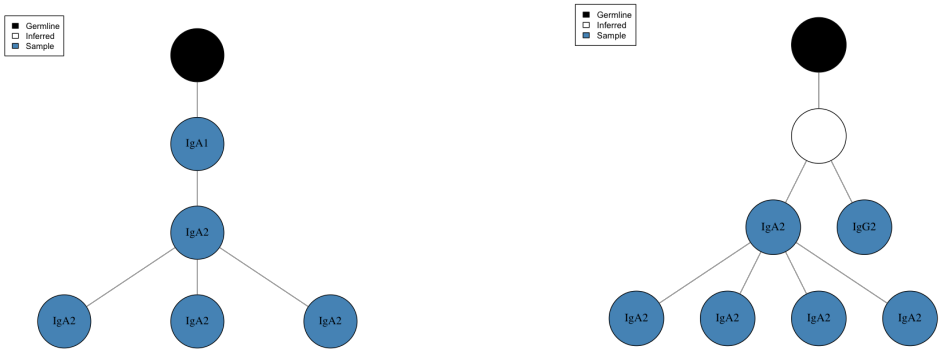


FIGURE 4. Boxplot with median of grouped mutation frequencies
(IgE+ severe asthma n=7, IgE- severe asthma n=7)

Lineage tree

We were able to identify clonally-related B cell sequences and use the patterns of somatic hypermutations and phylogenetic analysis to reconstruct lineage trees, such as clonally-related B cells that switch from IgA1 to IgA2 (Fig.5A). Using this analysis, we were able to identify, in one of the 7 severe asthma patient, specific lineage trees of interest containing both IgE and IgA2 in the clonal expansion (Fig.6). The obtained tree containing IgE and IgA2 shows two different class switches from a common ancestor but does not show a IgE to IgA2 class switch.



A. **B.**

FIGURE 5. Lineage tree build from one severe asthma patient data. **A.** This tree shows the evidence of an IgA1 switch to IgA2 with clonal expansion. **B.** This tree shows two different switches (to IgA2 and IgG2) from a same inferred sequence and a clonal expansion from the IgA2 isotype.

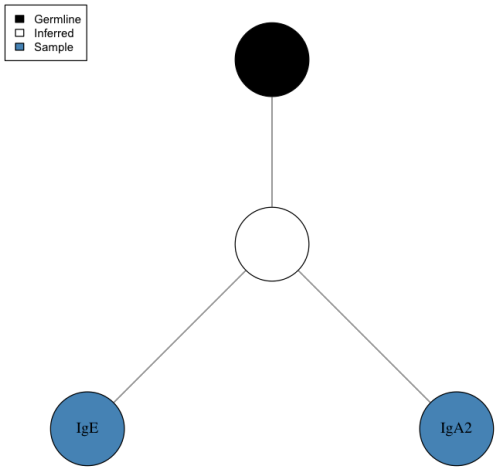


FIGURE 6. Lineage tree from one severe asthma patient containing IgE and IgA2 isotype from an inferred sequence.

Analysis of clonal abundance and diversity

The clonal abundance and diversity were tested for all isotypes from control, mild and severe asthma subjects. The only difference between controls and asthma patients was related to IgA2 clonal abundance, which washigher in asthma patients (mild and severe), approximately 6%, compared tocontrols (approximately 6% versus 1%).(Fig.7A-B).

Regarding clonal IgA2 diversity, it was found that control patient present a higher diversity that mild and severe asthma patient. This difference is present as well at the clonal richness level (q=0) as when index approaches the largest clone frequency (q=∝) (Fig.7C-D).

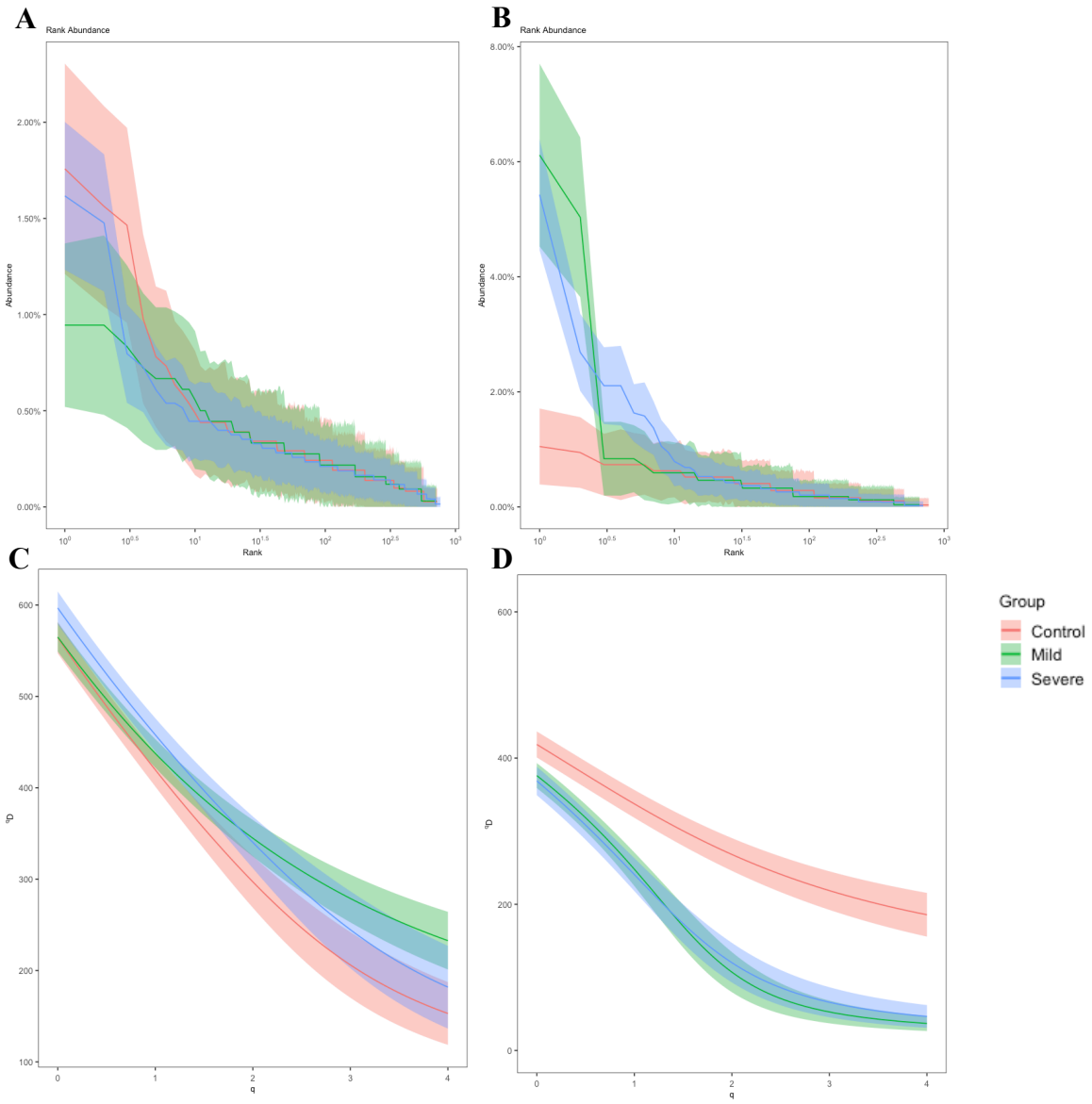


FIGURE 7. **A** and **B**: Clonal abundance of IgA1 and IgA2 respectively in control (red), mild (green) and severe asthma subjects (blue). **C** and **D**: Clonal diversity of IgA1 and IgA2 respectivaly in control (red), mild (green) and severe asthma subjects (blue).

Discussion

Our data using NGS for antibody repertoire sequencing of bronchial biopsies from 29 subjects with asthma compared to controls, enabled us to identify an increased amounts of IgA1 transcripts in patients with severe asthma.

These data differ from the pilot qPCR results showing decreases in IgA1 and IgA2 mRNA in asthma compared to controls. Of note, an increased production of IgA1 was found in our previous COPD study[4]. Due to technical difficulties in to designing qPCR primers that are highly specific to IgA1 and IgA2 (an issue overcome in NGS by comparison of the identified sequence with public databases) as well as to low variants resolution and the complexity to detect the expressed mRNA and not the germline transcripts, the new deep sequencing data can be considered as more accurate than the qPCR to quantify IgA1 and IgA2 synthesis.

Moreover, these data are consistent with our protein data observed in sputum, where IgA is increased in severe asthma compared to controls (data not shown).

Regarding the IgE class switch to IgA2, we were not able to detect IgE in control subjects (even though εmRNA was already detected in control lungs in a previous study by P. Takhar et al., 2007). Therefore, we investigated whether the switch of interest was detectable in samples where IgE switch was detected (7 severe asthma and 1 mild) but were unable to identify this switch. A subsequent sequencing at greater depth and re-sequencing of the cDNA library from IgE+ samples could be performed to increase the number of sequences, or a cDNA library could be produced from these IgE+ samples. With this reserve, one should aknowledge that, based on the current evidence confirmed in this study, this switch from IgE to IgA2 is not observed in the lung.

The analysis of clonal abundance and diversity shows interesting results, with a lower IgA2 diversity in asthma , suggesting a restricted array of antigen-driven responses when compared to healthy airway B cells.

Conclusion

This this 6-months fellowship was a fantastic opportunity for me and my project. First, I learnt molecular biology techniques, including bioinformatics analysis, that enrich both my project and my skills for the future. The bioinformatics basis that I have learnt with Dr King make me now more autonomous regarding the data analysis that still needs to be completed.

The data derived from this fellowship confirm local IgE synthesis in asthma and show that an IgE to IgA switch does not occur in the lung, even in controls, whilst further experiements should provide definite evidence for this. The other data bring many information on local antibody landscape in asthma lung, notably including at this stage of the analysis: upregulation of IgA and IgD synthesis (along IgG1 downregulation) in patients with evidence of IgE synthesis and a restricted antigenic diversity of IgA2 clones.

To understand the IgA1 increase in severe asthma, it can be interesting in the future to assess by qPCR (for the biopsies) or by ELISA for the sputum, the major pro-IgA factor/receptor (e.g. BAFF/APRIL, TACI, TGF-β, CD40L, IL-6, IL-10…).

Acknowledgements

I would like to warmly thank the EAACI and its scientic committee for granted me with this 6-month research fellowship, as well as my supervisors from the Host (Dr L. James) home Institutes (Prof. Charles Pilette and Dr. Antoine Froiure) for supporting me in this application. A special thank to Dr. Louisa K. James and Dr. Hamish King for the management and for sharing their knowledge. I also thank Dr Gras D. ad pr Chanez P. (University of Marseille), for their collaboration on this project by providing additional precious biopsy samples.

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