The Interaction between Grass Pollen Allergen and Respiratory Virus on the Immune Response in Allergic rhinitis and Asthma

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Abstract

Allergic rhinitis (AR) has a major impact on the health of Australians, with a prevalence of 19.3% in 2017-18 and costing the people $226.8 million to manage with the purchase of antihistamines (AIHW 2016). Globally, grass pollens (GP) are the major outdoor aeroallergens that triggers AR and asthma in sensitized patients (Bauchau et al 2004). A climatically diverse country like Australia has varied species of subtropical and temperate grasses, to which AR patients are exposed to in their local region (Davies et al. 2014). However, the patterns of sensitization in AR patient to the pollen of these grass subfamilies are poorly understood. To investigate this in chapter 1, participant sera from Queensland (n=15), Western Australia (n=11), New South Wales (n=8) and South Australia (n=11), were subjected to a cross-inhibition assay of specific IgE (sIgE) reactivity towards purified major GP allergens Pas n 1, Cyn d 1 and Lol p 1 with whole pollen extracts of subtropical grasses *Paspalum notatum*, *Sorghum halepense* (family Panicoideae), *Cynodon dactylon* (Chloridoideae) and temperate grasses *Lolium Perenne* and *Phleum pratense* (Pooideae). Participants from subtropical Queensland showed higher sensitisation to *P. notatum* and *C. dactylon* than *L. perenne* GP. sIgE was higher to Pas n 1 and Cyn d 1, and sIgE to Pas n 1 and Cyn d 1 was inhibited more by Panicoideae and Chloridoideae, respectively, than Pooideae GP. Conversely, participants from temperate regions showed highest sensitisation levels to *L. perenne* GP and Lol p 1, and sIgE to Lol p 1 was inhibited more by Pooideae than other GP. In summary, participants with GP allergy differed in patterns of allergic sensitisation to subtropical and temperate GP depending on biogeography and climate. Knowledge of the specificity of sensitisation to local allergens is important for optimal diagnosis and choice of allergen specific immunotherapy to maximize benefit.

AR is a co-morbid condition with asthma, with 30% of known AR patients having asthma and 80% of people with known asthma having AR (Bousquet et al. 2010). GP have been implicated as the cause of the recent thunderstorm-related asthma epidemic in Melbourne in 2016 (Thien et al. 2018). However, respiratory viruses are considered the major trigger of asthma exacerbations, with enteroviruses such as rhinovirus highly prevalent in children and influenza virus prevalent in adults with asthma (Zheng et al. 2018). While associations of asthma exacerbation with either aeroallergen exposure and respiratory virus infections have been made individually, recent studies have shown associations between sensitization to aeroallergen and increased respiratory virus-wheezing. A study demonstrated the interaction of house dust mite allergen specific IgE with the high affinity IgE receptor receptor expressed on plasmacytoid dendritic cells and the subsequent inhibition of type 1 interferons (Gill et al. 2010), showing a mechanism for the influence of aeroallergen exposure and a diminished antiviral response in asthma. To investigate the influence of GP allergy on antiviral responses, PBMC from 31 adult participants (15 allergic to *P. notatum* GP, 8 with other allergies, 9 with no allergies) were exposed with either *P. notatum* whole pollen extract (BaGP), human rhinovirus-16 (RV-16) or a combination of both and subsequently assayed for the expression of innate and adaptive cytokines and genes. The results showed an inhibition of the innate antiviral chemokine IP-10 induced by RV-16 following co-exposure with BaGP, and this inhibition was also observed in the non-atopic group. This finding suggests that the inhibition of the antiviral response following BaGP exposure may not solely rely on an indirect, IgE-mediated, late-stage adaptive immune mechanism, but can also occur at the innate level independent of IgE crosslinking.

Allergen sources have been previously shown to influence the immune response via non-IgE mediated mechanisms. GP allergen components have been shown to interact with dendritic cells directly through C-type lectin and DC-SIGN receptors and indirectly via adjuvant activities such as IgE induction. Thus, it would be important to understand the role of pollen components in its capacity to influence the immune response. To investigate this, PBMC from 42 teenage participants (19 with AR and asthma, 17 with AR only and 6 non-AR

controls) were treated with RV16 only or co-exposed with either whole BaGP or its purified major allergen Pas n 1. Results showed the inhibition of IP-10 occurred similarly to observations with adult PBMC from the previous chapter. However, this inhibition of RV16 induced IP10 was more pronounced in the whole *P. notatum* extract compared to purified Pas n 1. This confirms that there is an innate interaction between the pollen extract and the antiviral response, and that the presence of pollen components acts as an adjuvant to this interaction.

The key outcomes of this thesis inform the need for more consideration of AR in patients with asthma. It may aid management of patients with asthma to understand the specific allergen sensitization patterns and consideration of allergic status to be integrated into the clinical management of asthma.

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List of Abbreviations

AEC	Airway epithelial cells
AIT	Allergen immunotherapy
APC	Antigen-presenting cell
AR	Allergic rhinitis
AR+A	Allergic rhinitis with asthma
BaGP	*Paspalum notatum* pollen
BEC	Bronchial epithelial cells
BeGP	*Cynodon dactylon* pollen
BSA	Bovine serum albumin
CCD	Cross-reactive carbohydrate determinants
CO2	Carbon dioxide
EDTA	Ethylenediaminetetraacetic acid
ENT	Ear, Nose and Throat
FCS	Fetal calf serum
FFU/ml	Focus forming units per mililitre
GAAS	General Allergy and Asthma Study
GP	Grass pollen
GPA	Grass -pollen allergic
GPAS	Grass Pollen Allergy Study
HDM	House dust mite
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
ILC	Innate lymphoid cells
ILC2	Type 2 innate lymphoid cells
IRF	Interferon regulatory factor
JGP	*Sorghum halepense* pollen
$MgCl_2$	Magnesium chloride
MHC II	Major histocompatibility complex II
MOI	Multiplicity of infection
MPPBST	1% skim milk powder in PBS with 0.05% Tween-20
NA	Non-allergic
NEC	Nasal epithelial cells
NR	No rhinitis
OA	Other allergies
PAAS	Pollen Allergy and Asthma Study

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
QPIAS	Queensland Paediatric Immunology and Allergy Service
RGP	*Lolium perenne* pollen
RPN	Raw peanut extract
RV	Human rhinovirus
RV-16	Human rhinovirus-16
spIgE	Specific immunoglobulin E
SPT	Skin prick test
TCID50	Tissue culture infectious dose resulting in 50% cell death
TGP	*Phleum pratense* pollen
Th1	T helper cell type 1
Th2	T helper cell type 2
TRF	Time-resolved fluorescence
TSLP	Thymic stromal lymphopoeitin

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: QUT Verified Signature

Date: 20/04/2021

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Chapter 1: Literature Review

This literature review aims to establish the burden of grass-pollen induced allergic rhinitis in Australia and its immunological influence on asthma either directly or indirectly through impairment of antiviral immunity. Clinically, the management of asthma is performed by respiratory physicians and as such, the role of allergic rhinitis on asthma is often overlooked and the importance of allergic rhinitis is minimized. Current literature has largely investigated respiratory viruses and allergens as mutually exclusive triggers of asthma. This review will outline the multiple overlapping connections between the clinical manifestations of allergic rhinitis and asthma. It will examine aspects by which allergens per se, and sensitization to allergens, influence respiratory diseases. Finally, it will outline potential interactions between immunological responses to allergen and respiratory viruses, thus establishing a need to investigate these three disease states collectively.

1.1 BURDEN OF ALLERGIC RHINITIS AND ASTHMA IN AUSTRALIA

Allergic rhinitis (AR) is defined as the inflammation of the membrane lining the nose induced by an IgE-mediated immune response towards allergen exposure (Bousquet et al., 2008). The hallmark symptoms of this disorder are sneezing, nasal blockage, anterior or posterior rhinorrhea and itching of the nose, commonly accompanied by watery and itchy eyes. Following a revision of guidelines in 2008, allergic rhinitis is now subdivided based on the period of symptoms presentation to either “intermittent” or “persistent” (Bousquet et al., 2008). In Australia, the prevalence of allergic rhinitis in 2001 was 15.5% and that has increased to 19.3% between 2017 and 2018 (AIHW, 2018). This means that a large portion of the population have impaired quality of life, resulting in impaired productivity, psychosocial wellbeing and cognitive function (Fineman, 2002; Walls et al., 2005). AR also imposes a significant financial burden on the population, as reflected by the high total pharmacy transactions that include rhinitis therapy (e.g. antihistamines) of over $ 4.24 million, with single therapy options costing on average $19 and multiple therapies averaging $40. Despite its

significant social and economic burden, AR is still perceived by the public as an inconvenience rather than a credible threat to their health and quality of life.

Asthma is a chronic airway inflammatory disease, defined by respiratory symptoms such as wheezing, shortness of breath, chest tightness, as well as reduced lung function (National Asthma Council Australia 2015). In Australia, approximately 2.4 million people are affected by asthma accounting for 9.94% of the population (Blanchard et al., 2015). Asthma also accounted for approximately 117 000 disability adjusted life years (DALY), which accounts for years lived with a disability (YLD) and years of life lost (YLL) due to premature mortality (Health & Welfare, 2014). Furthermore, a cross-sectional study in community pharmacies in 2007 across 4 major Australian states found that 77% of 570 patients with asthma had poor asthma control identified by a symptom and activity tool, and only 19% of that cohort had an asthma action plan (Armour et al., 2011) . Asthma also has a significant psychological and emotional burden on its patients. A meta-synthesis of people's experience of living with asthma summarizes negative experiences, such as feelings of being judged by others, the frightening nature of its symptoms and fear of medication dependencies (Pickles et al., 2018). Apart from the individual and community health impact, asthma also inflicts a financial burden on the country. $655 million was spent on treating asthma between 2008 and 2009, and that number rose to $1.2 billion in 2015 (Health & Welfare, 2020).

Asthma is a complex, heterogeneous disease that varies in severity, symptoms and treatment response across individuals (Anderson, 2008). Currently, it is characterized based on a collection of varied and recurring symptoms, namely airway inflammation, airflow obstruction and bronchial hyperresponsiveness, making it more akin to a syndrome than a disease (Lötvall et al., 2011). This complex nature has made it difficult to accurately outline the underlying causes of the disease. It is established now that there is no single cause of asthma, rather a collection of factors that can be generally classified as external factors and internal factors. Internal factors are mainly a genetic predisposition of an individual towards an abnormal inflammatory response, leading to the hallmark airway hyperresponsiveness and subsequent obstruction. The external factors are typically environmental factors that can elicit airway

hyperresponsiveness via inhalation of respirable sized particulate triggers or respiratory infections.

1.2 ASSOCIATIONS BETWEEN ALLERGIC RHINITIS AND ASTHMA

There is increasing evidence that asthma has a strong association with allergic disease, especially AR. The most common association is described in the atopic march, which is the progression from atopic dermatitis in infancy and subsequent development of AR and asthma later in childhood (Bantz et al., 2014). A key paper discussing the impact of AR on asthma states that 80% of asthmatics were found to have AR while 30% of those with AR have asthma (Bousquet et al., 2008). Further investigations into the association of AR and asthma revealed the role of inhaled allergens (or aeroallergens) with increased risk of asthma. A cohort study (n=1314) on childhood allergic sensitisation showed an association between perennial allergens sensitization such as house dust mite (HDM) with loss of lung function reflected by low forced vital capacity (FVC) and forced expiratory volume-one second (FEV1) scores at school age (Illi et al., 2006).

While clinical associations between AR and asthma have been made, the underlying mechanisms are poorly understood. The biggest advance in understanding the pathogenesis of allergic disease is the discovery and characterization of immunoglobulin E or IgE (Ishizaka et al., 1966). AR is characterized as an IgE-mediated immediate hypersensitivity reaction, initiated when environmental allergens are deposited on the mucosal surface of the airway, where they contact antigen presenting cells (APCs) such as dendritic cells. Properties of the allergen source drive the innate immune system towards an allergic inflammatory response via induction of alarmins IL-25 and IL-33. With the assistance of major histocompatibility complex II (MHC II), the allergens are presented by APC to T cells which differentiate from naïve $CD4^+$ cells into allergen-specific T helper 2 (Th2) cells. These cells then secrete hallmark type 2 cytokines factors such as IL-4, IL-5 and IL-13 which promote basophil and eosinophil recruitment to mucosal site, stimulation of B cells to class switch to IgE, and finally mast cell degranulation (Min, 2010). Under conditions of allergic sensitisation, B cells are activated by allergen-specific Th2 cells, which within germinal centres of secondary lymphoid organs (e.g. local draining lymph nodes), the

B cell immunoglobulin heavy chain genes re-arrange toswitch class either directly from IgM in naïve B cells, or indirectly via IgG4, into allergen-specific IgE, during the allergic sensitisation phase (Davies et al., 2013). Upon re-exposure to that allergen, these IgE captured via the FcεR1 on the surface of inflammatory mucosal mast cells and blood basophils will then crosslink intracellular signalling domains releasing mediators such as histamine. The release of such pre-formed inflammatory mediators results in the manifestation of the symptoms in an early phase of an allergic reaction which typically are rhinorrhea, and sneezing (Walls et al., 2005). The discovery of IgE led to the development of diagnostics methods that could measure quantitatively an individual's reactivity to allergens by the IgE found in the sera (Hamilton et al., 2015). Specific IgE produced against grass pollens was recently found to precede the onset of AR and proven to be a good predictor of it as well (Hatzler et al., 2012). Further studies have also demonstrated that total IgE and specific IgE to grass pollens was associated with asthma onset, but not asthma severity (Buslau et al., 2014; Siroux et al., 2003). Thus, grass pollen specific IgE has potential as a prognostic biomarker of both AR and asthma.

High circulating levels of IgE in patient sera typically leads to allergic inflammation of the lungs, which occurs due to the development of a Th2 cytokine profile. A key phenomenon observed in asthma as well is the predisposition to a Th2 profile. Patients with asthma were found to have an inherently higher secretion of Th2 cytokines such as IL-4, IL-5 and IL-13, without allergen or viral stimulation, along with enhanced recruitment of eosinophils and mast cells observed in sputum, which account for the inflammatory conditions observed in asthma (Berry et al., 2004; Kabesch et al., 2006; Saha et al., 2008; Woodruff et al., 2009). Exposure to allergens in allergic patients generated a similar Th2-driven response in bronchial epithelial cells as observed in asthmatic patients, which heightens the severity of the inflammatory response leading to asthma exacerbation (Del Prete et al., 1993). While most studies have examined the adaptive phase of immune response leading to allergic inflammation, recent studies have revealed the role of innate lymphoid cell, specifically group 2 ILCs and their capacity to induce allergic inflammation at the innate level following exposure to Th2 inducing allergens such as HDM (Gold et al., 2014). Zissler and colleagues have summarized 161 potential shared biomarkers of respiratory inflammation, comprising cytokines related to not just Th1 and Th2

lymphocyte phenotypes but also Th17, Treg and 20 epithelium-derived cytokines (Zissler et al., 2016). Thus, it appears that there is a lot of immunological overlap, at both innate and adaptive level, in the pathogenesis of both asthma and allergic disease.

While both AR and asthma are complex conditions with heterogenous aetiology, they do share clinical manifestations and with recent genetic studies, possibly pathogenic pathways too. The earliest genetic links were made in a study in 2007 examining susceptibility to asthma in a cohort of twin children to asthma, eczema and rhinitis, with parentally reported frequencies of 8.7%, 16.8 % and 4.4% of the respective diseases and a heritability of approximately 90% (van Beijsterveldt & Boomsma, 2007). A 20-year study on a German cohort of 942 infants showed that at age 20, 18.5% of participants with allergic parents had two to three concurrent allergies compared to 6.3% in those with non-allergic parents. Also, in the same cohort, between a third to half of the participants with AR had concurrent asthma and/or eczema from school age onwards (Gough et al., 2015). With advancement in genetic sequencing methods, a large body of studies attempted to elucidate genetic links to AR and asthma, beginning with single nucleotide polymorphism (SNP) associations. While more than 100 of SNP associations were made, the reproducibility of these studies was low, prompting another approach (Nilsson et al., 2013). A recent genome-wide association study (n=360, 838) showed that asthma, AR and eczema are likely to coexist within a patient because they share 136 genetic risk variants that may dysregulate lymphocyte-mediated immunity, with six variants shown to have disease-specific effects (Ferreira et al., 2017).

1.3 GRASS POLLEN, A MAJOR ENVIRONMENTAL TRIGGER OF ALLERGIC RHINITIS AND ASTHMA

AR and asthma also share environmental triggers, typically resulting in an exaggerated hypersensitivity response. Dampness and mould indoors have been associated with a higher prevalence of asthma, AR and eczema in children (Wang et al., 2019). Further investigations into the indoor environment revealed the role of perennial aeroallergens, with HDM being the most associated with allergic airway diseases (Gandhi et al., 2013). Grass pollen (GP) has also been identified as one the most clinically important allergen worldwide by sensitization, associated most commonly with AR and asthma (Bauchau & Durham, 2004; Scala et al., 2010a).

Similarly, in Australia, AR is mostly associated with aeroallergens, primarily house dust mite, with grass pollen being the second most prevalent (AIHW, 2018).

Extreme weather conditions have been found to be associated with increases in the onset of AR and asthma, partially due to an increase in grass pollen concentrations in the air. Thunderstorm asthma is the most prominent of these extreme weather events, where there is a drastic increase in asthma-related hospital admissions, some proving to be fatal following a thunderstorm. In 2016, a thunderstorm event in Melbourne was associated with an unprecedented number of emergency department presentations (3365 admissions) related to asthma and 10 associated deaths (Thien et al., 2018). Surprisingly, a study that interviewed 1435 of thunderstorm-affected patients, for which 164 required hospital admission, found that AR was present in 87% of the patients while current asthma was only prevalent in 28% (Hew et al., 2019). In a study assessing 85 of the affected patients, 60% had no previous asthma diagnosis but 99% of the same cohort reported AR during the grass pollen season, with all patients showing ryegrass pollen sensitization (Lee et al., 2017), suggesting GP-related AR is strongly associated with, and may be a requirement for susceptibility tothunderstorm asthma, and further that some patients with AR may have "latent' or undiagnosed asthma. The most recent proposed causative mechanism for the onset of thunderstorm asthma is a high concentration of allergenic grass pollen (ryegrass pollen in the Melbourne case) produced by a thunderstorm outflow of cold air, which sweep up pollen grains and concentrate them in a shallow band of air at ground level (Davies et al., 2017). Thus, there is a need to monitor the concentration of allergenic grass pollen in the environment to better predict potential thunderstorm asthma-related events to better manage its burden on the healthcare system (Davies et al., 2017).

1.4 BIOGEOGRAPHICAL VARIATION IN SPECIFIC IGE RECOGNITION OF GRASS POLLEN ALLERGENS IN AUSTRALIA

Currently, literature surrounding the pollen of Poaceae grasses, and its relation to allergic disease and asthma has focused on temperate grasses. Temperate grasses belong to the subfamily of Pooideae, the most prominently studied allergenic species being Timothy grass (*Phleum pretense*), which has been identified as the most clinically relevant outdoor allergen source in temperate regions such as Europe, as tested by IgE sensitization (Ghunaim et al., 2005). In Australia, the most prominently identified allergenic species in southern regions such as Victoria and South Australia

is ryegrass (*Lolium perenne*) (Davies et al., 2011). Large countries such as Australia have a varied climate across the nation and hence, results in a varied distribution of grass species encompassing both temperate species and subtropical species, as illustrated in Figure 1.4.2 (Medek et al., 2016). In regions with a subtropical climate such as Queensland in Australia, the subtropical species of grasses are more abundant. These grasses arise from the subfamily of Panicoideae, which include Bahia grass (*Paspalum notatum*), Johnson grass (*Sorghum halepense*) and from the Chloridoideae subfamily, Bermuda grass (*Cynodon dactylon*) (Davies et al., 2012). In the south-east parts of Queensland, grass pollen was found to account for 40%-72% of the total pollen in the air, with pollen concentration positively associated with temperature, suggesting a correlation between grass pollen distribution and climate (Green et al., 2004; Rutherford et al., 1997).

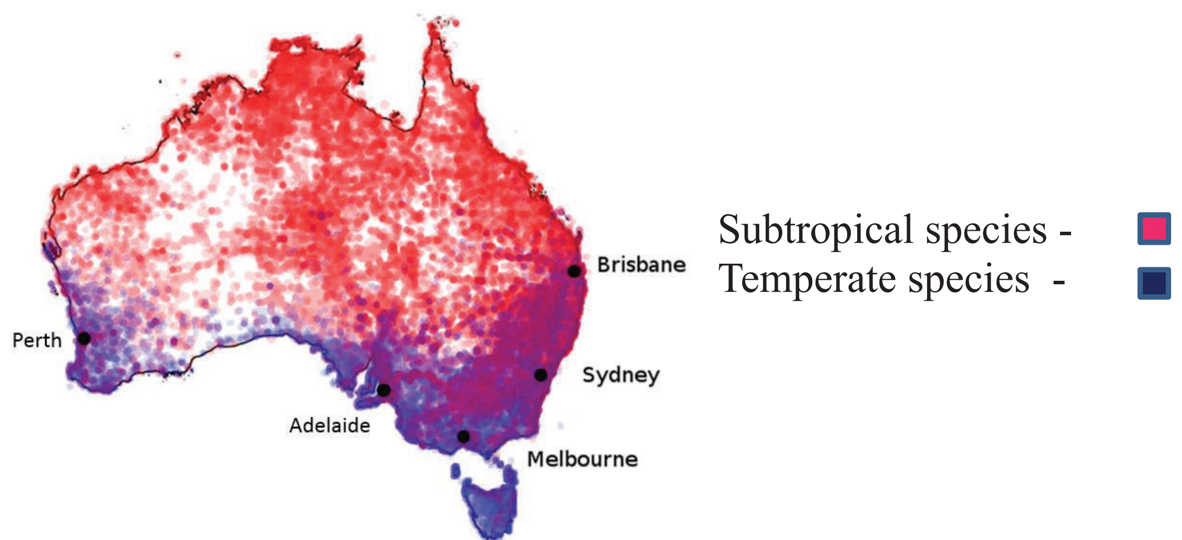


Figure 1.1.1 Distribution of subtropical and temperate grasses in Australia. Adapted from (Medek et al., 2016)

As grass species distribution is varied in a large country with heterogenous climates such as the United States of America and Australia, there is also variation in immunological recognition of grass pollen across regions. This results in varied clinical relevance of grass species as patients are primarily sensitized to the pollen of grass abundant in their state of origin. Patients from subtropical regions such as Queensland appear to have higher specific IgE concentrations to the subtropical grass pollens *Paspalum notatum* (Bahia), *Cynodon dactylon* (Bermuda) and *Sorghum halepense* (Johnson) than temperate

grass pollen (Nony et al., 2015). This trend is observed in other countries as well. A study on adult patients' allergic reactivity in Zimbabwe found a higher frequency of IgE reactivity with *C. dactylon* (38%) compared to *Phleum pratense* (Timothy grass) (26%) (Westritschnig et al., 2008). Similarly, in the temperate region of Melbourne, patients were found to have a higher specific IgE reactivity to the temperate grass *L. perenne* pollen and its purified allergen, Lol p 1 than levels of specific IgE with subtropical Bahia and *C. dactylon* grass pollen (Davies et al., 2011).

1.5 THE EMERGING IMPORTANCE OF SUBTROPICAL GRASS POLLEN AS AN ALLERGEN SOURCE

The burden of subtropical grasses as an allergen source may increase in the coming years. The biggest factor in this could be climate change, specifically the increase in temperature due to greenhouse gases such as carbon dioxide globally, conditions for which the C4 grass species of subtropical grasses thrive on (Norris et al., 2016; Polley et al., 2014). The increase in temperature globally is also causing the expansion of tropics pole wards, a condition which Australia is particularly vulnerable to given its proximity to the south pole, leading to expanded dry subtropical zones, decreased winter rainfall and rising temperatures (Turton, 2017). Furthermore, as half of the global population lives in or near subtropical, semi-arid zones, the widening of the tropics may result in most of the global population living in subtropical zones (Staten et al., 2018).

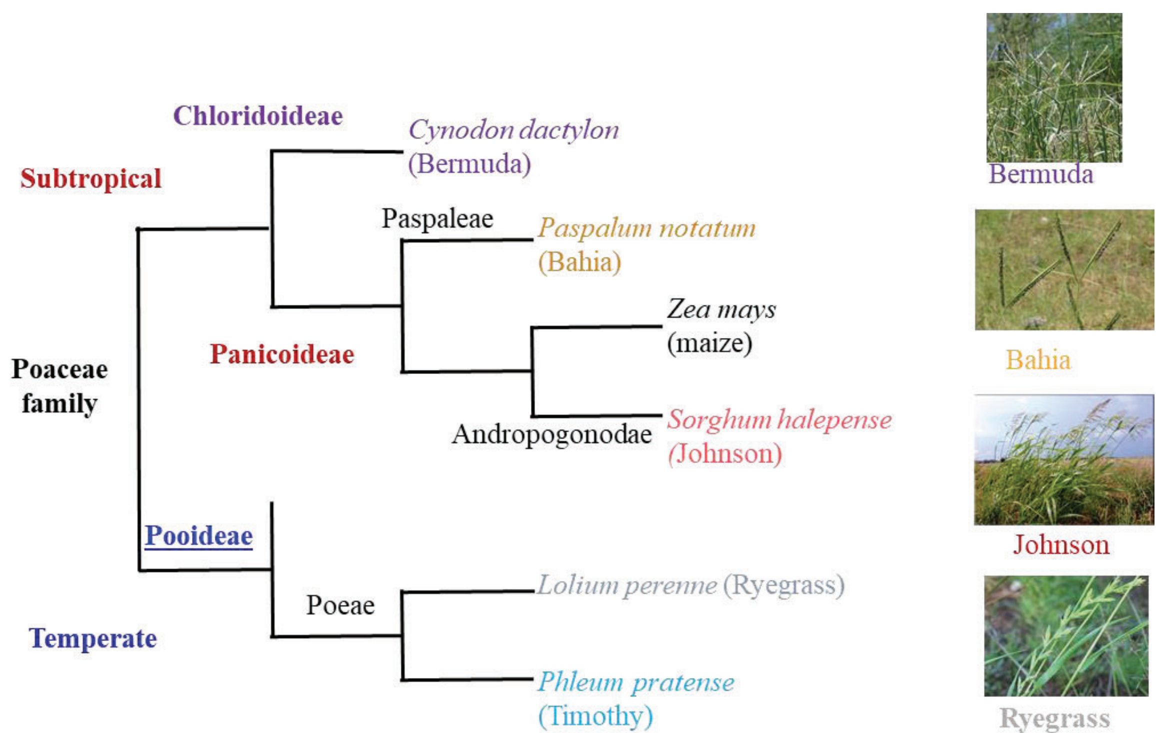


Figure 1.1.2 Phylogenetic trees of known allergenic grasses. Adapted from (Davies et al., 2012).

Due to the potential increase in the burden of subtropical grass pollen globally, more studies are conducted to further understand the allergology of subtropical grass species. Phylogenetic differences between the subtropical and temperate grasses are well established, as represented by the phylogenetic tree in Figure 1.5.1, with phylogenetic differences even observed between the Panicoideae and Chloridoideae subfamilies of subtropical grasses (Davies et al., 2012). A common misconception was that temperate grass species immunologically recognized similarly to subtropical grass species, but recent studies have disproved this. While showing some level of sensitization to all three species, patients with grass pollen allergy from Queensland (a subtropical region) showed higher skin prick test diameters towards subtropical grass pollen of *P. notatum, S. halepense* and *C. dactylon* compared to the temperate grass pollen of *L. perenne* (Davies et al., 2012). This cohort was further investigated by reciprocal cross-inhibition assays to examine IgE reactivity towards a solid-phase preparation of the four grass allergens when inhibited by their liquid-phase counterparts. The results show that the subtropical species were more specifically recognized by the patients, indicated by higher maximum inhibition, representing binding avidity and concentration of pollen inhibitor required to block 50% of IgE reactivity, compared to the temperate species. This suggests that there are unique

epitopes that are not shared between subtropical and temperate species. Furthermore, there are unique epitopes within Chloridoideae-derived *C. dactylon* that are not shared with the other Panicoideae subtropical species. This confirms that there are differential immune recognition not just between subtropical and temperate grass pollen species, but also within the two subfamilies of the subtropical species.

Further investigations into the composition of subtropical and temperate grass pollen have shown differences in allergen composition as well. Grass pollen have been shown to contain multiple allergenic proteins, the most frequently recognized in patients with known whole grass pollen IgE sensitization being the group 1 allergens, characterized as beta-expansins and constitute 10% of the total pollen content (Drew et al., 2011). While all allergenic grass pollen species share group 1 allergens, there are allergen groups that are unique to either subtropical or temperate species. Temperate grasses contain group 5 allergens, which are also frequently recognized though not to the same degree as group 1 allergens, while subtropical grass pollen lacks this group altogether (Kailaivasan & Davies, 2018; Westritschnig et al., 2008). As the recognition of subtropical grass pollens cannot be fully represented by temperate grass pollens, there is currently much debate surrounding the validity of utilizing diagnostic and treatment options based on temperate grasses.

Identifying the dominant allergen in a region is imperative for the development of specifically targeted therapy that can help reduce the burden of allergic disease in a community. One such method, allergen specific immunotherapy, involves administering gradually increasing doses of allergen extract, resulting in the reduction of symptom severity (Rolland et al., 2009). Studies on the administration of grass pollen immunotherapy have shown not just a reduction in allergic disease symptom severity but also, reduced the onset of asthma (Jacobsen et al., 2007; Möller et al., 2002). Meta-analysis of 88 previously conducted trials on immunotherapy administration for various allergens including house dust mite, animal dander, mould and pollen, have demonstrated a significant reduction in asthma symptoms and even asthma-related medication use (Abramson et al., 2010). However, a study has demonstrated that while there is some immunological cross-reactivity with pollen allergens of the temperate grass *L. perenne*, there is also species specific IgE reactivity observed for the pollen allergens of *P. notatum* (Bahia grass) (Davies et al., 2011). As such, grass pollen immunotherapy developed based on temperate grasses may have

less efficacy when applied for patients in subtropical regions, who are primarily sensitized to subtropical grasses.

1.6 HUMAN RHINOVIRUS, THE LEADING TRIGGER OF CHILDHOOD ASTHMA

Early-life exposure to respiratory viruses, leading to acute lower respiratory tract infections and bronchiolitis, is the most common cause of wheeze in children and subsequent development of asthma in later life. For children younger than the 1 years old, the primary associated cause is respiratory syncytial virus and for those older than 1 years old, it is human rhinovirus instead (Wark et al., 2018). A cause for the common cold, rhinoviruses are non-enveloped, positive-strand RNA viruses of the Picornaviridae family, with more than 100 antigenically distinct serotypes that are classified into species A and B based on their susceptibility to antiviral drugs and partial genetic sequences. A new C species was discovered in 2006, previously unidentified due to inability to culture it in the lab using conventional host cells at the time (Ashraf et al., 2015).

Associations between hospital admissions due to asthma exacerbation and human rhinovirus infections have been made in many studies. The hallmark study of 108 school-aged children showed that 80 to 85% of reported asthma exacerbations were associated with respiratory viruses, with rhinovirus as the most frequently detected virus, and a strong correlation between seasonal patterns of upper respiratory infections and asthma related hospital admissions was observed during periods of school attendance (Johnston et al., 1996). A prospective study following 285 children from birth to 3 years of age showed that respiratory illness with wheezing during infancy was a strong risk factor of 3rd year wheeze, with human rhinovirus at odds ratio of 10 and respiratory syncytial virus at odds ratio of 3 (Lemanske et al., 2005). Respiratory virus infections are also more closely associated to acute asthma exacerbations compared to chronic asthma. A study on children admitted to a hospital for asthma over a one-year period, found that 48% of children with acute asthma exacerbations had respiratory viral infections, compared to the 18% in stable asthmatic (Murray et al., 2006). In the same study, of the viral infections in the acute asthmatic group, 33% were identified as rhinovirus infections, significantly higher than other

virus groups such as RSV, coronavirus, and influenza, all below 5% (Murray et al., 2006).

The different serotypes of human rhinovirus are associated differently with disease and severity. Monitoring of infants through to age 1 have shown that human rhinovirus A and C species were 7 to 8 times more likely to cause moderate to severe respiratory illness (Lemanske et al., 2005). More recently, a study comparing 88 children with asthma exacerbations and 43 controls with stable asthma showed that while human rhinovirus A had a higher viral-load than that of human rhinovirus C in severe asthma exacerbation, it was human rhinovirus C that was most frequently detected in asthma exacerbation (Zheng et al., 2018). Thus, there is an established clinical association between asthma exacerbation and human rhinovirus infection. However, the mechanism behind this association is poorly understood.

1.7 IMPAIRED INNATE IMMUNE MECHANISMS AGAINST HUMAN RHINOVIRUS IN ASTHMATICS

The mechanism of infection of human rhinovirus involves first binding with intracellular adhesion molecule-1 (ICAM-1) on epithelial cells, which serves as the primary receptor for 90% of the identified serotypes (Greve et al., 1989). This interaction typically occurs at the basal and bronchial epithelial. Apart from human rhinovirus, other ligands of ICAM-1 include leukocyte function-associated antigen 1 (LFA-1) and macrophage adhesion ligand 1 (MAL-1), which upon engagement, results in the release of proinflammatory and pro-oxidative mediators. As such, this receptor has recently been proposed as a drug target in the treatment of lung inflammation due to asthma and rhinitis due to its capacity to traffic inflammatory cells (Mukhopadhyay et al., 2014; Traub et al., 2013).

The primary response to respiratory viral infections, including human rhinovirus, are the production of antiviral interferons, typically type 1 interferons (IFN); IFN-α and IFN-β, and type 3 interferons; IFN-λ1 and IFN-λ2/3 (Khaitov et al., 2009). Associations have been made between asthma in patients and the diminished antiviral response, characterized by deficient type 1 and 3 IFN production. Studies on primary bronchial epithelial cells (BEC) from asthmatic children have shown a deficiency in IFN-α and β production (Baraldo et al., 2012; Edwards et al., 2013). A

similar impairment of type 1 IFN production was also observed in primary BEC (Wark et al. 2005) and adult nasal epithelial cells (Pritchard et al., 2014).

Asthmatics also appear to mount a deficient response of type III interferons or IFN-λ, which are found to have similar properties to type I interferons (Ank et al., 2008). This group consists of IFN-λ subtypes 1-3, also known as IL28A/B and IL29. All subtypes of IFN-λ were found to be increased in the nasopharyngeal washes of infants infected with Respiratory Syncytial Virus (RSV) and a positive correlation was found between RSV infection severity and IFN-λ expression levels (Selvaggi et al., 2014). BEC and airway macrophages from asthmatic infants produced lower amounts of all 3 subtypes of IFN-λ compared to normal subjects, citing a possible impairment of this system by asthma (Contoli et al., 2006). Surprisingly, studies on asthmatic adults demonstrated high levels of IL28 mRNA only in sputum samples, with a positive correlation with eosinophils numbers present while IL29 mRNA was found to negatively correlate with asthma symptoms (Bullens et al., 2008). This suggests that IL28 and IL29 may have opposing or independent functions in immunity. Thus, with the impaired antiviral response, asthmatics are expected to be more susceptible to respiratory viral infections and as a result, are subject to more frequent and severe asthma exacerbations.

An earlier study demonstrated that PBMC obtained from asthmatics had impaired production of the Th1-cytokines IL-12 and IFN-γ compared to normal subjects (Papadopoulos et al., 2002). At the same time, this study also demonstrated that exposure of these PBMC to rhinovirus resulted in the induction of IL-4 exclusively by the asthmatic group. These findings suggest that the Th2 or proinflammatory cytokines may negatively impact the induction of an antiviral response, and thus, postulates that atopic or allergic status could influence innate antiviral immunity.

1.8 IMMUNE INTERACTIONS OF AEROALLERGEN AND RHINOVIRUS EXPOSURE IN ASTHMATICS

Sensitization to aeroallergens such as house dust mite and grass pollen have been shown to increase the risk of developing respiratory virus-induced wheezing and asthma exacerbations in children (Jackson et al., 2012; Jartti et al., 2010). Furthermore, weekly monitoring during common cold seasons in the US has revealed that children sensitized to at least one allergen had more viral associated illnesses with moderate to severe symptoms as compared to non-sensitized children (Olenec et al., 2010). More

recently, another study has demonstrated that wheeze and atopy together in children at high risk of developing asthma, were associated with a higher viral load of human metapneumovirus (hMPV) and Respiratory Syncytial Virus (RSV) (Spann et al., 2014). Collectively these data suggest that there may be an impairment of the antiviral response, modulated by allergen exposure *per se* or by sensitization to aeroallergens.

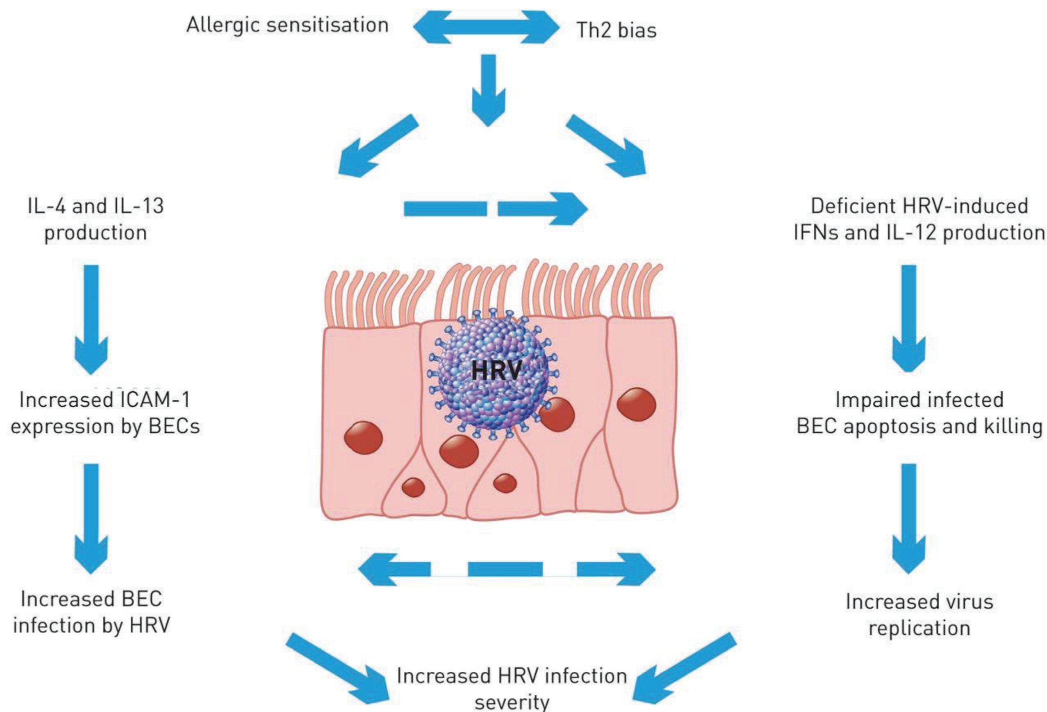


Figure 1.1.3 Immunological factors affecting human rhinovirus infection severity in allergic individuals. Adapted from Rossi and colleagues (Rossi & Colin, 2015)

There is a considerable immunological overlap between the pathogenesis of allergic response and asthma with antiviral immunity. A study on healthy, non-asthmatic adult PBMC showed that the type 1 interferons constrain the Th2 immune response towards human rhinovirus, a finding which could explain the Th2 driven response in asthmatics (Pritchard et al., 2012). As illustrated by Rossi and colleagues in Figure 1.8.1, both allergic sensitization and a Th2 bias arising from asthma leads to impaired antiviral immunity via deficient IFN production and increased infection by human rhinovirus in BEC, leading to increased human rhinovirus infection severity (Rossi & Colin, 2015). Together, these results suggest a possible synergistic interaction between asthma and allergic sensitization on impaired antiviral immunity.

Plasmacytoid dendritic cells (pDC) are the primary antigen presenting cells found in the mucosa that are the largest producers of type 1 interferon in the blood, especially in response to human rhinovirus (Xi et al., 2015). They were also found to have on its surface the high affinity receptor for IgE; FcεR1 receptor. In allergic patients, the crosslinking of allergen-specific IgE on pDC will result in a lowered production of IFN-α and IFN-λ in PBMC, which suggests a counter regulatory relationship between the FcεR1 pathway and the innate antiviral response (Durrani et al., 2012; Gill et al., 2010). pDC's from allergic asthmatic patients also had a diminished upregulation of Toll like receptor 7 (TLR7), a pathogen-associated molecular pattern (PAMP) receptor needed to mount an immune response against viruses (Roponen et al., 2009). TLR-7 is essential to the antiviral response as it detects single stranded RNA of viruses, such as influenza A and rhinovirus and in turn induces the secretion of large amounts of type I IFN, with IFN-α being secreted the most at 24 hours, by pDC (Liu, 2005). Thus, the pDC are the link between the allergic and antiviral response and thus, can indirectly influence the exacerbation of asthma.

1.9 SUMMARY AND IMPLICATIONS

Grass pollen is increasingly recognized as an important cause of AR and asthma exacerbation, associated with significant burden on healthcare systems especially through weather phenomena such as thunderstorms. However, efforts to mitigate this burden is limited by the lack of capacity to predict a sudden surge in hospital admissions such as that observed in thunderstorm asthma events (Thien et al., 2018). This issue is compounded by the temporal and spatial difference in grass pollen concentrations in regions across the world. Furthermore, there is a lack of knowledge surrounding regionally relevant allergens, which is important as studies have established genetic and immunological differences between grass pollen from species of different subfamilies (Davies, 2014a). Thus, there is a need to identify relevant allergens to a region, and specifically its population, to make more accurate diagnosis and targeted treatment.

Both asthma and AR have been individually associated with impaired antiviral immunity, but clinical associations have been made between both asthma and AR and the capacity to affect the antiviral response. It has been demonstrated that asthma alone can inhibit the antiviral response (M. Contoli et al., 2015; Papadopoulos et al., 2002; Spann et al., 2014). Moreover, the interaction of grass pollen as the allergen source and respiratory virus has not been investigated. Whilst HDM, the major indoor allergen is a known important allergen trigger in asthma, emerging evidence indicates an association with presentations of asthma and high levels of grass pollen exposure (Erbas et al., 2018; Erbas et al., 2013). However, most studies investigating this do not differentiate the effect of asthmatic status from that of AR when investigating the effect on antiviral immunity.

This thesis will address the immune interactions of respiratory viruses and GP allergy in asthma. By highlighting the importance of the role of allergic disease in the pathogenesis of asthma, guidelines for the management of asthma can be improved by incorporating elements of allergic disease management, including but not limited to antihistamine prescription and allergen avoidance or minimization strategies. Furthermore, this thesis will also highlight subtropical grasses as an emerging risk for allergic disease and asthma not just in Australia, but globally as well. By further

understanding the mechanistic interactions between asthma, AR and respiratory viruses, this thesis could potentially lead to the discovery of molecular targets that could lead to the development of novel medication for the management of these illnesses.

1.10 AIMS AND HYPOTHESIS

1.10.1 Aim

The aim of this thesis is to investigate the impact of a clinically relevant GP allergen, *Paspalum notatum* pollen on grass pollen sensitization profiles in AR in Australia and on the immune response towards human rhinovirus in adults with GP allergy and adolescents with AR and asthma

1.10.2 Hypotheses

1. I hypothesize that subtropical grass pollen allergens elicit higher level and more avid serum IgE reactivity than temperate grass pollen allergens for patients in subtropical regions. (Chapter 3)
2. I hypothesize that *P. notatum* pollen extract exposure will inhibit production of innate antiviral mediators towards human rhinovirus 16 (RV-16) by direct and indirect IgE-mediated effects on PBMC in grass pollen-allergic adult patients with AR. (Chapter 4)
3. I hypothesize that the innate effects of *P. notatum* pollen extract will be more pronounced than purified major allergen Pas n 1 (Chapter 4) and this effect will be stronger in patients with AR and asthma compared with those with AR and no asthma.

Chapter 2: Research Design

2.1 PARTICIPANTS

2.1.1 Grass Pollen Allergy Study (GPAS)

Participants in Chapter 3 were a subset of the Grass Pollen Allergy Study (GPAS; HREC/2009/QPAH/296) cohort, previously recruited with informed consent. This cohort consisted of adult participants; non-atopic; n=29, those with allergies other than grass pollen; n=54, and those with GP-allergy and AR (n=330) were recruited at clinical allergy and immunology specialist clinics in QLD; Cairns, Townsville, Brisbane, Toowoomba, and the Gold Coast (latitudes -21 to -27ºS), Western Australia (WA); Perth and Freemantle (latitude -32ºS), New South Wales (NSW); Sydney (latitude -33.9ºS) and South Australia (SA); Adelaide (latitude -35ºS). Patients who had prior GP immunotherapy were excluded.

Participant demographics, clinical history and SPT to ten environmental aeroallergen extracts including four GP; *P. notatum*, *S. halepense*, *C. dactylon*, and *L. perenne*, Southern GP mix (*Poa pratensis*, *Dactylis glomerata*, *Agrostis gigantea*, *Phleum pratense*, *Anthoxanthum odoratum*), as well as house dust mite, cat dander, *Alternaria*, *Aspergillus* moulds, and ragweed pollen (GreerLabs, USA) were assessed by skin prick testing. Serum total and spIgE to four GP extracts (g17, g10, g2 & g5 ImmunoCAPs, ThermoFisher Scientific, USA) were measured by Sullivan Nicolaides Pathology as part of the NHMRC Development project (GNT 1116107) "Development of an *in vitro* immunodiagnostic test for serum IgE specific to the major pollen allergen, Pas n 1, of the subtropical Bahia grass". Serum spIgE to natural allergen components; Pas n 1, Cyn d 1 and Lol p 1, that were purified (Drew et al., 2011) and biotinylated was measured using streptavidin ImmunoCAPs (Timbrell et al., 2014).

2.1.2 General Allergy and Asthma Study (GAAS)

Adult participants in Chapter 4 were a subset of the General Allergy and Asthma Study (GAAS) (HREC/07/QPAH/146) previously recruited with informed consent. Participants were assessed by questionnaire for clinical history of allergy and asthma as well as skin prick test for allergen sensitization towards a panel of 10 aeroallergen extracts; house dust mite (HDM), cat dander, *Alternaria* extract, and pollen of *P. notatum, S. halepense, C. dactylon, and L. perenne*. All clinical and demographic data were obtained from the GAAS database and used to stratify participants into three experimental groups as follows;

1. GP- allergic including sensitization to P. notatum pollen (GPA)
2. Other allergies and/or asthma (OA)
3. Non atopic (used as control) (NA)

2.1.3 Pollen Allergy and Asthma Study (PAAS)

The following study was designed to specifically address primary research question of this thesis. Adolescent participants in Chapter 4 were recruited as a core component of this research project with informed consent from themselves and a parent or guardian, as part of the Pollen Allergy and Asthma Study (HREC/17/QRCH/47). Participants were recruited from two departments within the Queensland Children's Hospital (QCH); Queensland Paediatric and Immunology Service (QPIAS) and Ear, Nose and Throat (ENT). Participant recruitment criteria were as follows:

Inclusion criteria:

- 12 to 18 years old
- Male or female
- Participants were assigned to the case groups if they presented with allergic rhinitis with or without asthma at QCH
- Participants were assigned to the control group if they do not have allergic rhinitis or asthma but have food allergies
- Scheduled for uncomplicated ENT surgery at QCH
- Written informed consent signed and dated by participant and parent/legal guardian, according to local regulations

Exclusion criteria

- o Have received allergen immunotherapy in the past
- o A significant medical disease or condition, other than asthma, that is likely to interfere with sample collection

A week prior to recruitment, potential eligible participants were identified with the assistance of QPIAS and ENT nurses, with clinical visit times and attending clinician notified. All participants were approached during their respective clinical visits, with no follow up required. The study was discussed with patients by the attending doctor who then introduced interested patients to the research team member (Mr Kailaivasan). Details of the study were explained to the participant and upon agreement to participate, were required to complete a consent form with parent/guardian supervision. The participant was then handed a questionnaire to be completed with the assistance of the parent/guardian. The questionnaire also contained a sheet for entry of the participant clinical AR and asthma status, medication usage and skin prick test results that was to be completed by attending medical doctor. Upon completion of questionnaire, participants were directed by the research team member to Queensland Pathology where blood collection was performed. Biological specimens and questionnaire of participants were deidentified by assigning them a 5 digit number; the first two digits indicate site of recruitment (01 for QPIAS, 02 for ENT) and the last 3 digits indicating the order in which they were recruited, starting at 001 for the first participant from that site.

Participants were then stratified into 3 experimental groups according to allergy and asthma status as determined by questionnaire and confirmed by clinician diagnosis. The experimental group were as follows:

i. Group 1 – Allergic rhinitis with asthma

ii. Group 2 – Allergic rhinitis only

iii. Group 3 – No rhinitis control

2.2 BIOLOGICAL SPECIMENS; SERUM, PLASMA AND PERIPHERAL BLOOD MONONUCLEAR CELLS

All whole blood specimens were collected from participants by collaborating healthcare professionals from participants presenting to the respective recruitment sites. For serum samples, 4 ml of whole blood was collected in 15 ml Vacuette serum separation tubes (Greiner, Austria) and centrifuged at 2000 g for 10 minutes within 2 hours of collection. Serum was transferred from above the separation gel to polypropylene tubes. Tubes were stored at -80°C.

18 ml of whole blood collected in two 9 ml Vacuette lithium heparin (Greiner, Austria) tubes were used to obtain peripheral blood mononuclear cells (PBMC) and plasma by density gradient separation using Lymphoprep (StemCell Technologies, Canada). 4 ml of warm Lymphoprep was transferred to a 15 ml tube and was layered over with up to 10 ml of blood with a serological pipette. Tubes were centrifuged at 600 g for 30 minutes at room temperature with no brakes to not disturb the gradient separation. Plasma was transferred from above the Lymphoprep to polypropylene tubes and stored at -20°C. Care was taken to leave 1 to 2 ml of plasma above the Lymphoprep layer so as to not aspirate the PBMC layer. PBMC were harvested from the interphase between the plasma and Lymphoprep layers using a sterile Pasteur pipette. PBMC were transferred to a 15 ml tube and topped up to capacity with RPMI (Merck-Sigma Aldrich, USA) with 2% FCS. Tubes were centrifuged at 600 g for 10 minutes, supernatant discarded and resuspended in fresh RPMI with 2% FCS. This wash step was repeated twice for 3 washes. PBMC were counted using trypan blue exclusion then washed and resuspended in freezing media consisting of 90% FCS and 10% DMSO before being stored in cryovials in liquid nitrogen.

2.3 PREPARATION OF WHOLE GRASS POLLEN EXTRACTS AND PURIFIED MAJOR ALLERGENS

Whole extracts from five different grass pollens were prepared by Allergy Research Group research assistant, Victoria Timbrell; three subtropical grasses *P. notatum* and *S. halapense* (subfamily *Panicoidieae*) and *C. dactylon,* as well as two temperate grasses, *Lolium perenne* (Ryegrass) and *Phleum pratense* (Timothy grass), from the Pooideae subfamily. Raw peanut extract was prepared as the negative control allergen source prepared by Allergy Research Group head Prof Janet Davies (de Leon et al., 2003). Commercially available non-defatted pollen (Greer, Lenoir, USA) were extracted in ammonium bicarbonate with a complete protease inhibitor cocktail (Nony et al., 2015). Commercially available raw peanuts were crushed and defatted with acetone. Contents were mixed and centrifuged at 1000 g, then resuspended in diethyl ether. This process was repeated five times. The suspension was vacuum filtered and dried for 10 minutes under vacuum. The produced powder was then dried with liquid nitrogen and incubated overnight in phosphate buffered saline (PBS) with Complete Protease Inhibitor Cocktail (Roche, Germany) at 4^0C. The mixture was again centrifuged at 1000 g to pellet debris and at 20 000 g to obtain a clear supernatant (de Leon et al., 2003).

2.4 PREPARATION OF HUMAN RHINOVIRUS-16

Human rhinovirus-16 (RV-16) stock was obtained from the Lung Allergy Research Centre, University of Queensland courtesy of Prof. John Upham. Propagation was performed by infection in HeLa cells. HeLa cells were thawed and cultured in 20 ml RPMI (Merck-Sigma Aldrich, USA) with 5% FCS (ThermoFisher, Sweden) and 1X Penicillin-streptomycin glutamine (ThermoFisher, Sweden) in a Nunclon Delta T75 flask (ThermoFisher, Sweden), incubated at 37°C for 24 hours until 70-80% confluent. HeLa cells were then split into two flasks using 1ml of Trypsin-EDTA (ThermoFisher, Sweden) and incubated at 37°C until 80-90% confluent. HeLa cells were then washed with warm PBS and infected with RV-16 at MOI of 0.25, diluted in RPMI without FCS, rocking the flask to ensure even coverage. Infected HeLa cells were

incubated for 2 hours at 32°C. Flasks were topped up with RPMI with 1% FCS to make 20 ml of media and incubated at 37°C until more than 60% cells have been lysed, which takes up to 48 hours. Flasks were then sealed with parafilm and stored at -80ºC for 24 hours.

Frozen infected HeLa cells were thawed and cell culture supernatants containing released virus were pooled into a 150 ml Erlenmeyer flask and kept on ice. Remaining attached cells were removed from flasks using a cell scraper and washed with RPMI with no FCS. Cell suspension were collected into 15 ml tubes and subjected to three freeze/thaw cycles to lyse cells by snap freezing in dry ice, thaw at 37°C water bath then vortexing twice for 30 seconds with 15 seconds rest on ice. Cell lysate was then clarified by centrifugation at 2000 *x* g for 10 minutes at 4°C and pooled into the Erlemeyer flask. Lysate were transferred into 50 ml tubes with a maximum of 30 ml per tube and snap frozen in dry ice before being stored at -80°C.

RV-16 purification and concentration were performed by spin filter purification, using the Amicon Ultra, Ultracell 15 ml spin columns (Merck-Sigma Aldrich, USA). Two filter units were prepared by filling with 70% ethanol and incubating at room temperature for 10 minutes. Tubes were then centrifuged at 1000 x g at 4°C for 5 minutes. The filter was then washed by adding sterile deionized water and centrifuged at the same conditions. Filter was lifted out and the flow through was discarded. Supplemented PBS (PBS with 30mM $MgCl_2$, 50mM HEPES and 0.05g BSA (Bovogen, Australia)) was added followed by centrifugation. Filter was then equilibrated by adding RPMI with no FCS and centrifuged with flow through discarded.

Cell lysate was thawed and clarified by centrifuging at 1000 x g at 4°C for 10 minutes. Clarified lysate was added to the filter and centrifuged at 2500 x g at 4°C for 25 minutes. This step was repeated for the remaining volume of lysate and centrifuged until a volume of 1 ml remained in the filter. Filter was washed with 1 ml of supplemented PBS without BSA to resuspend virus and aliquoted as 200 µl aliquots, snap frozen on dry ice and stored at -80°C.

One aliquot of virus suspension was used to determine virus titre. HeLa cells were prepared as described previously, trypsinised and seeded in RPMI with 2% FCS at 250 000 cells per well in a 96 well plate. 12 polypropelene tubes

were prepared and filled with RPMI with no FCS for serial dilution of virus suspension from 10^0 to 10^{-10} with one tube with just RPMI with no FCS as negative control. Plate was incubated for 6 days at 37°C. Wells were scored based on cell death, which was confirmed by removing the media and adding 50 µl/well 0.1% crystal violet solution. The TCID50 was calculated using Spearman-Karber algorithm and the titre of the virus was calculated with the following formula: 0.69 * TCID50/ml, and expressed as FFU/ml.

2.5 SERUM IGE REACTIVITY TITRATION TO PURIFIED MAJOR ALLERGEN OF GRASS EXTRACT BY ELISA

In Chapter 3, a titration ELISA was performed to determine the maximum serum IgE reactivity with each purified allergen. Purified major allergens were coated at 1 µg/ml as follows; Pas n 1 (from *Paspalum notatum*), Cyn d 1 (*Sorghum halepense*) and Lol p 1 (*Lolium Perenne*). Patient sera were then titrated at 1/10 four-fold serial dilutions. Titration was assayed by ELISA method, using polyclonal rabbit anti-human IgE (Dako, Denmark) at 1/2000 dilution, goat anti-rabbit horse radish peroxidase (Promega, USA) at 1/1000 dilution and SigmaFAST OPD for detection (Sigma Aldrich, USA). The midpoint of the linear phase of the titration curve was chosen for each individual as the serum dilution for the cross-inhibition assay for each of the three allergens.

2.6 DEVELOPMENT AND OPTIMIZATION OF 384-WELL MICROSCALE CROSS-INHIBITION ASSAY FOR SPECIFIC IGE REACTIVITY TOWARDS PURIFED GRASS POLLEN ALLERGENS

To investigate differences between serum IgE specificity and avidity towards three purified allergens derived from the major allergenic GP from the three subfamilies, Pas n 1, Cyn d 1 and Lol p 1, a microscale assay method needed to be developed to test the cross-inhibition of IgE reactivity with 5 allergenic GP extracts; *Paspalum notatum, Sorghum halepense, Cynodon dactylon, Lolium Perenne* and *Phleum pratense*. The protocol was adapted from existing IgE cross-inhibition protocol (Davies et al., 2012), but converted from 96 well format to a semi-robotic 384 well assay to conserve serum and antigen use.

The first step was establishing a baseline for cross-inhibition of the 5 GP extracts on IgE reactivity towards *P. notatum* pollen in a 96 well format. Nunc Maxisorp (ThermoFisher, USA) 96 well plates were coated with whole *P. notatum* pollen extract at 5 µg/ml. Plasma from one patient (GAAS 56) was pre-incubated for 90 minutes with GP extract inhibitors and raw peanut extract (RPN) serial diluted between 0.016 to 100 µg.ml before then being transferred to *P. notatum* pollen extract coated wells. IgE reactivity was detected with rabbit anti-human IgE (Dako, Denmark) at 1/1000 dilution in 1% skim milk powder in PBS with 0.05% Tween-20 (MPPBST) and subsequently goat anti-rabbit IgG conjugated with horseradish peroxidase (Promega, USA) at 1/2000 dilution in MPPBST. Visualization was performed SigmaFAST OPD (Sigma Aldrich, USA). The IgE reactivity observed was compared to that of 12 uninhibited wells to determine the percentage of inhibition.

The assay was then replicated using a new visualization method with higher dynamic range in Time-resolved fluorescence, using the DELFIA-TRF system (Perkin Elmer, USA). Previous studies have shown the utility of the DELFIA-TRF system to assay serum IgE responses in a smaller reaction volume (less than 20 µl) to house dust mite (Hales et al., 2006) and ant venom (van Eeden et al., 2011) with a wider dynamic assay range. The intention here is to develop an immunoassay method that utilizes a low reaction volume, enabling the testing of many combinations of antigen and inhibitor to conserve patient sera usage. For this trial, to conserve human sera use, two biotinylated monoclonal antibodies (mAb) were used; i) bio-RG2, that has high specificity to *P. notatum* extract and ii) bio-RE2, which has weak specificity.

Two 96 well plates were coated with 5 µg /ml of each of the five GP extracts in blocks of 9 rows in duplicates. Two sets of the blocks were applied in each plate, one block for bio-RE2 and the other for bio-RG2. The mAb were then added to the respective blocks and serial diluted down the plate with a dilution factor of 1:5 starting at 2µg/ml. One plate was analyzed using the ELISA method described previously and the other by TRF method for comparison of mAb detection. Detection of bio-RE2 and bio-RG2 as a representation of IgE reactivity was performed using DELFIA Streptavidin Eu-N1 (Perkin Elmer, USA) at 1/2000 dilution. Plate reading was performed and optimized on a CLARIOstar microplate reader (BMG Labtech, Germany) fitted with basic timed-resolved fluorescence filters, with data expressed as raw counts.

2.7 CROSS-INHIBITION OF SERUM SPECIFIC IGE REACTIVITY TO PURIFIED GRASS ALLERGENS BY GRASS POLLEN EXTRACTS

In Chapter 3, a subset of 8-15 GP-allergic participants were selected from each region based on availability of serum and sum of specific (spIgE) concentrations to four GP; *P. notatum, S. halepense, C. dactylon,* and *L. Perenne*. A total of 15 patients from QLD, 12 from WA, 8 from NSW and 15 from SA were selected.

The purified grass pollen group 1 allergens (Lol p 1, Cyn d 1 and Pas n 1) from each sub-family of grass will be used to coat the plate wells at 1 µg/ml. The five GP extracts *P. notatum, S. halepense, C. dactylon, L. Perenne* and *P. pratense* with RPN extract as an irrelevant plant allergen control, were tested as inhibitors at concentrations of 0.016 – 100 µg/ml.

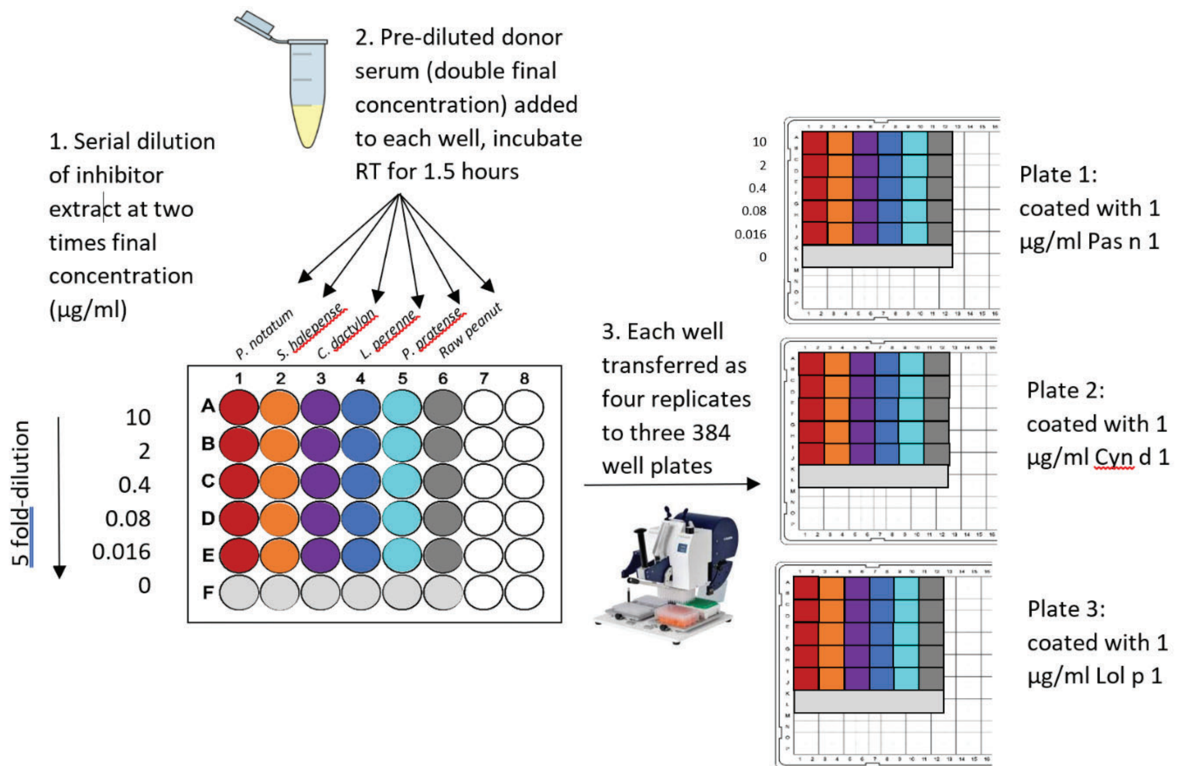


Figure 2.2.1 Experimental plate set up of the cross-inhibition assay of participant serum IgE reactivity towards three purified allergens, Pas n 1, Cyn d 1 and Lol p 1 by five inhibitor grass extracts, *P. notatum, S. halepense, C. dactylon, L. perenne, P. pretense* and a non-reactive extract, raw peanut.

First, three Spectraplate High-Binding 384 well plates (Perkin Elmer, USA) were coated with one of Pas n 1, Cyn d 1 and Lol p 1 at 1 µg/ml, diluted in 0.05M carbonate buffer and incubated overnight at 4°C.

A low protein binding 96 well plate was prepared with the six inhibitor extracts, serial diluted 1:2 with 1% skim milk powder in PBS with 0.05% Tween-20 (MPPBST), from 10 µg/ml to 0.016 µg/ml. Row F represents the uninhibited participant serum, that was mixed with only MPPBST and no inhibitor extract. Donor serum was thawed and diluted in MPPBST to the selected dilution based on the titration assay in 1.4.1. Serum was transferred to each well and pre-incubated for 90 minutes with extract inhibitors at room temperature while placed on a plate shaker.

Each well of pre-incubated serum and inhibitor extract mix was then transferred as four technical replicates to three purified allergen (Pas n 1, Cyn d 1, Lol p 1) coated 384 well plates, with a reaction volume of 10 µl. For example, well A1 from the 96 well dilution plate was delivered to wells A1, B1, A2 and

B2 of each (clockwise motion) of each of the three 384 well plate (one 96 well into a total of 12 384 wells). Delivery of the serum-inhibitor mix was performed using semi-automated liquid handling system, Gilson Platemaster (Gilson, USA), using 200 µl filter tips (Gilson, USA). Plates were incubated for 1 hour at room temperature with shaking.

Plates were washed three times using DELFIA wash buffer (Perkin Elmer, USA). For detection, polyclonal rabbit anti-human IgE (Dako, Denmark) was diluted 1/2000 in MPPBST and delivered to each well with the Platemaster. Plates were incubated for 1 hour at room temperature with shaking. Plates were then washed five times and DELFIA Eu-N1 goat anti-rabbit IgG (Perkin Elmer, USA) was diluted 1/1000 in MPPBST prior to delivery to each well. Plates were incubated for 30 minutes at room temperature with shaking. Plates were washed 8 times before DELFIA enhancement solution (Perkin Elmer, USA) was delivered to each well, followed by 5 minutes incubation at room temperature with shaking. Plate fluorescence were then read on a CLARIOstar microplate reader (BMG Labtech, Germany) fitted with basic timed-resolved fluorescence filters, with data expressed as raw counts (Appendix A).

Each of the five inhibitor extracts, and peanut extract as control, will be tested for inhibition of serum IgE reactivity towards the three purified allergens. The IgE reactivity observed will be compared to that of 12 uninhibited wells to determine the percentage of inhibition using the formula: 100% - ((raw counts Inhibitor/raw counts of No Inhibitor) * 100). The maximum inhibition of each serum-inhibitor combination for each purified allergen was determined and recorded.

2.8 ADULT PERIPHERAL BLOOD MONONUCLEAR CELL CULTURE FOR INNATE AND LATE MEDIATORS RESPONSE TOWARDS HUMAN RHINOVIRUS-16 AND WHOLE *P. NOTATUM* GRASS POLLEN EXTRACT

Adult PBMC from the GAAS cohort was previously collected and cryopreserved in liquid nitrogen in freezing media consisting of 90% FCS and 10% DMSO in liquid nitrogen. Aliquots of PBMC were thawed in a water bath and added dropwise to be resuspended in RPMI with no FCS in a 15 ml tube. Tubes were centrifuged at 600 g and supernatant discarded. Fresh RPMI

supplemented with 5% autologous plasma was used to resuspend the cell pellet and seeded in a Nunclon Delta surface 96-well plate (ThermoFisher, USA) at a density of 250 000 cells per well. They were co-cultured with filtered *P. notatum* pollen extract (BaGP) at 30 µg/mL and RV-16 at MOI of 1 according to the three patient groups (GPA, OA, NA). Experimental groups are as follows:

1. None
2. RV-16 only
3. BaGP only
4. RV-16 and BaGP

PBMC was cultured for 6, 24 and 120 hours to assess the early signaling and 120 hours for the late adaptive response. They were incubated at 37°C with 5% CO_2. After incubation, plates were centrifuged at 600 g for 5 minutes. Supernatants were transferred to low-protein binding 96 well plates, sealed and stored at -80°C. Cell pellets were resuspended in TRIsure (Bioline, USA) and stored at -80°C for future RNA extraction.

2.9 CYTOKINE ELISA FOR EARLY IMMUNE MEDIATOR RESPONSE IN ADULT COHORT

24-hour supernatants were analyzed for the early proinflammatory cytokines IL-6, TNF-α and early antiviral cytokines IP-10. The ABTS ELISA development kit (Peprotech, USA) was used according to manufacturer's protocol with one modification; the use of SigmaFAST OPD (Sigma-Aldrich, USA) as the visualization method.

Nunc Maxisorp (ThermoFisher, USA) 96 well plates were coated with capture antibody from kit, diluted in sterile filtered PBS and incubated overnight at room temperature. Plates were washed with PBS supplemented with 0.05% Tween-20 (PBS-Tween) and blocked for 1 hour at room temperature with PBS supplemented with 1% BSA (blocking buffer). Supernatant was diluted at least 1:2 (varies according to cytokine) in PBS-Tween supplemented with 0.1% BSA (diluent) and transferred to plates, along with standards. Plates were incubated for 2 hours at room temperature with shaking. Plates were washed and detection

antibody diluted in diluent were added to the plates. Plates were incubated for 2 hours at room temperature with shaking. Plates were washed 4 times and Avidin-HRP was added in 1:2000 dilution with diluent and incubated for another 30 minutes at room temperature with shaking. SigmaFAST OPD tablets were dissolved in MilliQ water and transferred to the plates, then incubated for 15 to 20 minutes, while examining colour development. Reaction was stopped using 4M hydrochloric acid and plates were read on CLARIOstar (BMG Labtech, Germany) microplate reader at 490nm wavelength.

2.10 PAAS QUESTIONNAIRE

A questionnaire (Appendix B) was used to determine if the participant had allergic rhinitis, asthma and food allergy. This was assessed by questions regarding symptom presentation, frequency and impairment of quality of life in the last 12 months. Participant's use of medication related to the illnesses will be recorded. Diagnosis was confirmed by a medical professional.

Questionnaire was adapted from the Core Questionnaires for 13 and 14-year olds from the International Study of Asthma and Allergies in Childhood for diagnosis and severity of allergic rhinitis and asthma. Asthma control score table was adapted from the Australian Asthma Handbook. Participant's age, gender, date of birth, ethnicity, exposure to cigarette smoke, hours spent outdoors per week, current and previous living locations were recorded using this questionnaire.

2.11 SKIN PRICK TEST AND SPECIFIC IGE TESTING

Skin Prick Test (SPT) data was obtained from participants presenting to the QPIAS clinics, only if considered to be indicated by the doctor and was performed by a healthcare professional at time of recruitment. This test examined the sensitivity of participants to the following allergens: the grasses *P. notatum, L. perenne*, Brome, Southern grass mix (Kentucky blue grass, Orchard, *P. pratense*, Redtop, Sweet Vernal, *C. dactylon* and *S. halepense* grasses), dog

hair and dander, cat pelt, feather mix, house dust mite (*Dermatophagoides pteronyssinus/farina)*, mould mix (*Aspergillus fumigatus, Hormodendrum cladsporiodes, Penicillum notatum)*, Acacia and Eucalyptus, plantain and ragweed.

Peripheral blood collected in the serum tube was used for specific IgE sensitivity testing via ImmunoCAP (Phadia). Sensitivity to the following panel of aeroallergens was tested; pollens from subtropical (*P. notatum, C. dactylon*) and temperate grass (*L. perenne*), house dust mite (HDM), cat dander, *Alternaria* spores and total IgE. Samples were sent to a third party, Sullivan Nicolaides Pathology for testing.

2.12 TEENAGERS PERIPHERAL BLOOD MONONUCLEAR CELLS CULTURE TO ASSESS INNATE ANTIVIRAL MEDIATOR RESPONSE TOWARDS HUMAN RHINOVIRUS-16, WHOLE *P. NOTATUM* EXTRACT AND PURIFIED PAS N 1

Peripheral blood was subjected to density gradient separation by Lymphoprep (Stem Cell Technologies, Canada) to isolate the peripheral blood mononuclear cells (PBMC) and to obtain the plasma, both which were subsequently cryopreserved prior to use in the study. Autologous plasma was used to supplement culture media at 5% during the culture of PBMC.

PBMC were seeded in a 96 well plate at 2.5 x 10^5 cells per well in duplicates and exposed to 6 treatment combinations of as follows:

1. No treatment control (N)
2. RV16 only (R)
3. BaGP only (B)
4. RV16 and BaGP (RB)
5. Pas n 1 only (P)
6. RV16 and Pas n 1 (RP)

RV16 is applied at MOI of 1, diafiltered *P. notatum* extract was applied at 30 µg/ml as in previous chapter and purified major allergen, Pas n 1, was applied at 3 µg/ml. Multiple plates were seeded for incubation times of 6 and 24 hours. Culture supernatants were harvested at 24 hours for cytokine assay and

cell pellets at 6 hours are preserved in RNAlater for RT-PCR as described in section 1.7.

2.13 BEAD-BASED ANTIVIRAL CYTOKINE ASSAY

24-hour PBMC supernatants were subjected to antiviral cytokine analysis using the Legendplex bead-based immunoassay (Biolegend, USA). Using the pre-defined anti-virus panel, 13 analytes were measured; IL-1β, IL-6, TNF-α, IP-10, IFN-λ1, IFN-λ2-3, IL-8, IFN-α2, GM-CSF, IFN-β, IL-10, IFN-γ, IL-12p70.

First, the lyophilized standard was reconstituted in the provided assay buffer and serial diluted 7 times in polypropylene tubes before being delivered to the provided V-bottom plate. Supernatants were thawed, 25 μl then transferred and diluted 1:2 in assay buffer. Pre-mixed beads containing the immobilized capture antibodies were vortexed for 1 minute, diluted 1:2 in assay buffer and 25 μl transferred to the plate using an electronic repeating pipette. Plates were sealed and incubated at room temperature on plate shaker at 800 rpm for 2 hours. The high speed was required to prevent the beads from settling. Plates were then centrifuged at 250 g at room temperature for 5 minutes. Plates were inverted and flicked to discard supernatant and blotted lightly on paper towel stack without disrupting bead pellet. Detection antibody cocktail was diluted 1:2 in assay buffer and 25 μl applied to each well with electronic repeating pipette before sealing the plate and incubating for 1 hour at room temperature, shaking at 800 rpm. Streptavidin-PE was diluted 1:2 in assay buffer and 25 μl delivered to each well with electronic repeating pipette before plates were sealed and incubated for 30 minutes at room temperature with shaking at 800 rpm. Plates were centrifuged at 250 g and supernatant discarded before being resuspended in the provided wash buffer.

All samples were assessed on the Cytoflex-S flow cytometer (Beckman Coulter, USA) and analysis by the CytExpert software. Prior to running the samples, a density plot with forward scatter (FSC-A) and side scatter (SSC-A) were set up to gate the two population of beads, the smaller A beads and larger B beads, within the bead mixture. A further two dot plots with PE for X-axis,

APC for Y-axis (Figure 25), gated on A Beads and B Beads, respectively. Then, the vial of Raw Beads was vortexed for 30 seconds and 400 µl was transferred into a fresh FACS tube and ran on the flow cytometer with flow rate set at low. The gain was adjusted until the A Beads and B Beads are well separated and the FSC and SSC readings are greater than $20x10^4$, to have enough separation from debris. The APC settings were adjusted to between 1 x 10^3 and 3 x 10^5 while the PE settings were adjusted to between 1 x 10^2 and 1 x 10^3, as shown in Figure 2.2.

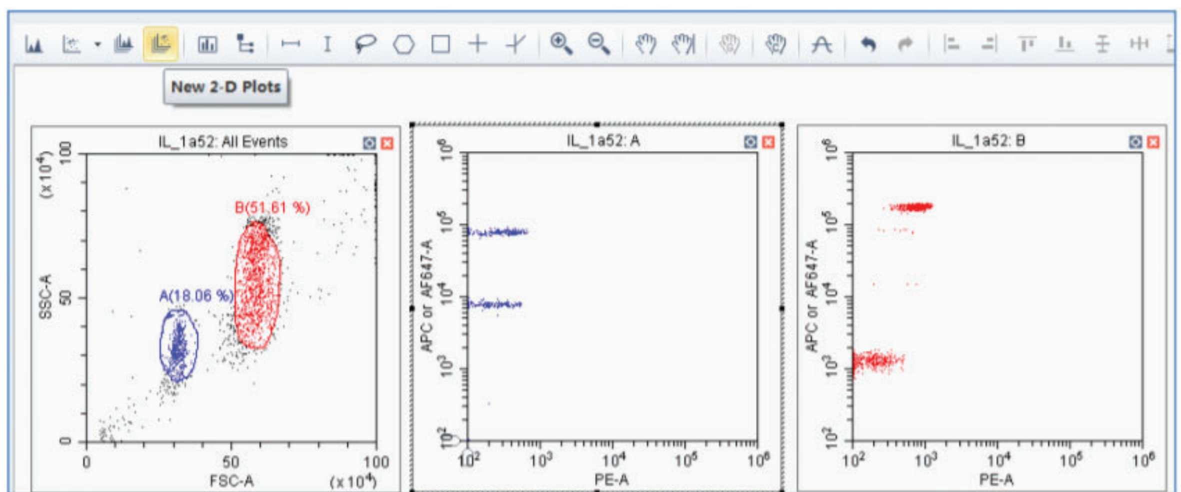


Figure 2.1.2 Template set up in CytExpert software for gating of A and B beads population for the Human Anti-Virus Response Panel for Legendplex bead based assay using the CytoFlex S flow cytometer.

The two populations of A beads and two populations of B beads (middle and right panels of Figure 2.2) were gated and PE channel gain adjusted until median fluorescence intensity for the 4 populations are between 200 to 800. The vial of PE beads was vortexed for 30 seconds, 400 µl transferred to a fresh FACS tube and ran on the flow cytometer, with flow rate set at low. The median PE signal of the beads was adjusted to be between $2.5x10^5$ and $3.0x10^5$. Finally, events to record was set at 4500 and the template was saved. Prior to each sample acquisition, the raw beads were run to verify that the FSC/SSC and PE fluorescence readings are within the described parameters above. The template was then set to 96 well plate acquisition mode, and the plate layout was filled in by inputting the treatment groups (N, R, RB, B, RP, P) and the corresponding participant ID. Using the 96 well plate tray, the plate was inserted, and the

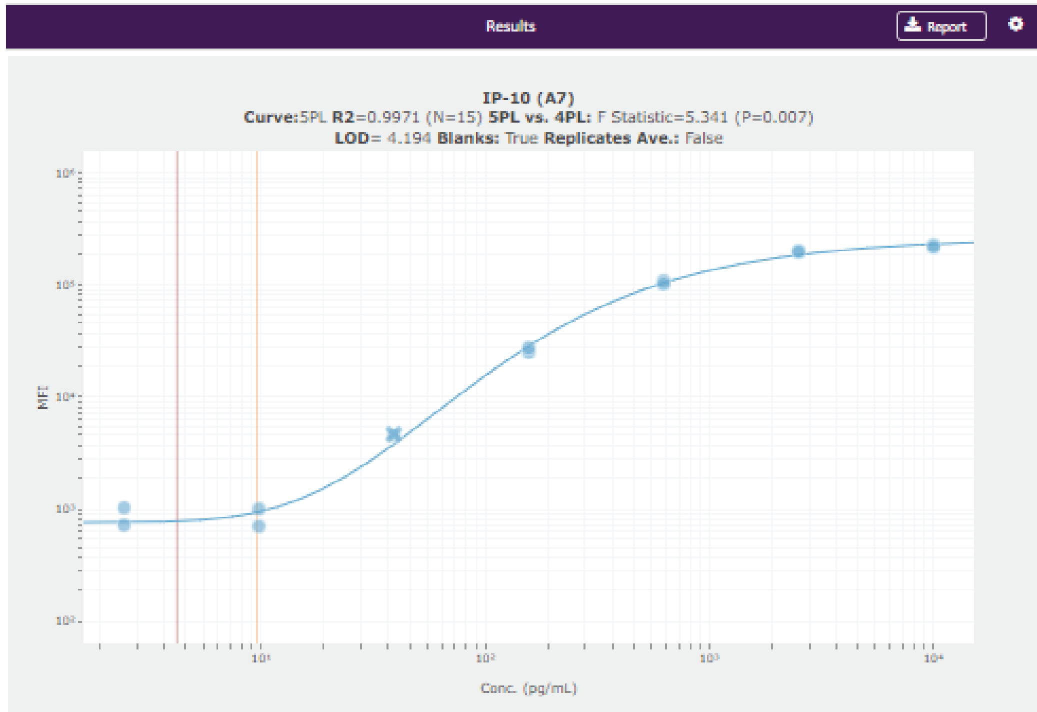
sample acquisition began, with flow rate set at high. The output of the readings were stored as FCS files.

The FCS files were uploaded onto the cloud based Legendplex Qognit analysis software (Biolegend, USA). All uploaded files were then reviewed for the gating of each population of beads as shown in Figure 2.2.

Figure 2.1.3 Gating of two populations of Legendplex beads (A and B) of the top standard (C7) with 4500 events recorded. Panel used was the Legendplex Human Anti-Virus Response Panel, tested with the Cytoflex S flow cytometer and analyzed using the Legendplex Qognit software.

Each individual bead population representing a tested cytokine is identified by their difference in APC intensity (refer A_beads and B_beads diagram in Figure 2.3). The software then generates a standard curve based on the standards and subsequently a predicted concentration for each sample as shown in Figure 2.4. These predicted concentrations were then organized in a

Microsoft Excel spreadsheet, the average of technical replicates calculated and then transferred to Graphpad Prism software for generation of graphs and further analysis.



Color By: ◉ Concentration ○ Count ○ MFI ○ CV

☐	Sample	Dilution	Replicat	Well	IL-1β (A4)	IL-6 (A5)	TNF-α (A6)	IP-10 (A7)	IFN-λ1 (A...	IL-8 (A10)	IL-12p70	IFN-α2	IFN-λ2/3	GM-CSF	IFN-β (B6)	IL-10 (B7)	IFN-γ (B9)
	C0		1	01 C1 G	<13.19	<0.61	<19.29	<4.19	<11.39	<2.88	<0.59	<0.24	<48.32	<1.23	<4.80	<1.03	<8.56
	C0		2	01 C1 G	<13.19	<0.61	<19.29	<4.19	<11.39	<2.88	<0.59	<0.24	<48.32	<1.23	<4.80	<1.03	<8.56
	C1		2	01 C0 H	<13.19	<0.61	<19.29	<4.19	<11.39	<2.88	<0.59	<0.24	<48.32	<1.23	<4.80	<1.03	<8.56
	C1		1	01 C2 F	<13.19	11.41	<19.29	11.39	<11.39	11.51	12.22	12.03	<48.32	9.78	10.67	9.95	<8.56
	C2		1	01 C0 H	<13.19	<0.61	<19.29	<4.19	<11.39	<2.88	<0.59	<0.24	<48.32	<1.23	<4.80	<1.03	<8.56
	C2		2	01 C2 F	<13.19	11.28	<19.29	11.04	<11.39	11.44	11.25	10.15	<48.32	9.47	8.73	10.96	12.52
	C3		1	01 C3 E	~~37.25~~	~~88.16~~	~~41.55~~	~~44.95~~	~~39.18~~	~~324.62~~	~~46.89~~	~~44.68~~	~~175.70~~	~~41.32~~	~~44.88~~	~~44.91~~	~~40.86~~
	C3		2	01 C3 E	38.69	43.78	36.24	44.26	36.92	41.68	48.87	44.64	189.68	41.09	36.88	43.11	40.86
	C4		1	01 C4 D	153.90	155.23	148.04	141.16	152.90	151.65	165.32	152.04	772.15	163.68	158.70	159.11	156.92
	C4		2	01 C4 D	160.18	172.68	165.05	151.69	166.88	164.49	171.44	167.54	811.19	178.73	168.70	169.58	162.22
	C5		1	01 C5 C	590.57	541.29	611.09	599.22	593.05	555.88	538.09	561.42	3057.49	552.86	553.44	532.33	560.61
	C5		2	01 C5 C	649.34	618.83	646.32	671.53	632.36	640.75	591.74	646.24	3244.40	637.09	632.02	630.67	650.36
	C6		1	01 C6 B	2312.82	2187.31	2263.58	3088.08	2271.16	2451.01	2014.78	2216.90	10701.7	2034.37	2409.56	2226.56	2276.83
	C6		2	01 C6 B	2840.18	2820.23	2709.13	3587.65	2814.13	3107.58	2621.52	2913.64	13036.9	2343.85	3116.74	2656.02	2997.55
	C7		1	01 C7 A	9496.74	11502.2	9551.59	8631.50	10329.5	8673.39	12424.1	8715.31	59987.7	11264.2	7894.26	10636.8	9388.70
	C7		2	01 C7 A	10036.8	11357.4	10677.0	5744.44	10417.6	9828.47	12636.8	10081.5	48084.8	12497.6	10994.1	12064.4	10135.9
☐	01004 B	1	1	02 0100	5923.45	14151.1	7112.70	<4.19	<11.39	934.35	<0.59	<0.24	<48.32	758.95	12.81	1360.12	58.61
☐	01004 B	1	1	02 0100	8713.86	18951.8	9626.73	42.32	12.18	980.14	<0.59	0.68	<48.32	827.56	16.80	1728.99	59.98
☐	01004 N	1	1	02 0100	17.21	36.37	<19.29	84.58	<11.39	5609.76	<0.59	<0.24	<48.32	<1.23	<4.80	2.80	<8.56
☐	01004 N	1	2	02 0100	<13.19	41.51	<19.29	12.72	<11.39	4392.25	<0.59	<0.24	<48.32	<1.23	<4.80	3.74	<8.56
☐	01004 P	1	1	02 0100	205.19	4616.80	144.20	4.63	<11.39	2538.17	<0.59	<0.24	<48.32	17.57	5.27	34.81	<8.56
☐	01004 P	1	2	02 0100	264.53	8282.06	169.33	5.93	<11.39	1806.12	<0.59	<0.24	<48.32	20.50	<4.80	41.18	<8.56

Figure 2.1.4 Generation of standard curve and predicted concentration of IP-10 in the Legendplex Human Anti-Virus Response Panel, tested with the Cytoflex S flow cytometer and analyzed using the Legendplex Qognit software

2.14 STATISTICAL ANALYSIS

Participant gender and percentage with AR and asthma were assessed by Fisher exact test for difference in frequencies between groups. Outcomes of continuous variables were tested for normality of distribution by the Kolmogorov-Smirnov test. Participant age, sum of SPT and total IgE between groups were then compared by Kruskal-Wallis ANOVA for difference between medians in groups with Dunn's pairwise comparisons. Differences between frequency of categorical data (gender, place of birth, frequency of asthma or AR etc.) between experimental groups were assessed by Fisher's exact test.

In Chapter 3, for spIgE cross-inhibition data, the maximum inhibition and area under curve were calculated. Self-inhibition was defined as inhibition of IgE reactivity with a purified allergen by the GP extract the allergen was derived from, such as the inhibition of Pas n 1 spIgE reactivity by *P. notatum*, Cyn d 1 by *C. dactylon* and Lol p 1 by *L. perenne*. Significant self-inhibition is achieved when self-inhibition is significantly higher than the inhibition of spIgE reactivity by the control RPN extract. The differences between the level of self-inhibition and inhibition by the other GP extracts were compared. Differences in median maximum inhibition between GP extracts assessed for significant by Kruskal-Wallis ANOVA for difference between medians in groups with Dunn's pairwise comparisons.

Data in Chapter 4 were tested for normality of distribution by the Kolmogorov-Smirnov test. Differences in cytokine levels between treatment conditions were assessed by Freidman's test ANOVA for difference between medians in treatment groups with Dunn's pairwise comparisons. Significant induction of antiviral mediators by RV-16 over no treatment was determined using Wilcoxon test. Significant induction of proinflammatory mediators over no treatment was determined using Wilcoxon test. Fold induction of cytokines were calculated as concentration of cytokine when PBMC culture was treated with a relevant stimulant divided by concentration of untreated PBMC culture. For example, the fold induction of IP-10 and IFN-α2 was calculated as the concentration of these cytokines when PBMC cultures were treated with RV-16 divided by the concentration of cytokines at no treatment. The percentage of

inhibition of antiviral mediators IP-10 and IFN-α2 by BaGP or Pas n 1 was calculated as follows:

$$\left(1 - \frac{concentration\ of\ mediators\ by\ RV-16 + BaGP\ or\ Pas\ n\ 1}{concentration\ of\ mediators\ by\ RV-16}\right) \times 100\%$$

Differences in cytokine levels, fold induction and percentage of inhibition of antiviral mediators between participant groups were assessed by Kruskal-Wallis ANOVA and Dunn's multiple comparison test.

For data generated by the Legendplex assay, there were samples that had an IL-6 predicted concentration above the maximum range of the assay (10 000 pg/ml). Ideally, a repeat on these samples would need to be performed, but due to financial and time restrictions, I was unable to do so. As such, for the purpose of analyzing IL-6 induction in treated samples compared to non-treated samples, all values greater than 10 000 pg/ml were assigned as 10 000 pg/ml. The lower limit of detection was defined by the Legendplex Qognit software and all values that fell below it were automatically assigned that concentration.

P-values denoted as; * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** = $p \leq 0.0001$; n.s. = not significant. All statistical analysis and graphical representation of data was performed in Graphpad Prism software.

Chapter 3: Biogeographical variation in specific IgE recognition of grass pollen allergens in Australia

3.1 BACKGROUND AND SIGNIFICANCE

While grass pollen has been acknowledged as a major aeroallergen globally, the patterns of sensitization to pollen of different grass subfamilies have been unclear. The distribution of grasses from different subfamilies have been shown to be influenced by climate, thus giving rise to the importance of understanding these patterns in large countries with diverse climatic regions such as Australia and the USA.

Recent studies have demonstrated that the IgE reactivity of grass pollen allergic patients vary according to the region of residence (Davies et al., 2011b, Nony et al., 2015). However, these studies only compare data from patients from a maximum of two regions with up to three grasses compared. This study will be the first to include patients from 4 regions of Australia, with differing climates and grass species distribution, with comparison of serum IgE reactivity with the pollen of 5 grass species from three subfamilies associated with pollen allergy in these regions. The regions from which patients were recruited as part of a multicenter cross-sectional Grass Pollen Allergy Survey (GPAS) were New South Wales, Western Australia, South Australia and Queensland.

By performing a cross-inhibition assay, we will be performing a multipoint assay defined by the multiple concentrations of inhibitors applied, to better define serum IgE reactivity towards the purified allergens by demonstrating both the magnitude and avidity of IgE reactivity. This assay method provides more robust and accurate results when compared to the standard, single point IgE reactivity assays (such as skin prick tests) that just indicate positive or negative reactivity. The outcomes of this chapter may contribute to the growing body of knowledge to inform the need to develop grass pollen immunotherapy and diagnostics based on subtropical grasses for the use of patients within subtropical region.

3.2 AIM

This chapter aims to evaluate allergic sensitisation levels to GP and IgE specificity for major GP allergens in biogeographically distinct regions in Australia. Sensitisation levels to three grass subfamilies will be examined; Chloridoideae (*Cynodon dactylon*) and Panicoideae (*Paspalum notatum*) that grow predominantly in subtropical climate, and Pooideae (*Lolium perenne*) which grow predominantly in temperate climate.

3.3 RESULTS

3.3.1. Conversion from 96-well to 384-well format using Time-Resolve Fluorescence ELISA

Experiment 1: Trial cross inhibition assay in 96 well format using a grass pollen allergic adult participant (n=1).

Trial experiment was set up as per the methodology in 2.6 to determine inhibition of the five inhibitor grass extracts on the IgE reactivity of one donor to Bahia grass alone.

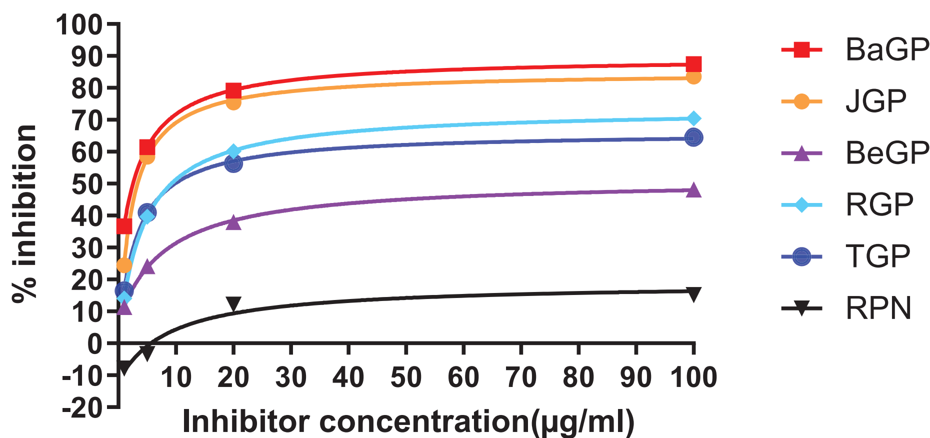


Figure 3.1 The cross-inhibition of five grasses; *S. halepense* (JGP), *P. notatum* (BaGP), *C. dactylon* (BeGP), *P. pratense* (TGP) and *L. perenne* (RGP) at range of concentration 0.016 to 100µg/ml on *P. notatum* pollen (specific IgE reactivity in the plasma of grass pollen allergic donor GAAS 56 (n=1), with raw peanut extract (RPN) as a negative control.

Table 3.1 Summary of IC50 and maximum inhibition achieved by the five grass pollen extracts on GAAS 56 plasma IgE reactivity to Bahia grass pollen.

Subfamily	Grass	IC_{50} (µg/ml)	Maximum inhibition (%)
Panicoideae	*P. notatum*	2.3	87.4
	S. halepense	4.4	83.5
Chloridoideae	*C. dactylon*	110.4	48.2
Pooideae	*L. perenne*	13.7	70.5
	P. pratense	16.8	64.5

From the results in Figure 3.1 and Table 3.1, the strongest inhibition of IgE reactivity to *P. notatum* grass pollen extract was by the Panicoideae subtropical grasses *P. notatum* and *S. halepense* grass, with an IC_{50} of 2.3 µg/ml and 4.4 µg/ml respectively. These inhibitions were stronger than both the Pooideae temperate grasses; *L. perenne* (13.7 µg/ml) and *P. pratense* grass (16.8 µg/ml). The inhibition by *P. notatum* and *S. halepense* grass pollens were also stronger than the other Chloridoideae subtropical grass, *C. dactylon* grass, which had the weakest inhibition and an IC_{50} of 110.4 µg/ml.

These results are consistent with the expected outcomes. *P. notatum* grass pollen is one of the major allergenic grass in Brisbane and thus exhibits high avidity of IgE reactivity, represented by the high maximum inhibition to itself and low IC_{50}. *S. halepense* grass pollen appears to have high immunological similarity to *P. notatum* grass pollen, which thus may explain its high inhibition (83.5%) of IgE reactivity and low IC_{50} of 4.4 µg/ml towards *P. notatum* grass. Conversely, *C. dactylon* grass has a very weak inhibition of IgE reactivity to *P. notatum* grass pollen despite being a subtropical grass. This could be attributed to *C. dactylon* arising from a separate subfamily, the Chloridoideae, which are phylogenetically different to the Panicoideae which both *P. notatum* and *S. halepense* arise from.

Experiment 2: Developing a TRF-based detection method for determining allergen-specific IgE concentration in patient sera.

This experiment was performed to compare the performance of ELISA and TRF as well as to optimize the plate reader settings.

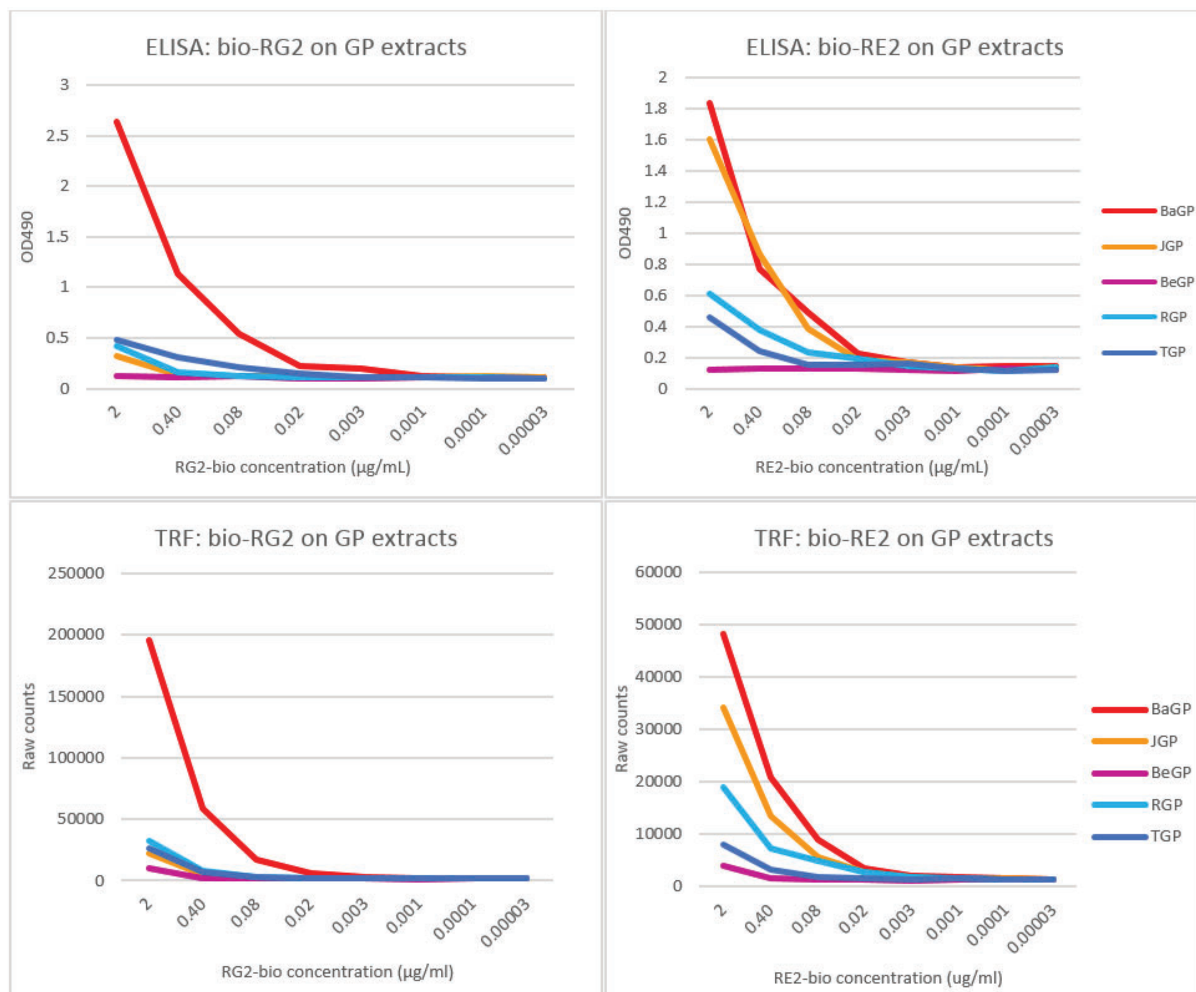


Figure 3.2 Biotinylated RG2 and RE2 titration against five grass pollen extracts *S. halepense* (JGP), *P. notatum* (BaGP), *C. dactylon* (BeGP), *P. pratense* (TGP) and *L. perenne* (RGP) on *P. notatum* pollen specific IgE reactivity in the plasma of grass pollen allergic donor GAAS 56 assayed using the ELISA and TRF methods.

From Figure 3.2, it appears that the results generated by the TRF method are similar with the results generated by the ELISA method. In both results, bio-RG2 was shown to have high specificity for BaGP extract and very weak reactivity for the other grass extracts. Bio-RE2 did have strong affinity for BaGP but also showed good reactivity for JGP and to a lesser degree RGP. Thus, it was not as specific as RG2. As seen in Figure 3.2, the TRF method can be used as a

detection method to replace the ELISA colorimetric approach yielding similar results.

Following consultation with a BMG Labtech technician and a Perkin Elmer product specialist, readings for the TRF method were performed at 5 minutes and 15 minutes as well as using the default reader settings (Integration start: 60µs, time: 400 µs) and recommended settings from Perkin Elmer (Enspire, integration start: 200µs, time: 500 µs) to determine optimal reading time and settings.

Table 3.2 The effect of plate reader settings and development time on TRF raw counts. The readings at the maximum mAb concentration at 2 µg/ml bio-RE2 and bio-RG2 concentration was compared with readings at no mAb. BaGP was selected because it had the lowest inhibition and therefore the highest reading, while BeGP was selected as it had the highest inhibition, hence the lowest readings.

Settings	Criteria	bio-RE2		bio-RG2	
		BaGP	BeGP	BaGP	BeGP
5 min (default)	Dynamic range	46240	2286	194112	8886
	Blank	2178	2178	2178	2178
15 min (default)	Dynamic range	45567	2059	193391	9118
	Blank	2106	2106	2106	2106
5 min (Enspire)	Dynamic range	43379	2196	182202	8569
	Blank	1480	1480	1480	1480
15 min (Enspire)	Dynamic range	41818	2784	177677	8271
	Blank	1415	1415	1415	1415

From Table 3.2, the dynamic range was calculated based on the difference between the reading at maximum mAb concentration (2µg/ml) and the reading at no mAb. In general, the Default settings appear to have a higher dynamic range compared to the Enspire settings. Thus, the Default reader setting is maintained for subsequent studies. Furthermore, the 15 minute timepoints were found to have lower raw counts compared to the 5 minute timepoint. Hence, 5 minute development time was selected for subsequent studies.

Experiment 3: Reproducibility of results in 384 well format TRF assay

This experiment was performed to evaluate the performance of a lower assay volume of 10µl in a 384 well format using TRF. A 384 well plate was coated in alternating blocks of 16 with either 5µg/ml of *P. notatum* grass pollen

or no coating. This was then incubated with 1µg/ml of bio-RE2. The assay volume used here was 20µl. Detection was performed as per the TRF method as described above using Streptavidin-conjugated with Europium.

While the assay itself showed good sensitivity, there was a lot of variation observed between the blocks coated with *P. notatum* grass pollen. The blocks of wells coated with BaGP and applied with bio-RE2 were analyzed by their means either according to the positions of the blocks across the plate (horizontally) or according to their rows (A to P) down the plate (vertically). Variability was displayed as the standard deviation (SD) of each block or row and by the overall coefficient of variation, COV, calculated as below;

COV = (average of SD across well or blocks /average of means across wells or blocks) * 100

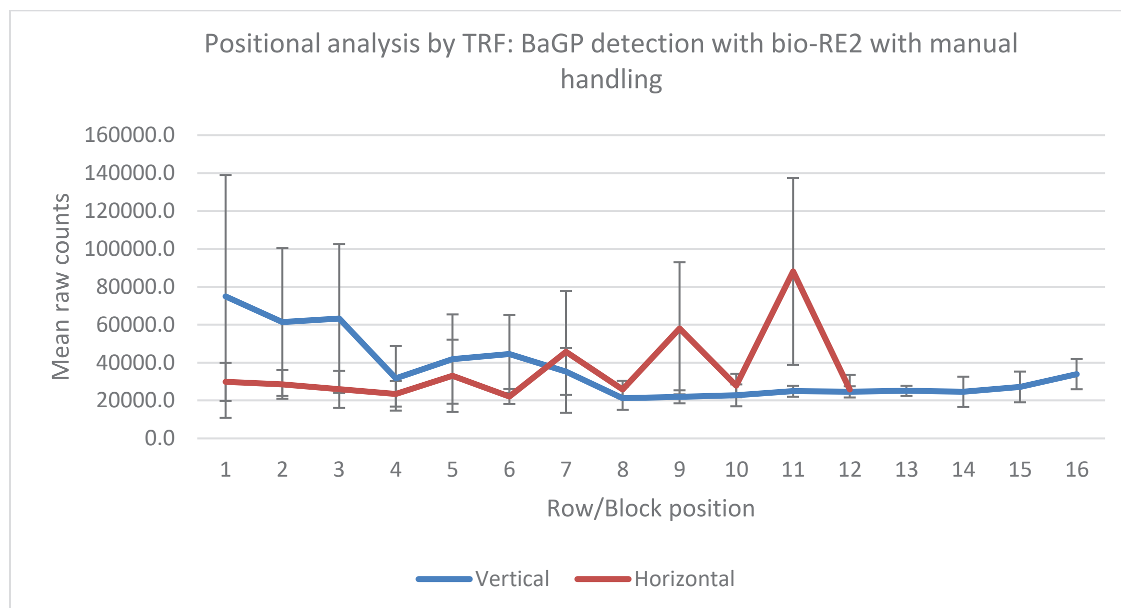


Figure 3.3 Raw counts for bio-RE2 detection of BaGP extract assayed in 384 well format using TRF. Data expressed as mean with SD as whiskers.

In Figure 3.3 above, it is observed that the mean raw counts start to vary drastically going down the plate and at the upper rows of the plate. This is reflected by the high SD and by the high overall COV of 38.3 horizontally and 37.0 vertically. The variation in raw counts could be due to fatigue as the bio-RE2 was delivered manually by a 12 channel multipipette. Thus, a repeat of this experiment was performed using a semi-automatic liquid handler (Gilson Platemaster).

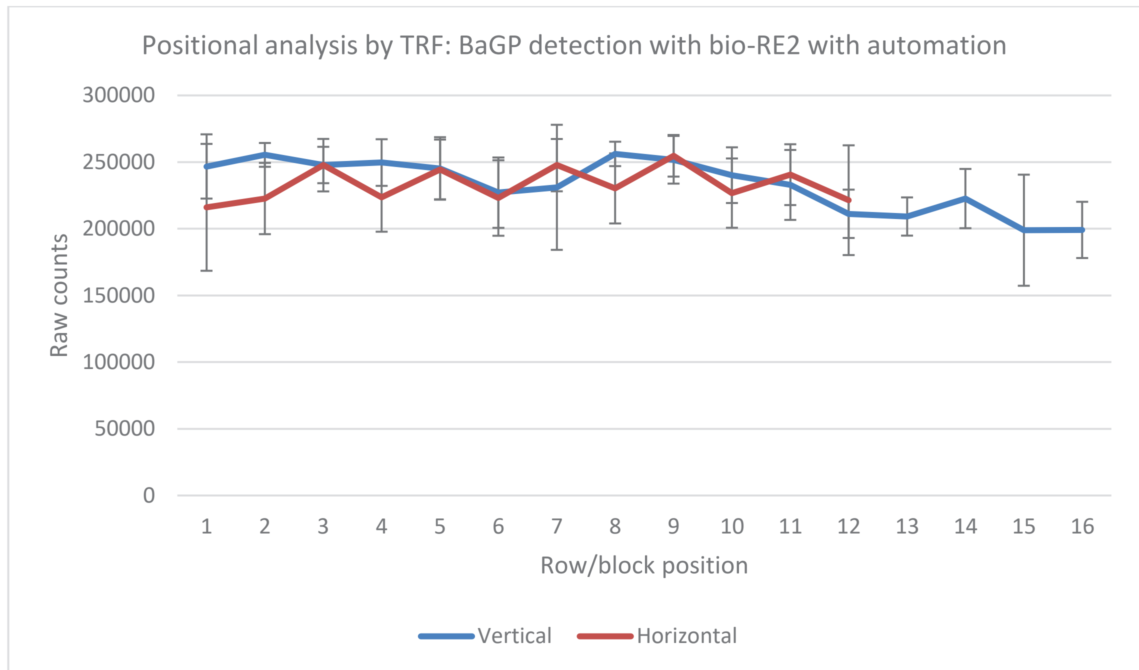


Figure 3.4 Raw counts for bio-RE2 detection of BaGP extract delivered with Gilson Platemaster assayed in 384 well format using TRF.

From Figure 3.4, there was lower variation in raw counts following delivery by the Gilson Platemaster, as reflected by the lower SD across and down the plates. There was also a much lower overall COV of 11.7 horizontally and 9.7 vertically. These findings necessitate the use of a semi-automatic liquid handler to ensure more consistent results throughout this experiment.

3.3.2. Participant demographics and sensitization profile

Table 3.3.3 Age, gender, total IgE, grass pollen specific and allergen specific IgE by ImmunoCAP of grass pollen allergic patients selected for cross inhibition assay. SPT and specific IgE data expressed as median (interquartile range).

Region	QLD	WA	NSW	SA	Statistical differences
Sample size	15	12	8	15	
No. of females (%)[1]	8 (53.3)	8 (72.7)	5 (62.5)	10 (66.7)	n.s.
Age; median (IQR) [2]	39 (37-56)	36 (23.5-40)	42.5 (38-48.25)	33 (22-44)	n.s.
Total IgE, kU/L[2]	213 (81-396)	285 (75.8-377)	149.5 (78.3-281.3)	127 (68-465)	n.s.
Sum of GP spIgE kU/L[2]	83.8 (50.5-180.3)	41.21 (29.4-115.5)	37.74 (23.6-50.7)	62.38 (28.9-154.7)	n.s.
Pas n 1 spIgE, kU/L[2]	26.8 (21.5-77.3)	9.5 (1.8-60.3)	8.1 (5.1-20.1)	18.1 (6.8-54.2)	n.s.
Cyn d 1 spIgE, kU/L[2]	37.4 (31.6-81.5)	23.6 (8.1-128.4)	6.3 (4.0-15.8)	13.1 (4.0-25.7)	QLD > NSW * QLD > SA *
Lol p 1 spIgE, kU/L[2]	19.5 (10.4-46.6)	56.8 (8.5-82.3)	23.9 (8.8-32.7)	30.2 (15.4-114.2)	n.s.

No significant differences were observed for number of females, age, total IgE, sum of GP specific IgE between patients of the four states selected for the cross-inhibition assay (Table 3.3). No significant differences were observed for specific IgE to Pas n 1 and Cyn d 1 between groups. However, there was a significant difference between Cyn d 1 specific IgE levels between QLD with both NSW and SA ($p \leq 0.05$).

3.3.3. Patient sensitization to GP by titration ELISA according to region

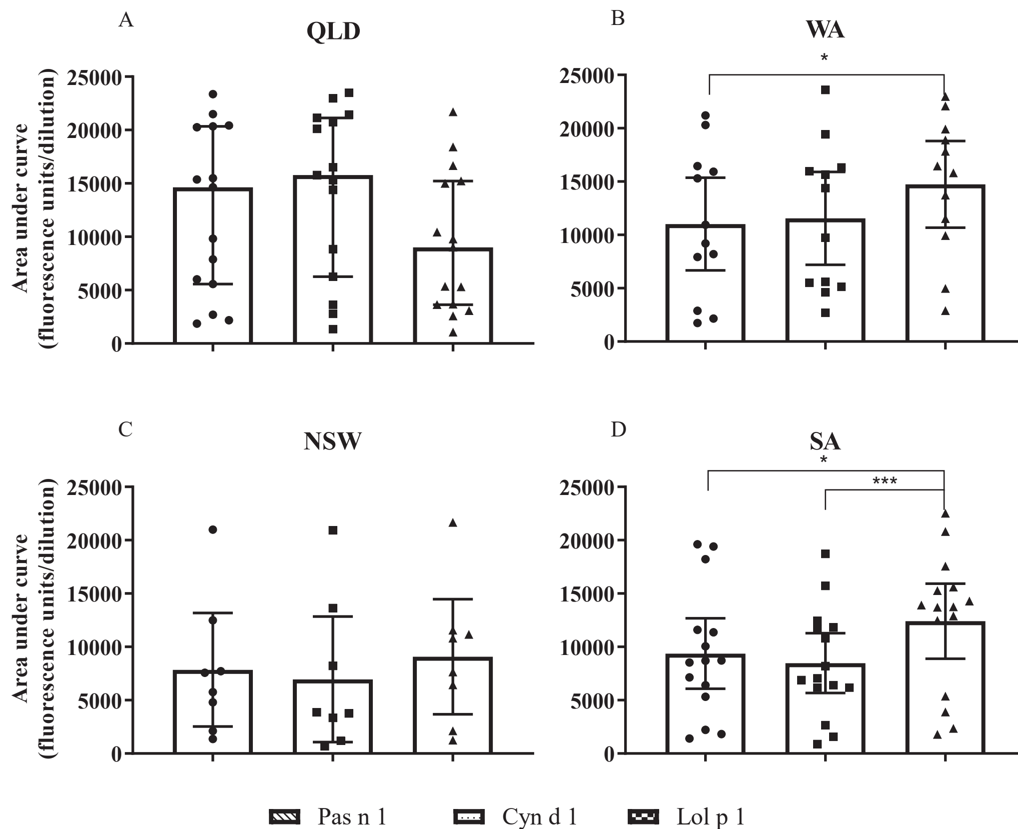


Figure 3.5 Area under the titration curve of participants selected for cross-inhibition sera IgE reactivity with purified allergen Pas n 1, Cyn d 1 and Lol p 1 from QLD (A), WA (B), NSW (C) and SA (D), expressed as median with interquartile range. Data expressed as median with whiskers representing interquartile range. P-values denoted; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

Figure 3.3 shows the titration curves of patient serum with purified major GP allergens Pas n 1, Cyn d 1 and Lol p 1 from the four regions tested as per the methodology described in 2.5. Data was expressed as area under the titration curve as a measure of IgE reactivity. The results here showed concordance in trends with the specific IgE reactivity towards purified allergen as reported in Table 3.2.1. For example, the highest sera IgE reactivity with Lol p 1 was observed in WA and SA, and the highest sera IgE reactivity with Pas n 1 and Cyn d 1 was observed in QLD. In SA, the IgE reactivity towards Lol p 1 was significantly higher than Cyn d 1 and Pas n 1, while in WA, Lol p 1 reactivity was only significantly higher than Pas n 1. In QLD, no differences were observed in the reactivity towards all 3 allergens, with trends indicating higher reactivity towards Pas n 1 and Lol p 1. In NSW, there is lower reactivity to all three

allergens compared to the other three regions, with no significant differences between them. There was a high incidence of allergen immunotherapy, which is an exclusion criteria for this study. Consequently, most of the patients with high reactivity towards the grass pollen allergens were excluded and therefore, only the patients with lower reactivity were available for inclusion.

3.3.4. Patient reactivity to purified major grass pollen allergen varies according to grass subfamily and region

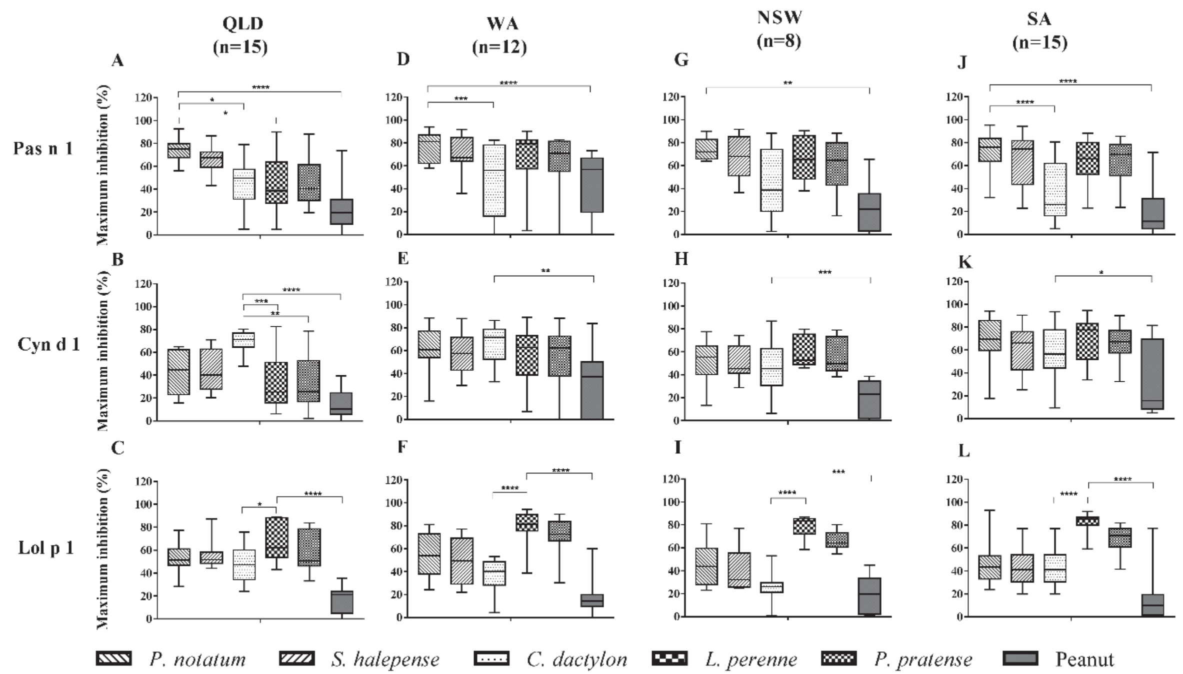


Figure 3.6 Maximum cross inhibition of serum IgE reactivity with purified allergen components Pas n 1, Cyn d 1 and Lol p 1 by inhibitor extracts of (from left to right) P. notatum, S. halepense, C. dactylon, L. perenne, P. pratense and raw peanut for patients from Queensland (QLD, A-C), Western Australia (WA, D-F), New South Wales (NSW, G-I) and South Australia (SA, J-L). Maximum inhibition expressed as median and interquartile range. P-values denoted; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

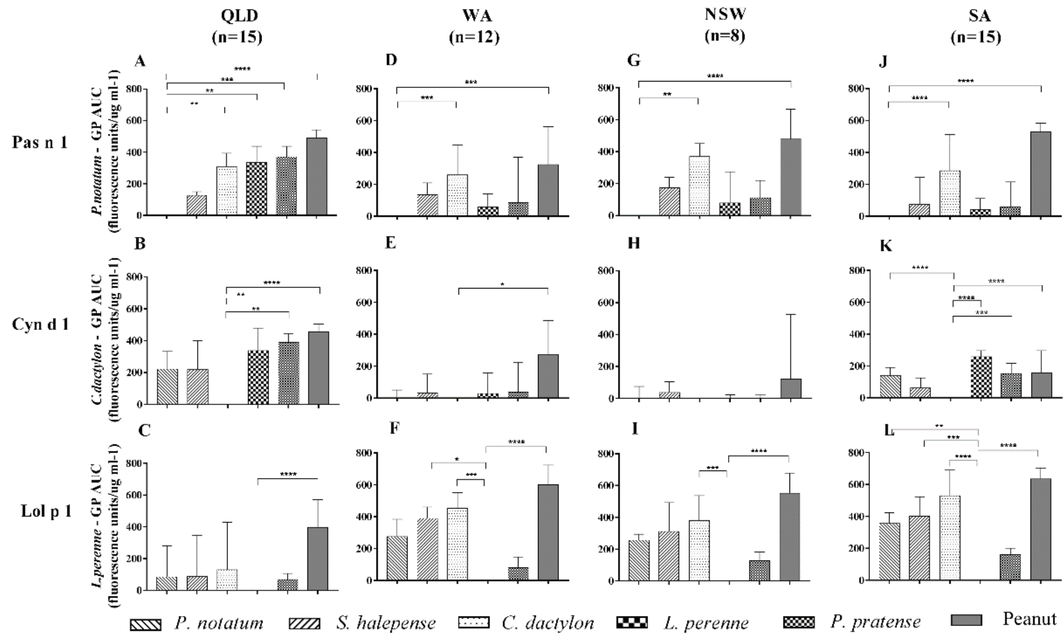


Figure 3.7 Difference in area under the curve between cross-inhibition of specific IgE reactivity with purified allergen components Pas n 1, Cyn d 1 and Lol p 1 by inhibitor extracts (from left to right); *P. notatum*, *S. halepense*, *C. dactylon*, *L. perenne*, *P. pratense* and raw peanut negative control, for a subset of patients in Queensland (QLD, A-C), Western Australia (WA, D-F), New South Wales (NSW, G-I) and South Australia (SA, J-L). Data expressed as median and upper quartile (whiskers) difference in area under the curve (AUC) relative to the self-inhibitor grass pollen (GP) extract and other inhibitor extracts. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Figure 3.6 shows the maximum inhibition achieved by the five GP extracts in cross-inhibiting serum IgE reactivity towards the three purified allergens, which provides a comparison of specificity of IgE reactivity. Figure 3.7 shows the difference in area-under the cross-inhibition curves of spIgE reactivity towards purified allergen between the five GP and provides a comparison of avidity. Methodology was as described in 2.7.

In QLD, self-inhibition by *P. notatum* pollen extract was significantly higher than the RPN control for Pas n 1, Cyn d 1 and Lol p 1 (Figure 3.6 A,B,C and 3, A,B,C). Maximum inhibition of spIgE reactivity with Pas n 1 by *C. dactylon* and *L. perenne* was significantly lower than self-inhibition by *P. notatum* GP (Figure 3.6 A). The area under the curve for spIgE of reactivity with Pas n 1 was significantly different than self-inhibition by *P. notatum* GP, and *L. perenne*, *P. pratense*, *C. dactylon* GP, but not *S. halepense* GP, indicating specific and avid IgE binding to Pas n 1 (Figure 3.7 A). Maximum inhibition of spIgE reactivity with Cyn d 1 by *L. perenne* and *P. pratense* GP were

significantly lower than self-inhibition by *C. dactylon* GP (Figure 3.6 B). Similarly, the area under the curves for spIgE reactivity with Cyn d 1 was significant different than self-inhibition by *C. dactylon* GP for *L. perenne* and *P. pratense*, indicating specific and avid IgE reactivity with Cyn d 1 (Figure 3.7 B). Maximum inhibition of spIgE reactivity with Lol p 1 by *C. dactylon* was significantly lower than self-inhibition by *L. perenne* GP (Figure 3.6 C). However, no difference in the area under the curve of spIgE reactivity to Lol p 1 was observed for any GP (Figure 3.7 C).

In WA, significant self-inhibition was observed for Pas n 1, Cyn d 1 and Lol p 1 (Figure 3.6 E,F,G and 3.7 E,F,G). spIgE reactivity with Pas n 1 by *C. dactylon* was significantly lower than self-inhibition by *P. notatum* by maximum inhibition and area under the curve, exemplifying the difference in IgE specificity between Cyn d 1 and Pas n 1 (Figure 3.6 E, 3.7 E). No significant difference in the area under the curve of IgE reactivity with Cyn d 1 between self-inhibition by *C. dactylon* and other GP was observed (Figure 3.7 F). Maximum inhibition of spIgE reactivity with Lol p 1 by *C. dactylon* was significantly lower than self-inhibition by *L. perenne* GP (Figure 3.6 G). A large difference in the area under the curve of spIgE reactivity to Lol p 1 between self-inhibition by *L. perenne* and *C. dactylon* and *S. halepense* were observed (Figure 3.7 G).

In NSW, self-inhibition compared with RPN was observed for Pas n 1, Cyn d 1 and Lol p 1 based on maximum inhibition (Figure 3.6 G,H,I) and for Pas n 1 and Lol p 1 by difference in the area under the curve (Figure 3.7 G,I). However, the area under the curve between self-inhibition of Cyn d 1 spIgE by *C. dactylon* GP did not differ from RPN indicating that IgE binding to Cyn d 1 in these subjects may not have been specific for Cyn d 1 (Figure 3.7 H). spIgE reactivity to Pas n 1 was inhibited less by *C. dactylon* GP than *P. notatum* GP based on the area under the curve, but the maximum inhibition did not differ (Figure 3.6 G, 3.7 G). spIgE reactivity with Lol p 1 was inhibited less by *C. dactylon* GP than self-inhibition by *L. perenne* based on maximum inhibition and difference in area under the curve ($p < 0.001$; Figure 3.6 I, 3.7 I).

In SA, significant self-inhibition compared to RPN was observed for spIgE reactivity with Pas n 1 , Cyn d 1 and Lol p 1(Figure 3.6 J, K, L and 3.7 J,

K, L). Pas n 1 spIgE reactivity by *C. dactylon* GP was significantly lower than self-inhibition by *P. notatum* GP by maximum inhibition and difference in area under the curve (Figure 3.6 J, 3J). The maximum inhibition of spIgE reactivity of Cyn d 1 did not differ between any of the GP (Figure 3.6 K), but differences in self-inhibition by *C. dactylon* GP by area under the inhibition curve were observed with *P.notatum* GP , *L. perenne* G and *P.pratense* GP. Maximum inhibition of Lol p 1 spIgE reactivity by *C. dactylon* GP was significantly lower than self-inhibition by *L. perenne* GP (Figure 3.6 L). The area under the inhibition curve of spIgE reactivity with Lol p 1 was lower than self-inhibition by *L. perenne* GP for *C. dactylon* GP *P. notatum* GP and *S. halepense* GP, whilst that for *P. pratense* GP was similar to *L. perenne* GP (Figure 3.7 L).

Within the QLD cohort, significant self-inhibition of Lol p 1 spIgE reactivity was observed (Figure 3.6 C) and significant difference in the area under the curve between self-inhibition with raw peanut (Figure 3.7 C) was observed. This indicates both strong affinity and avidity for *L. perenne* within the QLD cohort, which is rather unexpected for participants from a subtropical region. Upon further examination, 4 participants within the cohort showed relatively higher SPT response towards *L. perenne* and consequently, had higher spIgE affinity and avidity for Lol p 1. These participants were found to originate from known temperate regions NSW, WA and the United Kingdom.

Why IC50 was not analyzed as an output

The data from the cross-inhibition ELISA was initially assessed by IC50 i.e. the concentration of GP inhibitor extract that resulted in 50% inhibition of IgE reactivity to purified GP allergen. However, there were many inhibitor-allergen combinations that did not yield an IC50 below the maximum inhibitor concentration of 100 µg/ml, primarily due to weak inhibitory activity on IgE reactivity towards purified allergen by GP from different subfamilies. Although statistical methods have suggested utilizing a nominal value as an upper limit (e.g. maximum inhibitor concentration), there will be an inability to account for differences of inhibitory activity at those higher ranges, resulting in a loss of data resolution.

3.4 DISCUSSION

GP is the major aeroallergen trigger for allergic rhinitis worldwide and there is considerable biological diversity amongst grass subfamilies that influences the allergen composition and degree of similarity between allergens of GP (Davies, 2014b; Matricardi et al., 2016). Biogeographical variation in the level of sensitisation to Panicoideae, Chloridoideae and Pooideae GP was observed over four regions in Australia with different climates; the subtropical regions QLD, and WA and the more temperate NSW and SA, as determined by skin prick test diameter and serum spIgE concentration, and purified group 1 allergen components.

The observed difference in concentrations of spIgE to GP group 1 allergens appeared to be more pronounced than the differences between spIgE to whole GP extracts, as shown by the differences between spIgE to Lol p 1 compared to *L. Perenne* pollen extract in WA, NSW and SA. This suggests that measuring spIgE to allergen components may offer better diagnostic precision than GP extracts (Matricardi et al., 2016). Currently, there are no commercially available diagnostic tests for spIgE to Pas n 1 and Sor h 1. There is a diagnostic test for Cyn d 1, however it is only available as a natural purified allergen component, which may show false positives due to cross-reactive carbohydrate determinants (CCD) in populations where grass is not an important aeroallergen source.

This study is the first to compare GP allergen sensitization profiles from multiple states in Australia across a wide geographical range, separated by 4300 kilometers east to west and 1100 kilometers north to south, and diverse climates. This was also the first study to investigate GP sensitization profiles in two understudied states; SA and WA. The IgE cross-inhibition assays confirmed regional variation in specificity of IgE reactivity established in literature (Davies et al., 2012; Davies et al., 2011) and revealed regional variation in the avidity of spIgE for purified group 1 allergen components subtropical and temperate GP. Specific and unique recognition of Pas n 1 and Cyn d 1 by participant spIgE was observed for individuals from the subtropical region of QLD that could not be blocked by *L. perenne* or *P. pratense* GP. Conversely in the temperate region of

SA, spIgE reactivity with Lol p 1 was inhibited less by Panicoideae and Chloridoideae GP.

The use of cross-inhibition assays allowed for the investigation of both specificity and avidity of spIgE reactivity for the group 1 allergens Pas n 1, Cyn d 1 and Lol p 1, by enabling the comparison of maximum inhibition and the area under the inhibition curve between GP from different subfamilies. This method is more informative than single point spIgE assays that only allow for comparisons of specificity as avidity informs the magnitude of IgE reactivity. The use of microscale cross-inhibition assay format coupled with semi-robotic liquid handling allowed simultaneous testing of a large combination of spIgE reactivity towards three purified allergens cross-inhibited with 5 GP extracts for each donor at the same time, enabling comparisons of spIgE specificity and avidity across these test conditions. The comparisons of difference in the area under the inhibition curves between other GP and self-inhibitor GP extracts indicate differences in spIgE avidity. In previous studies, investigators have compared the inhibitor concentration at which 50% (IC50) inhibition is reached as a measure of avidity (Nony et al., 2015). In this study however, there were cases where GP extracts and the control RPN extract did not reach the IC50, thus preventing this method of quantative analysis. For example, only *C. dactylon* and *L. perenne* showed significantly different maximum inhibition to *P. notatum* self-inhibition of spIgE to Pas n 1, whereas the area under inhibition curves for *C. dactylon*, *L. perenne* and *P. pratense* GP revealed differences between each of these GP and *P. notatum*.

Across all regions, GP from the same subfamily showed comparable spIgE reactivity to each other, with the Panicoideae *P. notatum* and *S. halepense* showing similar maximum inhibition and area under the cross-inhibition curve, and similarly for the Pooideae *L. perenne* and *P. Pratense* GP. *C. dactylon* GP showed unique spIgE reactivity indicating the presence of some distinct Cyn d 1 epitopes compared to allergens of either Panicoideae or Pooideae GP. The phylogenetic relationship between grasses within subfamilies is closer than between subfamilies (Davies et al., 2012), and consequently there is higher primary sequence similarities of major allergens within subfamilies (Davies et al., 2008). Structural differences between epitopes of allergens of separate grass

subfamilies are likely to account for the observed region-dependent, species-specific IgE recognition of GP allergens in sensitized GP allergic participants, although there has been little comparative B cell epitope analysis of GP allergens to date (Ball et al., 1999; Yuan et al., 2012).

Allergen immunotherapy (AIT) offers effective reduction in symptoms, reduces new sensitizations and the risk of progression to asthma in children (Dhami et al., 2017). Assessment of patients receiving temperate GP sublingual immunotherapy (SLIT) tablets showed lowered mean AR medication prescriptions and lower proportions of new-onset of asthma compared to the non-AIT treated groups (Devillier et al., 2017). However, currently available AIT is not standardized for subtropical GP, which may be important for efficacious treatment of people who are primarily allergic to Panicoideae and/or Chloridoideae GP, given that only one of three T cell epitopes are shared between major allergens Pas n 1, Cyn d 1 and Lol p 1 (Burton et al., 2002a; Etto, de, et al., 2012; N. P. Eusebius et al., 2002). Therefore, more specific assessment of GP sensitization profiles, particularly in countries with multiple climatic zones, may serve to precisely identify the relevant GP to achieve the most efficacious treatment for an individual patient (Matricardi et al., 2016).

An interesting observation made in the QLD cohort was that 4 participants were observed to have relatively high affinity and avidity towards *L. perenne* based on the cross-inhibition assay (Figure 3.6 C, Figure 3.7 C). They were found from their reported history to be migrants from temperate regions NSW, SA and the United Kingdom. This observation indicated an important role of migration in influencing participant allergen sensitization profile. Leung and colleagues were the first to discuss the effects of immigration on both atopy and asthma in Australia by demonstrating that Australians (n=109) and Australian born Asians (n=424) were more likely to develop both hayfever and asthma relative to Asian immigrants of Chinese origin (n=636) in Melbourne. Furthermore, they also found a strong association ($p < 0.01$) between the length of stay of Asian immigrants in Australia and prevalence of hayfever and a weaker association with history of wheeze or asthma ($p = 0.02$) (Leung et al., 1994). A global study utilizing the International Study of Asthma and Allergies in Childhood (ISAAC) cohort found that immigration was associated with lower

prevalence of asthma, rhinoconjunctivitis and eczema in adolescents of both 13 to 14 years old from 48 countries (n=326 691) and 6 to 7 year olds from 31 countries (n=208 523) (Garcia-Marcos et al., 2014). Another key finding Garcia-Marcos and colleagues made was that the protective effect on asthma, rhinoconjunctivits and eczema in adolescents was confined to migration to more affluent countries with high prevalence of those diseases, citing a potential socio-economic influence as well. However, the protective effects of the pre-migration environment decreased as the length of stay in the host country increased, a trend observed by Leung and colleagues as well (Garcia-Marcos et al., 2014). Another study involving participants with diagnosed allergic rhinitis and/or asthma in Northern Italy found that new immigrants (n=395) demonstrated a time-dependent increase in the number of sensitized aeroallergens such as HDM, *Alternaria alternata, P. pratense* and more, as determined by SPT, compared to residents (n=1395) (Burastero et al., 2011). While the association between migration and clinical prevalence of allergic disease and asthma is well studied, there is currently a lack of immunological and serological studies to determine differences in allergen sensitization profiles between migrants and residents of a country. Tham and colleagues recently reviewed the effects of migration on allergic disease and outlined six key factors that influence allergic risk in migrant populations; immigration generation, duration of residence in host country, affluence of host country, age at the time of migration, period of migration in relation to birth (i.e. birth before or after migration) and rural childhood environment (Tham et al., 2019). As several studies have shown that there is time-dependent loss of allergic disease and asthma in migrants, there is a distinct possibility that extended periods of stay in foreign countries may also influence the allergen sensitization profile (Burastero et al., 2011; Garcia-Marcos et al., 2014; Leung et al., 1994). Thus, there is a need to account for both migration and mobility-associated factors when examining the allergen sensitization profiles within a country.

The findings of this chapter were incorporated as part of a journal based on the GPAS cohort data (Appendix C) (Kailaivasan et al., 2020).

3.5 STUDY LIMITATIONS

A limitation of this study was the small number of NSW participants for the cross-inhibition analysis (n=8). This was due to the high incidence of allergen immunotherapy in the original GPAS cohort of NSW, resulting in most of the participants being excluded due to the study exclusion criteria. There were also lower serum IgE reactivity with grass pollen allergens observed in the titration assay (Figure 3.5 C) for patients from NSW for whom sera was available. This low GP-specific IgE reactivity may have consequently affected the lack of differences in serum IgE affinity and avidity observed for the inhibitor grass extracts.

Of the 15 participants living in QLD who were selected for the cross-inhibition assays based on sum of GP specific IgE and serum availability, 4 were born outside QLD, in regions with a predominance of *L. Perenne* (i.e. NSW, WA and the United Kingdom), contributing to variation in the range of inhibition of spIgE with Pas n 1 and Lol p 1 by *L. perenne* and *P. pretense* GP. This suggests that the place of origin where sensitization occurred and migration may diversify the allergen sensitization profiles of individuals within a population. Regional differences in sensitization rates, even for closely related taxa, based on analysis of large Japanese IgE datasets have been recently reported (Minami et al., 2019). These findings reinforce that clinical interpretation of spIgE responses need to be correlated with symptoms upon exposure and the allergens likely to be encountered by the patient within their usual environment. As discussed in section 3.4, migration and mobility of participants are potential confounding factors in the study, thus factors such as length of stay in host country, socio-economic background and rural nature of environment need to be considered. However, due to the relatively small sample sizes in this study, we were not able to elucidate fully the influence migration and mobility on this cohort.

To improve the design of future studies, participant questionnaires should account for factors that could affect the allergen sensitization profiles. Migration associated factors can be accounted through information such as country of origin, period of stay in host country, age at migration and nature of childhood environment (i.e. urban or rural) while accounting for affluence of native country and host country. Extended periods of stay in other countries

should also be recorded, especially that of differing climates which will be particularly relevant to this study. Other demographic factors not related to migration and mobility yet could potentially influence allergen sensitization include childhood environment (i.e. rural or urban), socio-economic status by SEIFA index based on postcode of residence and ethnicity should also be accounted for. However, to properly stratify for these factors, the sample size in each group will need to be substantially larger than what was recruited for this study.

3.6 CONCLUSION

The results of this chapter show that sensitivity to known allergenic grasses in grass pollen allergic patients vary according to the region they reside in. Patients showed more specific and avid IgE reactivity to the most biogeographically abundant grasses within their respective regions.

GP of the same subfamily mostly showed similar levels of inhibition to each other indicating subfamily specific allergic sensitivity. Allergens of the two subtropical Panicoideae and Chloridoideae grass subfamilies show separate IgE reactivity patterns.

Regional variation in allergic sensitivity with diverse GP allergens is clinically important for optimal diagnosis and targeted allergen specific immunotherapy for Australian patients with allergic rhinitis due to grass pollen allergy.

Chapter 4: The innate antiviral interactions in the PBMC of a high risk of pollen allergy group following co-exposure of rhinovirus-16 with whole *Paspalum notatum* extract and purified major allergen Pas n 1

4.1 BACKGROUND

Preliminary studies previously conducted by visiting Occupational Trainee, Hamid Nouri, have demonstrated a counterregulatory effect between *P. notatum* exposure and the innate antiviral response in adult PBMC. This study examined the response of the antiviral chemokine IP-10 (also known as CXCL10) and the pro-inflammatory cytokine IL-6 in the PBMC healthy patients and patients allergic to *P. notatum* when treated with RV-16. PBMC were obtained from the GAAS cohort described in 1.1. Results are summarized in Figure 4.1.6 and 4.1.7 below.

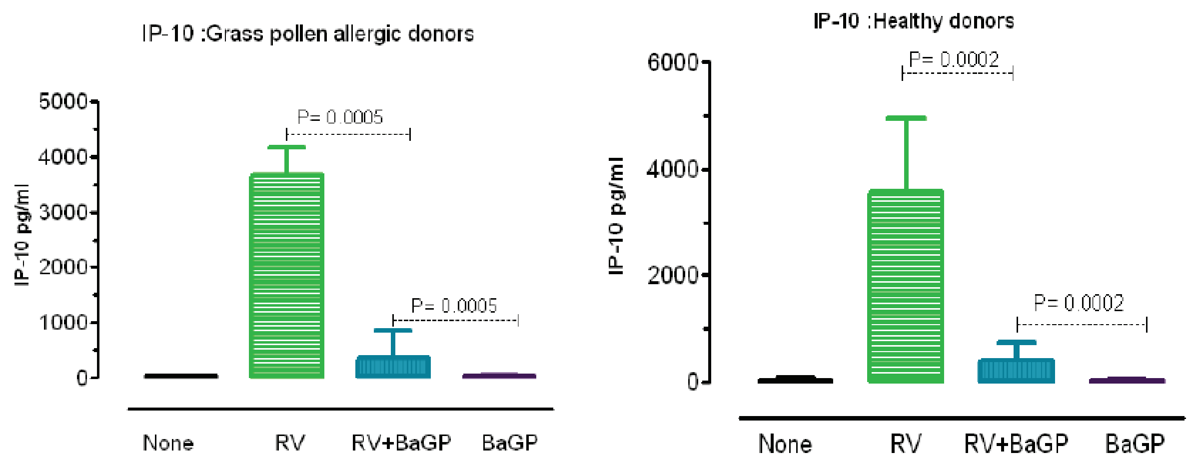


Figure 4.1.6 IP-10 secretion in PBMC of healthy (n=7) and GP-allergic patients (n=12) when treated with *P. notatum* (BaGP) extract and RV16. (data provided by visiting Occupational Trainee, Hamid Nouri). ELISA assays performed on 24-hour culture supernatants.

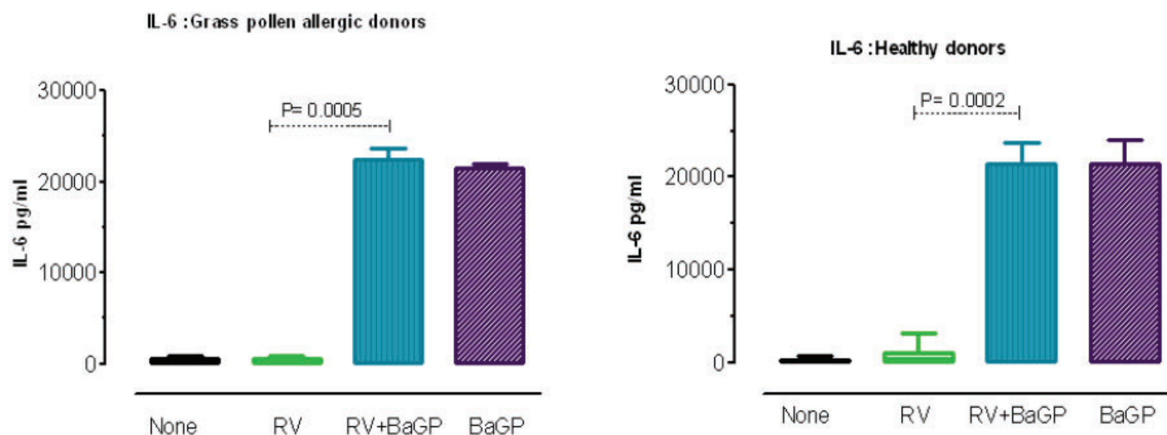


Figure 4.1.7 IL-6 secretion of healthy (n=7) and GP-allergic patients (n=12) when treated with *P. notatum* pollen (BaGP) extract and RV16. data provided by visiting Occupational Trainee, Hamid Nouri) ELISA assays performed on 24 hour culture supernatants.

In Figure 4.1.6, both healthy and *P. notatum* pollen (BaGP) allergic donors, it was found that co-exposure with BaGP resulted in a significant decrease of IP-10 secretion following RV-16 infection. This suggest that there is a direct interaction between *P. notatum* and the innate immune response to RV-16, independent of allergy status. In Figure 4.1.7, there was no significant difference in IL-6 secretion when *P. notatum* was co-exposed with RV compared to BaGP alone. It is also observed that there was no stimulation of IL-6 following exposure to only RV-16. Thus, no such modulation of the inflammatory response was observed in the co-exposure of *P. notatum* and RV-16 as observed with the innate immune response.

Apart from IgE-mediated effects on innate and adaptive immunity other investigations have revealed non-IgE mechanisms mediated by allergen sources and their components. Allergen sources and their component molecules can indirectly influence the activity of DC. Studies have shown pollen grains demonstrate IgE-inducing adjuvant activity by inducing the maturation of dendritic cells and inhibiting the secretion of IL-12 (Allakhverdi et al., 2005, Kamijo et al., 2013). Allergens are also able to interact with DCs via surface receptors such as the C-type lectin receptors. A common receptor associated with allergens is the mannose receptor, which are known to interact with HDM, dog and peanut allergens by facilitating allergen internalization and the induction of a Th2 immune response (Li et al., 2010, Royer et al., 2010). Another C-type lectin receptor more closely associated with grass pollen is the dendritic cell-

specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), which has been shown to bind strongly to Bermuda grass pollen and induce tumor necrosis factor alpha (TNF-α) secretion on myeloid dendritic cells (Hsu et al., 2010).

With a range of adjuvant activities observed in multiple components of a whole pollen extract, the preparation of the extract for studies becomes a key issue. For example, only using the purified allergen removes the adjuvant activity of the other pollen components. The key question here is whether the adjuvant properties are essential to the induction of an allergic response or potentially, has a greater influence than the purified allergen alone. Thus, it would be interesting to compare different fractions of the grass pollen to determine the influence on innate immunity.

Studies examining the immune response in allergic and/or asthmatic participants tend to emphasize on adults (age 18 and above) and adolescents (age 5 and below). However, there is an age group that is understudied that are highly affected by AR and asthma, teenagers. The German Health Interview and Examination Survey for Children and Adolescents (KiGGs) study indicated that children between 11 to 17 years old showed the highest prevalence of allergic conjunctivitis and asthma (Schmitz et al., 2011). Another study on children between 7 to 18 years old demonstrated a correlation between grass pollen count with higher allergic symptom severity and a lower health related quality of life (HRQL) scores (Kiotseridis et al., 2013). An observational, case-control study on wheezing-related hospital admissions of children demonstrated that 77% of wheezing subjects between 3 to 18 years old that tested positive for a respiratory virus was accounted by rhinovirus (Heymann et al., 2004). Apart from health, the National Young People and Asthma Survey conducted in Australia also showed that young people within the age of 15 to 17 years old experienced the highest amount of psychiatric distress as a result of asthma (Blanchard et al., 2014).

This chapter will be the first study to investigate the interaction of *P. notatum* on the early immune responses induced by RV-16 in PBMC of adolescents at risk of both GP induced and respiratory viral induced airway inflammation. This will also be the first immunological based study to account

for the effect of asthma on the immune response towards two of its major triggers i.e. aeroallergens and respiratory viruses. This study will also investigate the presence of direct interactions of a whole pollen extract compared to its purified major allergen in its capacity to inhibit the innate antiviral response.

This outcome may inform the need for consideration of allergic status and allergen exposure in the clinical management of asthma. Furthermore, if a mechanistic relationship between asthma and allergy is discovered, it may indicate the need for use of allergen-specific immunotherapy to manage allergic responses to pollen allergens, and the upper airway inflammation that adversely affects asthma (Bousquet et al., 2008).

4.2 AIM

To investigate the effect of asthma on the innate and adaptive immune response in PBMC of adults and adolescents with grass pollen allergy following Bahia grass pollen and rhinovirus exposure.

4.3 HYPOTHESIS

We hypothesize that *P. notatum* mediated inhibition of the innate antiviral response to RV-16 will be evident in PBMC of both participants with AR and without, and that this effect will be heightened in asthmatics.

4.4 RESULTS: INHIBITION OF ANTIVIRAL RESPONSE INDUCED BY HUMAN RHINOVIRUS-16 BY WHOLE *PASPALUM NOTATUM* POLLEN EXTRACT IN GRASS-POLLEN ALLERGIC AND NON-ALLERGIC ADULTS

4.4.1 Adult participant demographics

Table 4.1Adult participant demographics and skin prick test data according to experimental groups

	Grass pollen-allergic (GPA)	Other allergies (OA)	Non-allergic (NA)	Statistical differences
No. of participants	15	8	8	n.s.
Mean Age (SD)	37.5 (11.8)	53.5 (9.4)	38.1 (14.9)	n.s.
Mean Weight, kg (SD)	77.3 (14.5)	74.8 (18.2)	81.0 (25.6)	n.s.
Mean Height, kg (SD)	168.1 (4.5)	169.2 (7.5)	172.8 (10.2)	n.s.
% females (number)	73.3 (11)	75.0 (6)	75 (6)	n.s.
% current asthma (number)	66.7 (10)	87.5 (7)	87.5 (7)	n.s.
Total SPT, mm (IQR)	47.5 (40.0-60.5)	11.5 (6.6-19.5)	0 (0)	GPA > OA * GPA > NA ***
P. notatum SPT, mm (IQR)	9 (6.5-10.5)	0 (0)	0 (0)	GPA > OA *** GPA > NA ***

PBMC from a total of 31 participants were utilized in this study and the demographics are as shown in Table 4.1. 15 participants in the GP-allergic group, 8 in the other allergies group and 8 in non-allergic groups. No significant differences were observed between age, weight, height, and gender distribution between groups. The GP-allergic showed significantly higher total (median 47.5 mm, IQR 40.0-60.5) and *P. notatum* (median 9 mm, IQR 6.5-10.5) skin prick test wheal size compared to the other two groups. No significant differences between the percentage of participants with current asthma was observed between the three groups as well, with the lowest percentage being in the GP-allergic group (66.7%) and both other allergies and non-allergic group having 87.5% each.

4.4.2 Innate antiviral cytokine IP-10 induced by RV-16 inhibited by co-exposure with whole P. notatum extract in grass pollen-allergic adult participant PBMC

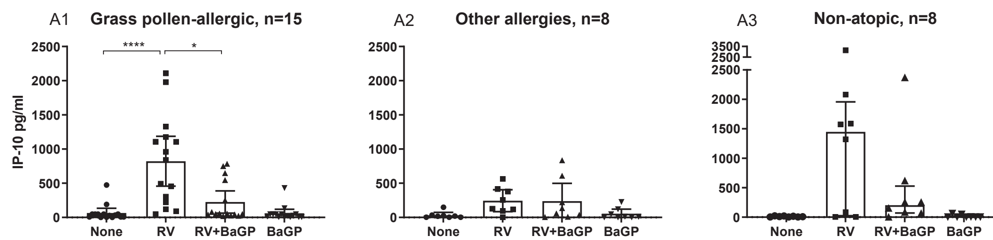


Figure 4.1 Innate antiviral cytokine IP-10 and inflammatory cytokines IL-6 and TNF-α in 24-hour PBMC supernatant of grass-pollen allergic (n=15), non-grass pollen allergic (n=8) and non-atopic (n=8) adult participants as examined by ELISA. Data expressed as individual points overlayed with median and interquartile range. P-values are denoted as p<0.0332, ** p<0.0021, *** p<0.0002, ****p<0.0001

There was significant induction of IP-10 following RV-16 exposure in the short term PBMC cultures of participants from the grass allergic group, with median IP-10 concentrations of 839.9 pg/ml (Figure 4.1; A1, IQR 222.8-1175.0;). No significant induction of IP-10 was observed in the other allergies group or the non-allergic group, though the IP-10 induction in the non-allergic group at median 1575 pg/ml (IQR 85.2-2081) was close to significant (p = 0.0580). When PBMC were co-exposed to both RV-16 and whole *P. notatum* extract, a significantly lower level of IP-10 was observed, with median 53.2 pg/ml (IQR 38.3-544.1). This represented a 93.7% inhibition of RV-16 induced IP-10 following co-exposure with whole *P. notatum* extract. No significant difference between these two treatments were observed in the other allergies group. In the non-allergic group, a lower level of IP-10 was observed following co-exposure of both stimuli, with median 254 pg/ml (IQR 85.2-620.2), however the difference was not statistically significant (p = 0.0580). While the differences in IP-10 levels between RV-16 exposed and co-exposed PBMC in the non-allergic group were not statistically significant, chiefly due to the lower number of participants (8 vs 15 in the GP-allergic group) there appeared a similar trend observed in the grass pollen allergic group of lower IP-10 levels following co-exposure of RV-16 with whole *P. notatum* extract. These findings suggest that co-exposure of RV-16 with whole *P. notatum* extract results in a diminished IP-10 response, and trends in the non-allergic group suggest that this interaction could be independent of allergy status.

4.4.3 Innate inflammatory cytokines IL-6 and TNF-α induced by whole P. notatum extract in adult participant PBMC regardless of allergy status

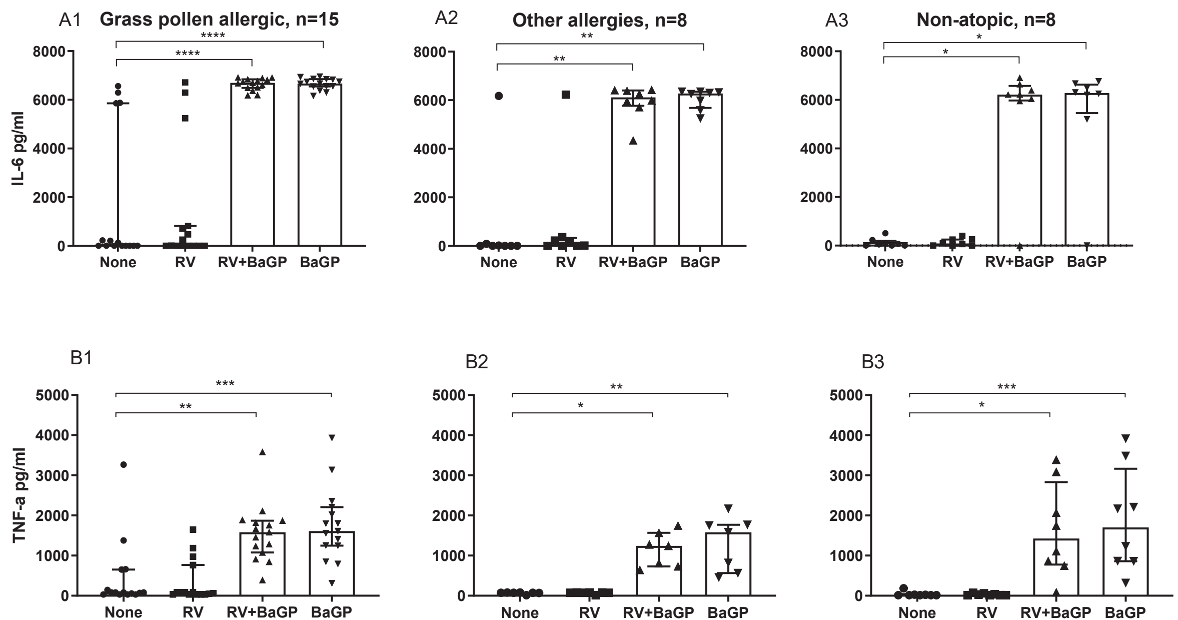


Figure 4.2 Innate inflammatory cytokines IL-6 and TNF-α in 24-hour PBMC supernatant of grass-pollen allergic (n=15), non-grass pollen allergic (n=8) and non-atopic (n=8) adult participants as examined by ELISA. Data expressed as individual points overlayed with median and interquartile range. P-values are denoted as p<0.0332, ** p<0.0021, *** p<0.0002, ****p<0.0001

There was significant induction of the innate inflammatory cytokine IL-6 following *P. notatum* extract exposure in the 24-hour PBMC cultures of GAAS participants in all three groups (Figure 4.2). Participants in the GP-allergic group had IL-6 levels with median 6668 pg/ml (IQR 6550-6852), other allergies with 6278 pg/ml (IQR 5683-6348) and non-allergic with 6310 pg/ml (6210-6676), with the GP-allergic group having significantly higher induction compared to the other allergies group. There was no significant difference between IL-6 levels of PBMC exposed to *P. notatum* extract alone and co-exposure of extract with RV-16 across all three groups. There was also significant induction of TNF-α following *P. notatum* extract exposure in all three groups with median levels 1612 pg/ml (IQR 1249-2208) in GP-allergic group, 1580 pg/ml (IQR 564.4-1770) in other allergies group and 1701 pg/ml (860.7-3166) in the non-allergic group. No significant differences in cytokine levels following *P. notatum* extract exposure between these groups were observed. Following co-exposure of *P. notatum* extract with RV-16, there was no significant differences observed when compared to *P. notatum* extract exposed across all three groups. These findings

show that *P. notatum* extract exposure significantly induces innate inflammatory cytokines but do not change with RV-16 co-exposure. Taken together, these findings show that allergen exposure can diminish the innate antiviral response, but the innate inflammatory response is not affected by allergen exposure.

4.4.4 Late inflammatory cytokines for grass-pollen allergic and non-allergic adults

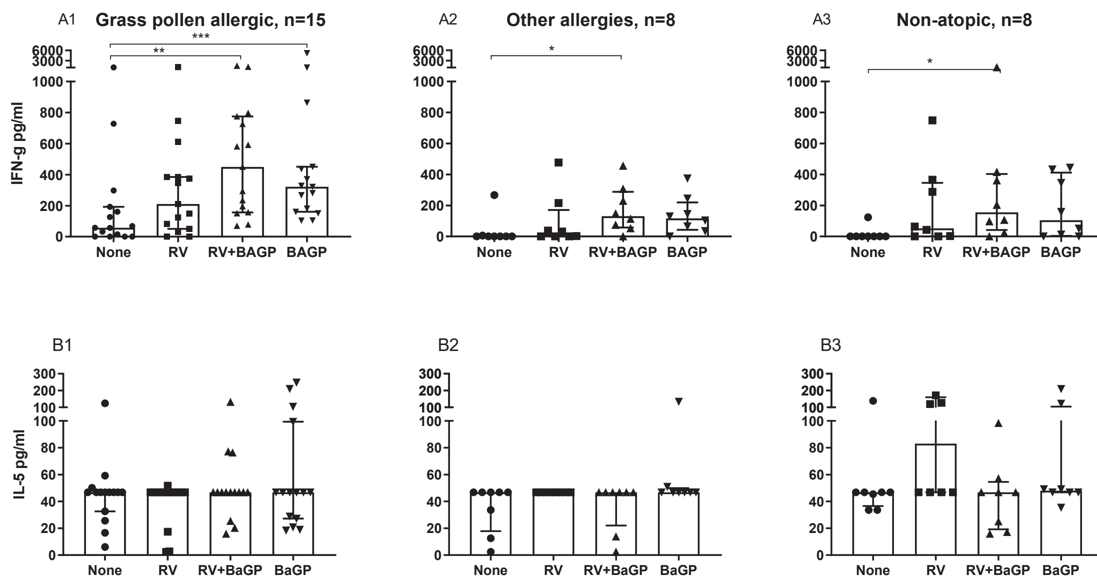


Figure 4.3 Late inflammatory cytokines IFN-γ and IL-5 in 120-hour PBMC supernatant of grass-pollen allergic (n=15), non-grass pollen allergic (n=8) and non-atopic (n=8) adult participants as examined by ELISA. Data expressed as individual points overlayed with median and interquartile range. P-values are denoted as p<0.0332, ** p<0.0021, *** p<0.0002, ****p<0.0001

Significant induction of IFN-γ was observed in the 120-hour PBMC supernatant of adult participants allergic to GP when exposed to *P. notatum* pollen extract only and co-exposed RV-16 with pollen extract, with respective medians 450.4 pg/ml (Figure 4.3 A1, IQR 156.6-775) and 322.2 pg/ml (A1, IQR 160.2-451.2). No statistical difference between the IFN-γ levels of *P. notatum* GP extract only and co-exposed extract and RV-16 were observed. In both the other allergies and non-atopic group, significant induction of IFN-γ was only observed in PBMC co-exposed with both RV-16 and whole *P. notatum* pollen extract, with median 130.9 pg/ml (Figure 4.3 A2, IQR 58.0-287.9) and 155.5 pg/ml (A3, IQR 41.27-402.8) respectively. When comparing the induction of IFN-γ by co-exposure with RV-16 and whole *P. notatum* pollen extract across

the three groups, no significant difference was observed. No significant induction of IL-5 by RV-16 or whole *P. notatum* pollen extract was observed in all three experimental groups. No significant difference in IL-5 levels was observed following co-exposure with both RV-16 and *P. notatum* pollen extract either. Most of the participants expressed very low levels of IL-5 that were below the assay detection limit.

4.4.5 Limitations of the adult participant cohort

The major limitation of the cohort was the high incidence of asthma in all three experimental groups (Table 4.1). As studies have shown that asthma can diminish the antiviral response, we are unable to distinguish the effect of asthma on the differential induction of IP-10 by RV-16 from that of AR within the grass-pollen allergic and other allergies groups. There was also a low number of non-allergic and other allergies participants (n=8 each) relative to the grass-pollen allergic group (n=15). There was also a lack of serum IgE data for the cohort, with skin prick test being the indicator for allergic sensitivity towards *P. notatum* pollen extract. Serum IgE is a much more specific and sensitive indicator of allergen sensitivity compared to skin prick test (Bignardi et al., 2019). As IgE-linked interactions in the PBMC could be observed in this study, it will provide more immunological context to the experimental findings based on the participant biological specimens.

Thus, in the subsequent study (4.5), recruitment for a new cohort would include asthma status in the stratification process, distinguishing participants with AR alone from those with both AR and asthma as described in 2.10. All recruited participants will also have their serum tested for serum IgE towards a panel of aeroallergens as described in 2.11.

We expected to see an IL-5 response following *P. notatum* exposure of PBMC cultures at 120 hours, however no significant induction was observed (Figure 4.3 A1-3 BaGP). Based on a study by Eusebius and colleagues that examined IL-5 response induced by Cyn d 1 in purified T cell cultures, there was a marked IL-5 response observed at 72 hours (N. Eusebius et al., 2002). As we

assayed for late inflammatory cytokines with supernatant that was harvested at 120 hours, it is possible that the timepoint was too late and there could have been degradation of the cytokine. It may also be possible that in our cultures, there were too few allergen-specific precursor T cells, to detect an adaptive cellular response to allergen at this time point.

4.4.6 Summary of results for adult cohort

The results of the adult cohort show that whole *P. notatum* pollen extract inhibits the production of the innate antiviral mediator IP-10 produced in response to RV-16 in PBMC cultures, suggesting that components of grass pollen allergen extract may inhibit rhinovirus-induced antiviral response at the innate level. This inhibition was observed in both allergic and non-allergic participant groups, which suggests that this inhibition could be driven by a non-IgE driven mechanism. No changes in the induction of innate inflammatory cytokines IL-6 and TNF-α were observed following co-exposure with RV-16, which suggests that human rhinovirus does not affect the innate inflammatory response induced by grass pollen.

4.5 RESULTS: INNATE ANTIVIRAL RESPONSE INDUCED BY RV-16 FOLLOWING CO-EXPOSURE WITH EITHER WHOLE *PASPALUM NOTATUM* POLLEN EXTRACT OR ITS PURIFIED MAJOR ALLERGEN PAS N 1 IN PBMC OF ADOLESCENTS WITH OR WITHOUT ALLERGIC RHINITIS OR ASTHMA.

4.5.1 Adolescent participant demographics

Table 4.2 Adolescent participant demographics based on recruitment sites.

Recruitment sites	**QPIAS**	**ENT**	**Statistical differences**
Number of participants	26	17*	-
Age (SD)	15 (1.65)	14.7 (0.44)	n.s.
Weight, kg (SD)	59.9 (10.98)	62.5 (16.45)	n.s.
Height, cm (SD)	166.7 (9.91)	170.8 (9.89)	n.s.
BMI (SD)	21.4 (3.10)	21.5 (4.69)	n.s.
Number of females (%)	12 (44.4)	2 (21.4)	n.s.
Number of Caucasian (%)	23 (85.2)	9 (50)	n.s.
Number living in QLD (%)	27 (100)	18 (100)	n.s.
Number born in QLD (%)	22 (81.5)	15 (83.3)	n.s.
Number that spend >20 hours outside per week (%)	8 (29.6)	3 (16.7)	n.s.
Number with parent/guardian that smokes (%)	2 (7.4)	7 (38.9)	n.s.
Number with rhinitis (%)	25 (92.6)	14 (77.8)	n.s.
Number with current asthma (%)	17 (63)	13 (72.2)	n.s.
Number previously hospitalized for asthma (%)	12 (44.4)	4 (22.2)	n.s.
Number with food allergy (%)	23 (88.5)	0 (0)	****

*note that 1 patient in ENT was previously recruited from QPIAS

A total of 45 participants were recruited from both locations within Queensland Children's Hospital (QCH) as part of the Pollen Allergy and Asthma (PAAS) study to date, with 27 being from the Queensland Paediatric Immunology and Allergy Service (QPIAS) and 18 from Ear Nose and Throat (ENT) departments, with one patient recruited first from QPIAS and later ENT. No significant differences were observed for age, weight, height and body mass index (BMI) between both groups (Figure 4.2). All participants recruited are currently living in QLD, only two participants born outside of Australia (one in New Zealand from QPIAS and another from Russia from ENT). Participants in

neither group have reported smoking. QPIAS had a higher percentage of food allergy (88.5%) compared to ENT (0%) respectively.

Table 4.3 Adolescent participant demographics based on experimental groups, allergic rhinitis with asthma (AR+A), allergic rhinitis only (AR) and no rhinitis (NR)

Experimental group	AR+A	AR	NR	Statistical differences
Number of participants	19*	17	6	
Age (SEM)	15.2 (0.37)	14.4 (0.49)	14.8 (0.40)	n.s.
BMI (SEM)	22 (1.0)	21.3 (0.71)	19.9 (1.99)	n.s.
Number of females (%)	8 (38.0)	5 (29)	2 (33)	n.s.
Number of Caucasians (%)	16 (71.4)	11 (64.7)	5 (83.3)	n.s.
Number born in QLD (%)	18 (86.0)	12 (71.0)	6 (100)	n.s.
Number with food allergy (%)	13 (61.9)	7 (41.2)	3 (50.0)	n.s.
Number severe rhinitis (%)	9 (42.9)	4 (23.5)	NA	n.s.
Number severe asthma (%)	3 (14.3)	NA	NA	-
Number hospitalized for asthma (%)	16 (76.2)	NA	NA	-

*number of participants in AR+A appears as one less as one participant was recruited twice (once from QPIAS then later in ENT)

Participants were stratified into the 2 case groups; AR with asthma, AR only and a third control group if they did not present with rhinitis (no rhinitis, NR), which are either non-atopic or presenting with food allergy only. Group 3 had the lowest number of participants with 6, while groups 1 and 2 had similar number of participants (19 and 17) respectively. No significant differences were observed for age, BMI, ethnicity and proportion born in QLD between groups.

4.5.2 Adolescent participant serum IgE data

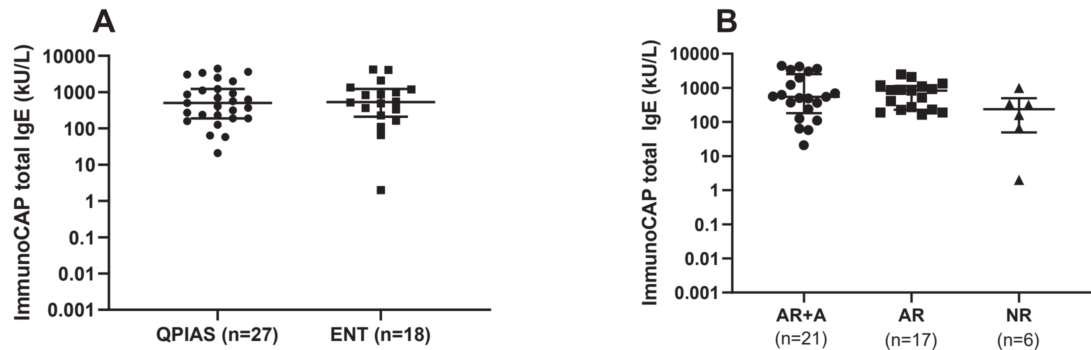


Figure 4.4 Total serum IgE data for adolescent participants according to A) recruitment sites and B) experimental groups; allergic rhinitis and asthma (AR+A), allergic rhinitis only (AR) and no rhinitis group (NR), as tested by ImmunoCAP (ThermoFisher Scientific, Sweden). Data expressed as individual points overlaid with median and interquartile range.

No significant differences in total serum IgE was observed between participants recruited from QPIAS (Figure 4.4 A QPIAS; median 501.0 kU/L, IQR 190.0-1222.0) and ENT (A ENT; median 531.5 kU/L, IQR 211.0-1229). As there were no differences in demographics and serum total IgE levels, participants from both recruitment sites can be merged into a single cohort before stratification into experimental groups based on AR and asthma status. When stratified into the experimental groups, no significant differences were observed in serum total IgE between the AR and asthma (Figure 4.4 B AR+A, median 549.0, IQR 182.0-2528.0), AR only (B AR, median 823.0, IQR 229.5-1148.0) and no rhinitis groups (B NR, median 236.5.0, IQR 49.3-495.5).

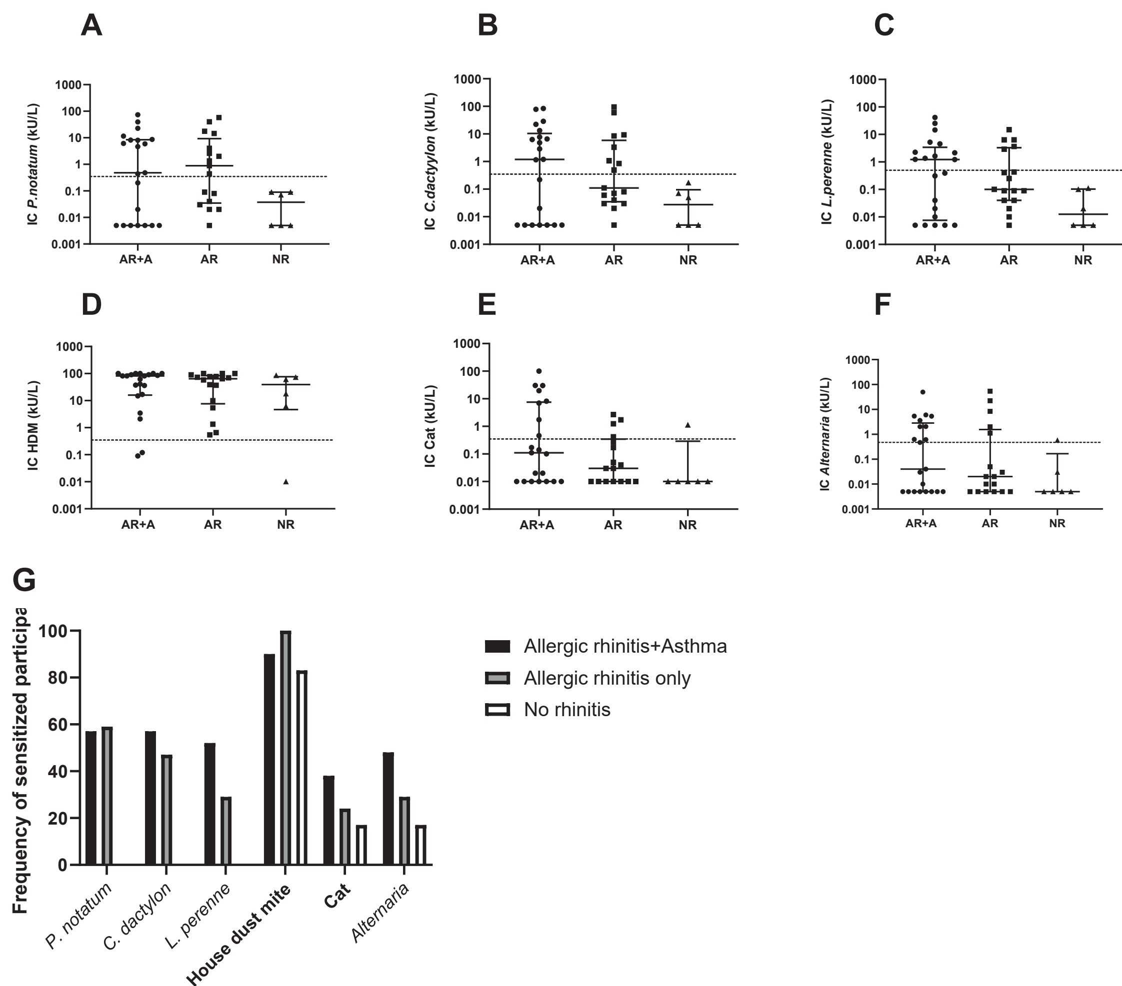


Figure 4.5 Participant serum specific IgE reactivity to aeroallergens tested aeroallergens pollen extracts of grasses A) *P. notatum,* B) *C. dactylon,* C) *L. perenne*, D) house dust mite, E) cat dander and F) *Alternaria* fungal spores as tested by ImmunoCAP, (ThermoFisher Scientific, Sweden) , according to experimental groups; allergic rhinitis and asthma (AR+A), allergic rhinitis only (AR) and no rhinitis group (NR). Serum IgE readings expressed on a logarithmic scale. Line at 0.35 kU/L represents serum IgE level that indicates sensitization to allergen. Data expressed as individual points overlaid with median and interquartile range. G) Relative frequency (expressed as %) of participants with sensitization to aeroallergens (serum specific IgE > 0.35Ku/L) within experimental groups.

No significant differences were observed between total serum IgE levels (Figure 4.5) between the allergic rhinitis and asthma (AR+A), allergic rhinitis only (AR) and no rhinitis (NR) groups for the tested aeroallergens *P. notatum* pollen (A), *C. dactylon* pollen (B), *L. perenne* pollen (C), HDM (D), cat dander (E) and *Alternaria* (F). The median serum IgE towards *P. notatum* pollen for the allergic rhinitis and asthma (A AR+A, median 0.48 kU/L, IQR 0.005-8.4) and allergic rhinitis only (A AR, median 0.89 kU/L, IQR 0.035-9.3) were above the sensitization level of 0.35 kU/L while that of the no rhinitis group (A NR, median 0.038 kU/L, IQR 0.005-0.009). Furthermore, the number of participants with

sensitization to *P. notatum* extract (above 0.35 kU/L) for the allergic rhinitis and asthma group (12 out of 19 participants) and allergic rhinitis only group (9 out of 16 participants) were higher than the no rhinitis groups (0 out of 16 participants). Compared to the other grass pollen extracts, the median serum IgE levels for only the allergic rhinitis and asthma groups for *C. dactylon* (B AR+A, median 1.19 kU/L, IQR 0.005-10.5) and *L. perenne* pollen (C AR+A, median 1.22 kU/L, IQR 0.0075-3.4) were above the sensitization level of 0.35 kU/L. Thus, it appears that a larger proportion of the cohort is sensitized towards *P. notatum* compared *C. dactylon* and *L. perenne*. The median serum IgE levels for all aeroallergens except HDM (Figure 4.4 in the no rhinitis group (NR) was lower than the allergic rhinitis and asthma (AR+A) and allergic rhinitis only (AR) groups but no significance was achieved possibly due to the lower participant number (n=6) in the no rhinitis groups compared to the other two (n=19 and 17 respectively). The median serum IgE levels for HDM in all three groups were high, the highest in allergic rhinitis and asthma (D AR+A, median 81.6 kU/L, IQR 15.9-95.3), followed by allergic rhinitis only (D AR, median 64.4, IQR 7.7-83.7) and no rhinitis (D NR, median 39.25 kU/L, IQR 4.6-76.4). Only 2 participants in the allergic rhinitis and asthma group and one participant from the no rhinitis group showed no sensitization (serum IgE < 0.35 kU/L) towards HDM. All median serum IgE levels towards cat dander (E) and *Alternaria* spores (F) for the three experimental groups were below sensitization level. There appears to be a high frequency of sensitization towards HDM (Figure 4.5 G) in all three experimental groups (above 80%). The second most recognized aeroallergens are the pollen of subtropical grasses *P. notatum* and *C. dactylon*, followed by the temperate grass *L. perenne*. These findings correspond to the conclusion made in Chapter 3 where patients with grass pollen allergy have higher IgE reactivity towards pollen of grasses that are abundant in their region and climate of origin.

4.5.3 1Selection of purified allergen Pas n 1 experimental concentration and pilot study

A pilot study utilizing the methodology described in 2.8 was performed with test conditions expanded to include purified Pas n 1 at three concentrations 1, 3, 5 and 10 µg/ml. This was to ascertain if the major allergen showed a similar capacity to inhibit RV-16 as in the whole grass pollen extract, and if so, how it is affected by dose.

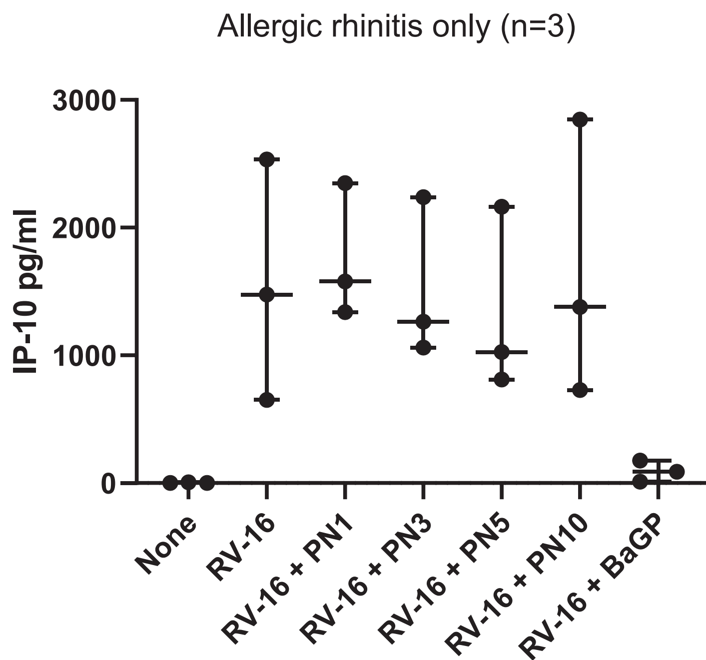


Figure 4.6 IP-10 production by PBMC of adolescent participants with allergic rhinitis and asthma following exposure with RV-16 alone, RV-16 co-exposed with purified *P. notatum* allergen (PN) at 1,3,5 or 10 µg/ml, RV-16 and *P. notatum* whole *P. notatum* pollen extract (PNGP). Data expressed as median with interquartile range.

Induction of IP-10 was observed following exposure of participants PBMC to RV-16 (Figure 4.6 RV-16). As observed in the adult participant study (Figure 4.1 A1), inhibition of IP-10 production was observed following co-exposure of PBMC with RV-16 and *P. notatum* pollen extract and allergen (Figure 4.6 RV-16+BaGP). The inhibition induced by the purified allergen appears weaker compared to the whole extract across all concentrations. Furthermore, the inhibition induced by the purified allergen appears to be a dose-dependent response as there was a trend of stronger inhibition of IP-10 as Pas n 1 concentration increased, with a change in trend observed at 10 µg/ml.

However, the differences in IP-10 inhibition between the Pas n 1 concentrations were not statistically different.

A study on the mass quantification of major allergens in whole pollen extract by sandwich ELISA show that group 1 allergens comprise of approximately 5 to 10% of whole pollen extract, as exemplified by 1.25 µg/ml of *C. dactylon* extract containing less than 1 µg/mg of Cyn d 1 (Arilla et al., 2001). With that as a guideline, the 30 µg/ml of whole *P. notatum* extract would be expected to have between 1 to 3 µg/ml of Pas n 1. Thus, 3 µg/ml was selected as the experimental concentration of Pas n 1 as it was shown to have the highest possible inhibitory activity of IP-10 while realistically being within the range of expected composition within a whole pollen extract.

4.5.4 Inhibition of antiviral mediators IP-10 and IFN-α2 by whole P. notatum extract but not purified major allergen in participants with allergic rhinitis and asthma and no rhinitis

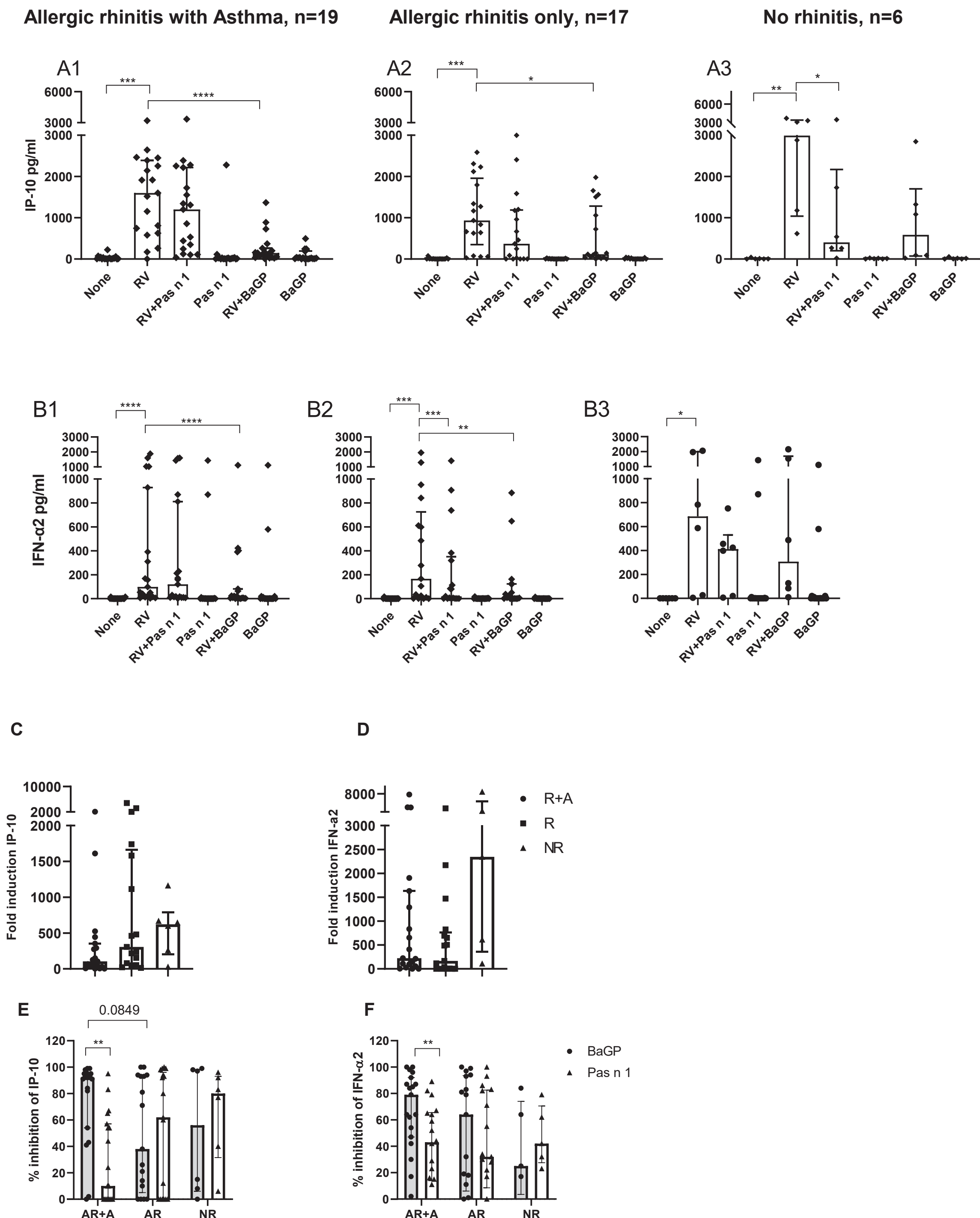


Figure 4.7 Concentration of antiviral mediators IP-10 (A1-3) and IFN-α2 (B1-3) expressed by PAAS participant PBMC when cultured for 24 hours with human rhinovirus-16 (RV; MOI 1) and either purified Pas n 1 (3 µg/ml) or whole *P. notatum* pollen extract (BaGP; 30 µg/ml). Fold induction of IP-10 (C) and IFN-α2 (D) by RV-16 treated PBMC and percentage of inhibition of RV-16 induced IP-10 (D) and IFN-α2 (E) by whole *P. notatum* extract and Pas n 1 in adolescent participants with allergic rhinitis and asthma (AR+A), allergic rhinitis only (AR) and no rhinitis (NR). Data presented as individual points, overlayed with median and interquartile range. P-values are denoted as $p<0.0332$, ** $p<0.0021$, *** $p<0.0002$, ****$p<0.0001$

A significant induction of IP-10 by RV-16 was observed in 24-hour PBMC cultures of PAAS participants in all three participants groups (Figure 4.7). The highest median IP-10 induction was observed for the no rhinitis group (A3 RV; median 2991 pg/ml, IQR 1037-3424), followed by allergic rhinitis and asthma (A1 RV; median 1599 pg/ml, IQR 626.5-2389) and allergic rhinitis only group (A2 RV; median 933 pg/ml, IQR 351.1-1959.0). Within each group, there appeared to be participants with lower induction of IP-10 by RV-16 than others, resulting in the high interquartile ranges observed. In the allergic rhinitis and asthma group (A1 RV), 11 participants had IP-10 concentration above 1500 pg/ml and 8 had less than 1500 pg/ml (IQR 626.5-2389). Similarly in the allergic rhinitis only group (A2 RV), there were 5 participants with IP-10 concentrations above 1500 pg/ml and 11 had less than 1500 pg/ml (IQR 351.1-1959.0). Significant induction of IFN-α2 by RV-16 was observed in all three participant groups (Figure 4.7 B1-3 RV) with the highest median observed in the no rhinitis group (B3; median 685.1 pg/ml, IQR 20.8-1993), similar to the observation in IP-10. This was followed by allergic rhinitis only group (B2; median 166.5 pg/ml, IQR 6.0-726.3) and allergic rhinitis and asthma group (B1; median 98.3 pg/ml,IQR 14.7-928.5). There also appears to be large variations in the induction of IFN-α2, as observed in the allergic rhinitis and asthma group (B1) with 4 participants above 1000 pg/ml and the remaining 14 below 400 pg/ml (IQR 14.7-928.5). No significant differences in the fold induction of IP-10 (Figure 4.7 C) and IFN-α2 (Figure 4.7 D) between experimental groups were observed, despite the median for the no rhinitis groups were higher than the other two groups for both IP-10 (C NR median 623.1, IQR 203.0-793.2) and IFN-α2 (D NR median 2347, IQR 359.9-5918).

After co-exposure with whole *P. notatum* extract, there was significantly reduced IP-10 concentration (Figure 4.7) in the allergic rhinitis and asthma group (A1 RV+BaGP; median 145.8 pg/ml, IQR 43.3-260.4) and allergic rhinitis only group (A2 RV+BaGP; median 117.3 pg/ml, IQR 41.1-1282.0). No significant difference was observed in the no rhinitis group (A3 RV+BaGP). Significantly lower IFN-α2 concentration by RV-16 co-exposed with whole extract was observed in the allergic rhinitis with asthma group (B1 RV+BaGP; median 8.6 pg/ml, IQR 78.4) and allergic rhinitis only group (B2 RV+BaGP; median 11.3

and IQR 121.5. No significantly lower IFN-α2 induction was observed in the no rhinitis group (B3 RV+BaGP). Following co-exposure with purified Pas n 1, there was significantly reduced IP-10 induction by RV-16 observed in the no rhinitis group (A3 RV+Pas n 1; median 404.0 pg/ml, IQR 201.0-2170). No significantly lower concentration of IP-10 by RV-16 co-exposed with Pas n 1 observed for the other two groups. Significantly lower IFN-α2 concentration by RV-16 co-exposed with Pas n 1 was only observed in the allergic rhinitis only group (B2 RV+Pas n 1; median 20.2 pg/ml, IQR 1.8-350.7).

To examine the influence of asthma status on innate antiviral mediators, comparisons of both the fold induction of innate antiviral mediators and subsequent inhibition by whole *P. notatum* pollen extract and Pas n 1 were made. No significant differences in the induction of both IP-10 and IFN-α2 by RV-16 was observed between the AR and asthma and AR only groups (Figure 4.7 C and D; R+A and R). The AR and asthma group appeared to have a stronger inhibition of IP-10 by whole *P. notatum* pollen extract (Figure 4.7 E AR+A BaGP; median 92%, IQR 54.0-95.0) compared to AR only (Figure 4.7 E; AR BaGP; median 38%, IQR 5.0-93.5), though it was not statistically significant (p=0.0849). This trend appeared to be reversed for inhibition by Pas n 1, with the AR and asthma group having a lower inhibition (Figure 4.7 E; AR+A Pas n 1; median 10%, IQR 0.0-57.0) compared to AR only (Figure 4.7 E; AR+A Pas n 1; median 62%, IQR 0.0-96.0) while not statistically significant (p=0.7885). No significant difference or trends were observed for the inhibition of IFN-α2 by either whole *P. notatum* pollen extract or Pas n 1 (Figure 4.7, D). However, it appears that asthma status influenced the inhibitory capacity of whole *P. notatum* pollen extract compared with purified allergen alone on RV-16 induced IP-10 and IFN-α2 (Figure 4.7, E and F), an effect not observed in the AR only or no rhinitis groups.

To determine if there was a difference in immune interactions induced by allergen source, comparisons were made between the percentage of inhibition of the innate antiviral mediators IP-10 and IFN-α2 by either whole *P. notatum* extract and purified Pas n 1 (Figure 4.7 E, F). The percentage of inhibition of RV-16 induced IP-10 by *P. notatum* pollen extract (E AR+A BaGP; median 93%, IQR 86.7-93.4) was significantly higher compared to Pas n 1 in the allergic

rhinitis and asthma group (E AR+A Pas n 1; median 80.8%, IQR 51.2-93.0). Similarly, the percentage of inhibition of RV-16 induced IFN-α2 by whole *P. notatum* extract (F AR+A BaGP, median 79%, IQR 47.0-92.0) was higher than that by Pas n 1 (F AR+A Pas n 1, median 43%, IQR 14.0-65.5). No significant differences in percentage of inhibition of IP-10 between whole extract and purified allergen in the allergic rhinitis only (E AR) and no rhinitis groups (E NR). No significant differences were observed between percentage of inhibitions of IFN-α2 between whole *P. notatum* extract and purified Pas n 1 in the allergic rhinitis (E,F AR) only and no rhinitis (E,F NR) groups. However, when examining the individual participants within these groups, there were varying proportions of participants that did not exhibit an inhibition of IP-10 or IFN-α2. For IP-10, 2 out of the 19 participants in the allergic rhinitis and asthma group did not exhibit inhibition by *P. notatum* and 7 did not for Pas n 1. Similarly, 4 participants in the allergic rhinitis only group did not exhibit inhibition of IP-10 by whole extract and 6 did not for Pas n 1. Finally, 3 participants did not exhibit inhibition of IP-10 by whole extract, and all did for Pas n 1.

4.5.5 Induction of inflammatory mediators IL-6, TNF-α and GM-CSF by P. notatum extract but not Pas n 1

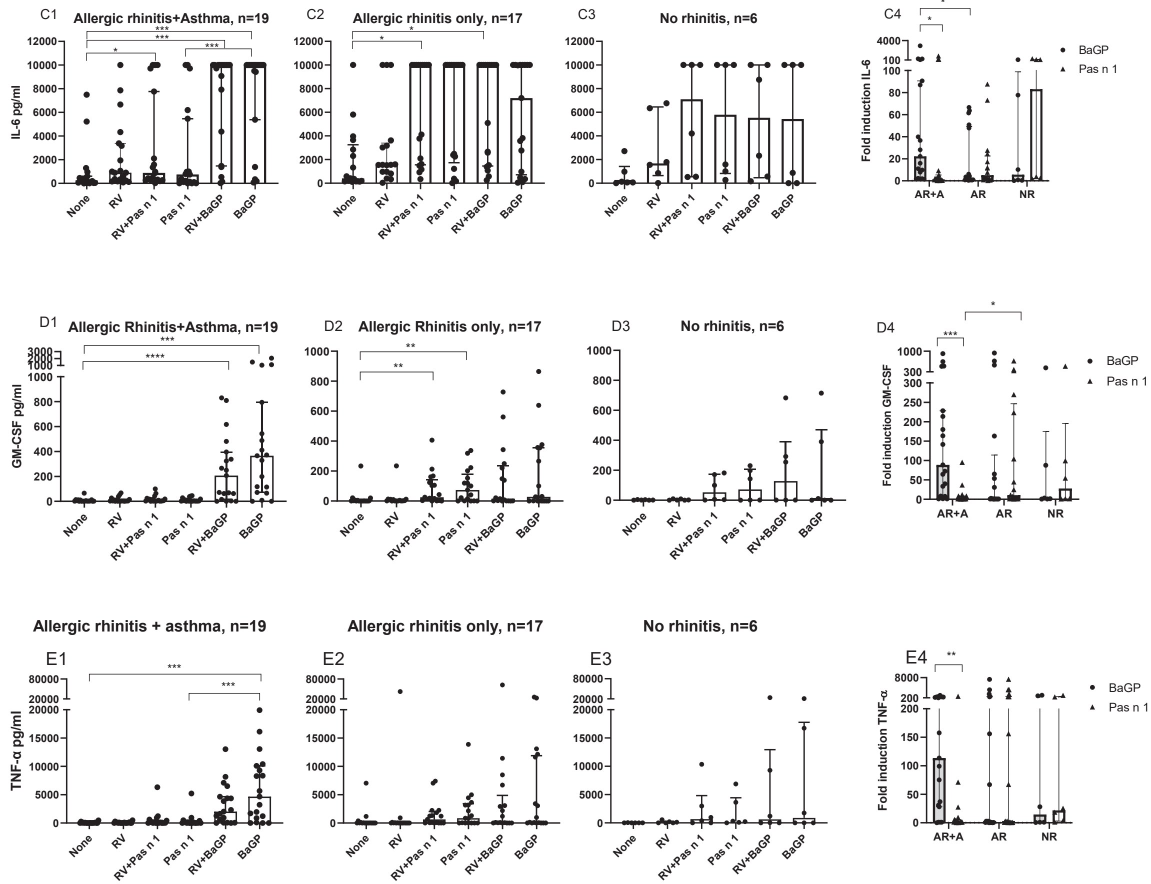


Figure 4.8 Concentration of inflammatory mediators IL-6 (C1-C3), GM-CSF (D1-D3) and TNF-α (E1-E3) as expressed by PAAS participant PBMC when cultured for 24 hours with human rhinovirus-16 (RV, MOI=1) and either purified Pas n 1 (3 µg/ml) or whole *P. notatum* extract (BaGP, 30 µg/ml). Fold induction of inflammatory mediators IL-6 (C4), GM-CSF (D4) and TNF-α (E4) over non treated in adolescent participants with allergic rhinitis and asthma (AR+A), allergic rhinitis only (AR) and no rhinitis (NR). Data presented as individual points, overlayed with median an interquartile range. P-values are denoted as p<0.0332, ** p<0.0021, *** p<0.0002, ****p<0.0001

Significant induction of IL-6 induced by whole *P. notatum* pollen extract observed in the allergic rhinitis with asthma group (Figure 4.8 C1 BaGP; median 10000 pg/ml, IQR 5386-10000), but not in the allergic rhinitis only or no rhinitis groups. When co-exposed with both RV-16 and whole *P. notatum* pollen extract, there was significant induction of IL-6 (C1 RV+BaGP, median 10000 pg/ml, IQR 1473-10000), but this did not differ significantly from IL-6 levels induced by whole *P. notatum* pollen extract only. No significant induction of IL-6 by Pas n 1 and whole *P. notatum* extract was observed in all three groups, though induction by Pas n 1 in the allergic rhinitis only group (C2 Pas n 1 median 10000,

IQR 1552-10000) was close to significance. However, significant induction of IL-6 in the allergic rhinitis only group was observed with co-exposure of RV-16 with Pas n 1 (C2 RV+Pas n 1; median 886.9 pg/m, IQR 287.5-7765) and *P. notatum* pollen extract (C2 RV+BaGP; median 10000, IQR 1473-10000). The fold induction of IL-6 by whole *P. notatum* pollen extract in the allergic rhinitis and asthma group (C4 BaGP, AR+A) was higher than that observed in the allergic rhinitis only group (C4 BaGP, AR). The fold induction of IL-6 in the allergic rhinitis and asthma by whole *P. notatum* extract (C4 AR+A BaGP; median 22.2, IQR 2.3-90.6) was significantly higher than by Pas n 1 (C4 AR+A Pas n 1; median 1.3, IQR 0.3-5.4). A large proportion of the IL-6 concentration data, especially following induction by whole *P. notatum* extract was above the assay detection limit, which was 10 000 pg/ml. As such, there is a loss of resolution at the high end, resulting in comparisons of induction levels between experimental groups and allergen sources unreliable.

Significant induction of GM-CSF by whole *P. notatum* pollen extract was observed only in the allergic rhinitis and asthma group (Figure 4.8 D1; median 365.8 pg/ml IQR 72.0-794.2). When whole *P. notatum* pollen extract was co-exposed with RV-16, there was a significantly lowered induction of GM-CSF (D1; median 206.9 pg/ml, IQR 15.4-396.4). Significant induction of GM-CSF was observed by Pas n 1 in the allergic rhinitis only group (D2; median 73.7, IQR 1.4-177.7). This induction of GM-CSF was not significantly different following co-exposure of Pas n 1 with RV-16 (D2; median 25.5, IQR 3.1-142.2). The fold induction of GM-CSF by Pas n 1 in the allergic rhinitis only group (D4 AR Pas n 1; median 10.7, IQR 1.1-246.5) was higher than that of the allergic rhinitis and asthma group (D4 AR+A Pas n 1; median 1.0, IQR 0.7-8.0). No differences between the fold induction of GM-CSF by whole *P. notatum* pollen extract was observed between the allergic rhinitis and asthma (D4 AR+A BaGP) group and allergic rhinitis only group (D4 AR BaGP).The fold induction of GM-CSF in the allergic rhinitis and asthma group by whole *P. notatum* pollen extract (D4 AR+A BaGP; median 88.7, IQR 8.2-228.2) was higher than Pas n 1 (D4 AR+A Pas n 1; median 1.0, IQR 0.7-8), and no significant differences between whole *P. notatum* pollen extract and Pas n 1 was observed in the other groups.

Significant induction of TNF-α by whole *P. notatum* pollen extract was observed only in the allergic rhinitis and asthma group (Figure 4.8 E1 BaGP; median 4666 pg/ml IQR 876.8-10092). No significant induction of TNF-α was observed for the allergic rhinitis only and no rhinitis groups (E2, E3). When examining TNF-α induction by Pas n 1 (E4) and whole *P. notatum* pollen extract (E5) across all three experimental groups, no significant differences was observed. No significant differences were observed between experimental groups in fold induction of TNF-α by whole *P. notatum* pollen extract (D4 BaGP) and Pas n 1 (D4 Pas n 1). The fold induction of TNF-α by whole *P. notatum* (D4 AR+A BaGP; median 28.1, IQR 28.1-113.6) was significantly higher than Pas n 1 (D4 AR+A Pas n 1; median 1.0, IQR 1.0-5.5).

To examine the influence of asthma status on innate inflammatory mediators, comparisons were made between the fold induction of IL-6, GM-CSF and TNF-α by both whole *P. notatum* pollen extract and Pas n 1. Fold induction of IL-6 by whole *P. notatum* pollen extract was significantly higher in the allergic rhinitis and asthma group (Figure 4.8 C4 AR+A BaGP; median 22.2, IQR 2.3-90.6) compared to allergic rhinitis only group (Figure 4.8 C4; AR BaGP, median 1.3, IQR 0.9-49.3). This trend was observed for GM-CSF and TNF-α (D4 and E4, Figure 4.8). Conversely for Pas n 1, there was significantly higher fold induction of GM-CSF in the allergic rhinitis only group (Figure 4.8 D4 AR Pas n 1, median 10.7, IQR 1.1-246.5) compared to allergic rhinitis and asthma group (Figure 4.8 D4 AR+A Pas n 1, median 1.0, IQR 0.7-8.0). Notably, for those with asthma and AR, the fold induction of IL-6, GM-CSF and TNF-α by whole pollen extract was higher than by the purified allergen component, this difference was not apparent in those with AR or no rhinitis (C4, D4, E4, Figure 4.8).

4.5.6 Summary of results for adolescent cohort

To summarize the findings of the adolescent cohort, the innate inflammatory mediators (Figure 4.8) IL-6 (C1), TNF-α (E1) and GM-CSF (D1) were induced significantly in the allergic rhinitis only group by whole *P. notatum* pollen extract, but not in the allergic rhinitis only or no rhinitis groups. These findings corroborate the findings in the grass pollen allergic adult cohort (Figure

4.2), where IL-6 (A1) and TNF-α (B1) were induced by whole *P. notatum* pollen extract. When whole *P. notatum* pollen extract was co-exposed with RV-16 in both these cohorts, no significant differences in innate inflammatory mediators were observed. These suggest that whole *P. notatum* pollen extract has the capacity to induce an innate inflammatory response, and that this effect could be influenced by asthma status as in the PAAS cohort, no significant induction was observed in the two groups with no asthma participants (allergic rhinitis only and no rhinitis groups).

The effect of asthma status is highlighted by a trend (p=0.0849) of stronger inhibition of IP-10 induced by whole *P. notatum* pollen extract (Figure 4.7 E) and significantly lower induction of GM-CSF by purified allergen Pas n 1 (Figure 4.8 D4), though these patterns were not consistent across the other innate antiviral or inflammatory mediators, respectively. Thus, there appears to an influence of asthma on the immune response towards *P. notatum* allergen source, with differing effect on innate inflammatory mediator induction between whole extract and Pas n 1, as well as subsequent inhibition of innate antiviral mediators by whole extract only. However, the findings of the study were limited due to the failure to achieve the recruitment target as determined by power calculations, and particularly by the low number of non-atopic participants recruited. Thus, it was difficult to determine conclusively the influence of asthma.

4.5.7 Alternate analysis of adolescent cohort data independent of allergic rhinitis and asthma status

There appeared to be large variations in the induction of antiviral mediators by RV-16 in the PBMC of the adolescent cohort across the three experimental groups as reflected in IP-10 (Figure 4.7 A1-A3) and IFN-α2 (B1-B3). Thus, there appears to be differences in immune response between adolescent participants that is not related to allergic rhinitis and asthma status. To investigate the difference in antiviral response, fold induction of IP-10 and IFN-α2 were examined as continuous variables independent of disease status to determine if there are correlations with the fold induction of other cytokines, serum IgE or gender differences.

Induction of innate antiviral mediators did not show significant correlation with inflammatory mediators

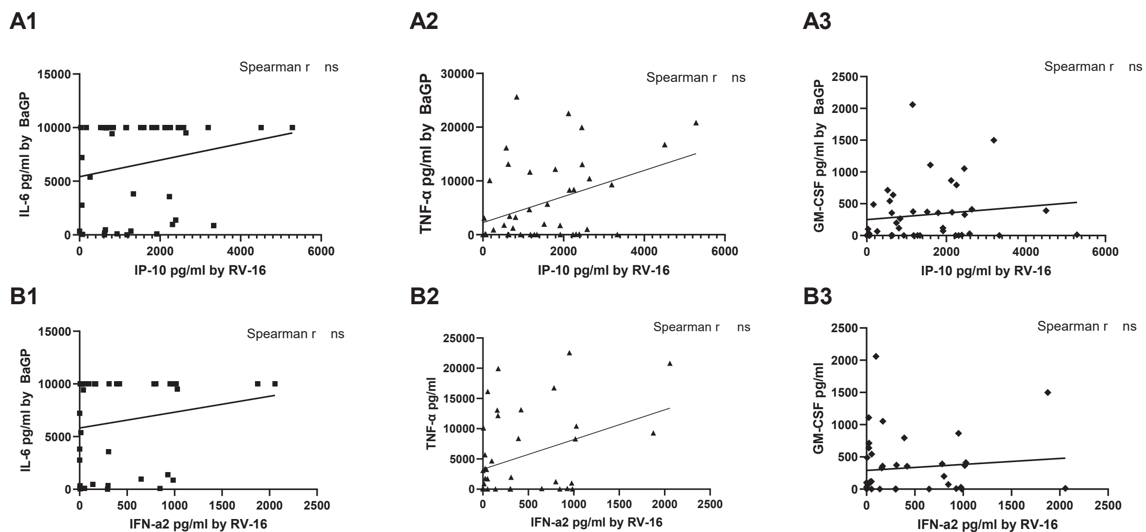


Figure 4.9 Correlation of antiviral mediators IP-10 and IFN-α2 induced by human rhinovirus-16 (RV-16) with innate inflammatory mediators IL-6 (A1, B1), TNF-α (A2, B2) and GM-CSF (A3, B3). Data expressed as individual points overlaid with line of best fit.

IP-10 concentration induced by RV-16 was plot against concentration of inflammatory mediators IL-6, TNF-α and GM-CSF induced by whole *P. notatum* pollen extract and assessed by non-parametric Spearman r correlation (Figure 4.9). No significant correlation was observed between both antiviral mediators and all three inflammatory mediators.

Fold induction of innate antiviral mediators did not show significant correlation with total IgE, serum specific IgE towards house dust mite and Paspalum notatum pollen

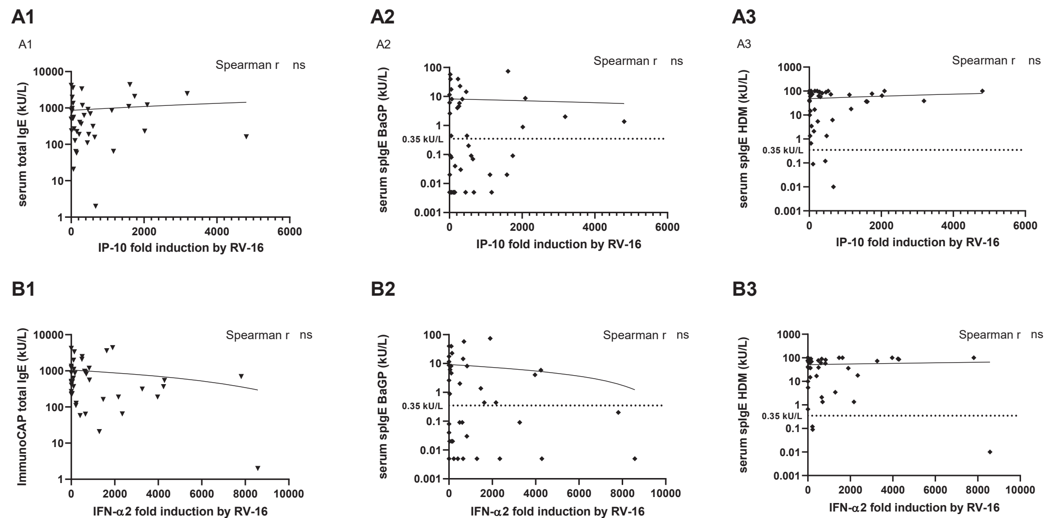


Figure 4.10 Correlation of fold induction of IP-10 (A) and IFN-α2 (B) by human rhinovirus-16 with total IgE (1), serum specific IgE to *P. notatum* pollen (2) and house dust mite (3).

Next, the induction of IP-10 was assessed against allergen sensitization, characterized by total IgE and serum specific IgE towards *P. notatum* pollen (Figure 4.10). When assessed by non-parametric Spearman r correlation, no significant correlation was observed between both antiviral mediators and each of total IgE and specific IgE to *P. notatum* pollen and HDM.

Induction of innate antiviral mediators did not differ between genders

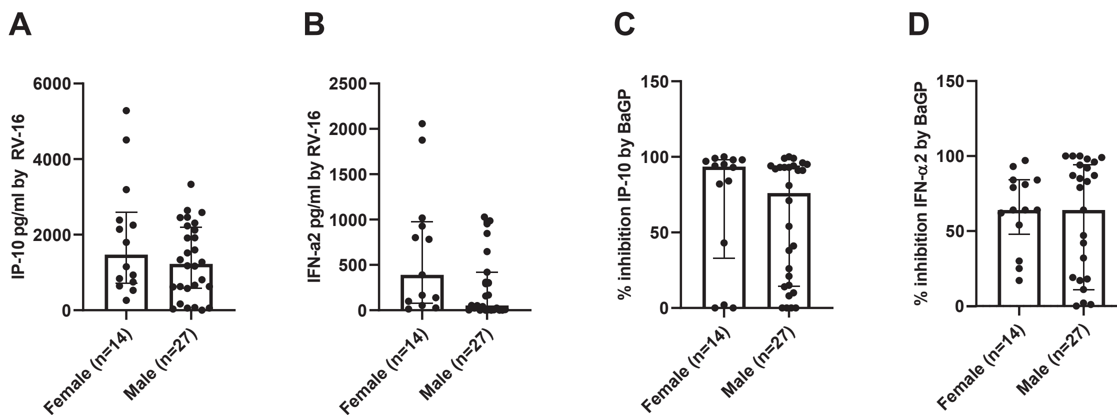


Figure 4.11 Induction of innate antiviral mediators IP-10 (A) and IFN-α2 (B) by human rhinovirus-16 and percentage inhibition of IP-10 (C) and IFN-α2 (D) by whole *P. notatum* pollen extract by gender of adolescent participants. Data expressed as individual points overlaid with median and interquartile range.

When participants were stratified according to gender (Figure 4.11) there was no significant difference between RV-16 induced concentrations IP-10 (A) and IFN-α2 (B) between male and female participants. No significant difference in the percentage of inhibition of RV-16 induced IP-10 (C) and IFN-α2 by whole *P. notatum* pollen extract was observed between genders.

These findings suggest that the large variations in participant antiviral response could neither be explained by their inflammatory response towards *P. notatum* pollen extract nor their sensitization towards it or total IgE as a whole. Sensitization towards HDM also could not explain this difference in antiviral response, despite the much higher levels of sensitization of HDM compared to *P. notatum* pollen. By further examining the PAAS cohort, there was a high incidence of food allergy, which in most cases was the primary reason for presenting to the QPIAS recruitment site. As participant food allergy was not assessed in all participants by Skin Prick Test or serum specific IgE, it remains a confounding variable and, in most cases, could account for a larger proportion of total IgE when compared to specific IgE to *P. notatum* pollen or HDM, which was shown to be much higher than *P. notatum*. As for those presenting to the ENT recruitment site, most participants had other underlying conditions that necessitated the turbinate reduction, not just allergic rhinitis, that were not reported for the purpose of this study.

Significant correlation between inflammatory mediators induced by whole P. notatum extract with percentage of inhibition of IP-10 but not IFN-α2 by extract.

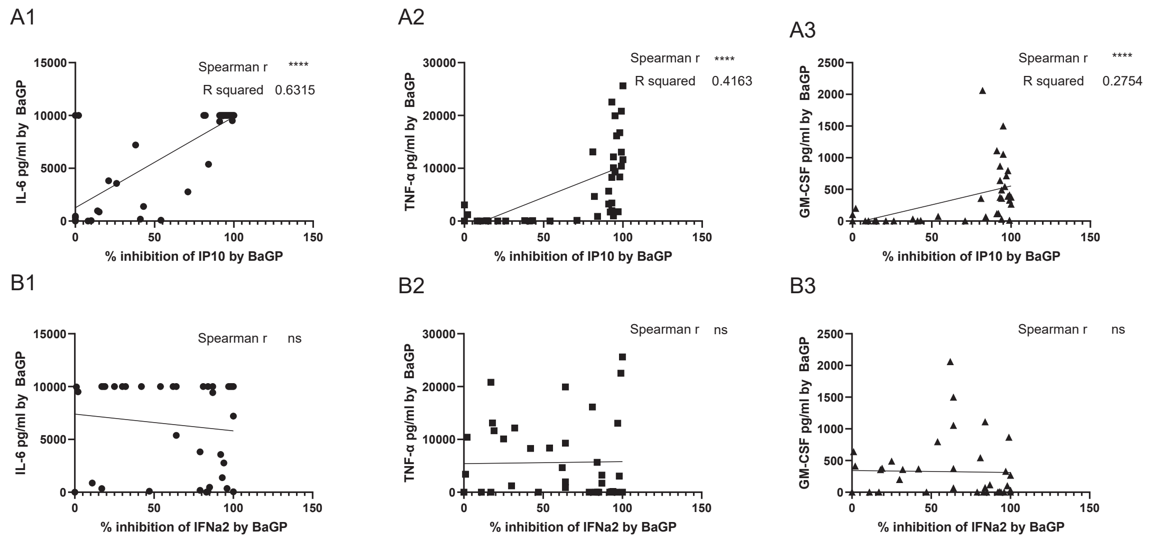


Figure 4.12 Correlation of percentage of inhibition by whole *P. notatum* pollen extract on innate antiviral mediators IP-10 (A) and IFN-α2 (B) induced by human rhinovirus-16 with innate inflammatory mediators IL-6 (A1, B1), TNF-α (A2, B2) and GM-CSF (A3, B3). Data expressed as individual points overlaid with line of best fit.

Significant correlation (Spearman r ****) and simple linear regression ($p<0.0001$) were found between the percentage of inhibition by whole *P. notatum* pollen extract (Figure 4.12) on RV-16 induced IP-10 and all three inflammatory mediators, with TNF-α having the strongest correlation (Spearman rho value 0.82, linear regression R^2 0.42), followed by IL-6 (Spearman rho value 0.72, linear regression R^2 0.63) and GM-CSF (Spearman rho value 0.72, linear regression R^2 0.28). No significant correlation was observed between percentage of inhibition by whole *P. notatum* pollen extract on RV-16 induced IFN-α2 with the innate inflammatory mediators was observed.

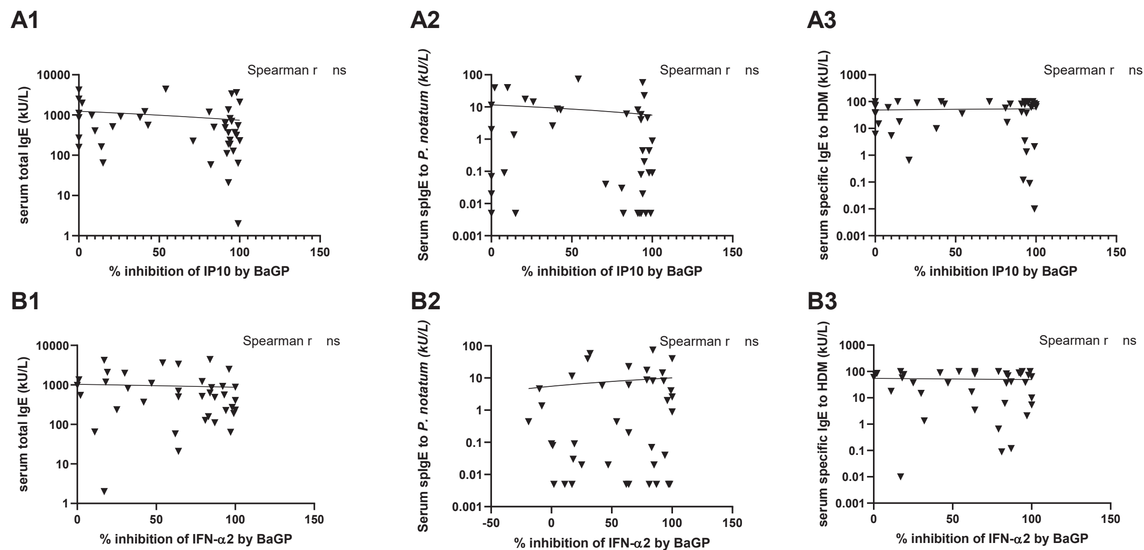


Figure 4.13 Correlation of percentage of inhibition by whole P. notatum pollen extract on innate antiviral mediators IP-10 (A) and IFN-α2 (B) induced by human rhinovirus-16 with serum total IgE (A1, B1), specific IgE to P. notatum pollen (A2, B2) and house dust mite (A3, B3). Data expressed as individual points overlaid with line of best fit.

No significant correlation was observed between the percentage of inhibition by whole *P. notatum* pollen of IP-10 (Figure 4.13 A) and IFN-α2 (B) induced by RV-16 with total IgE (A1, B1), serum specific IgE to *P. notatum* pollen (A2, B2) and serum specific IgE to HDM (A3, B3).

To summarize the findings of the alternative analysis performed, the induction of innate antiviral mediators IP-10 and IFN-α2 by RV-16 did not correlate with the induction of innate inflammatory cytokines IL-6, TNF-α and GM-CSF induced by whole *P. notatum* pollen extract. However, the percentage of inhibition of the innate antiviral mediator IP-10 by whole *P. notatum* pollen extract did correlate with all three inflammatory cytokines induced by whole *P. notatum* pollen extract. This correlation was not observed for IFN-α2. No correlation was observed between the induction or percentage of inhibition of the innate antiviral mediators with serum total IgE, serum specific IgE to *P. notatum* pollen and serum specific IgE to house dust mite. No gender-related differences were observed for the induction or percentage of inhibition of the innate antiviral mediators.

4.5.8 Discussion

The key finding in this chapter is that there was a significant inhibition of the antiviral mediator IP-10 induced by RV-16 by the whole pollen extract of the allergenic grass, *P. notatum*, in the PBMC of both GP-allergic adults (Figure 4.1, A1 RV+BaGP) and adolescents with allergic rhinitis and asthma (A1 RV+BaGP). When examining the adolescent cohort, there was significant inhibition of IP-10 observed in the allergic rhinitis and asthma (Figure 4.7 A1 RV+BaGP) and allergic rhinitis only groups (A2 RV+BaGP). Significant inhibition of IFN-α2 was also observed in the allergic rhinitis and asthma (Figure 4.7 B1 RV+BaGP) and allergic rhinitis only groups (B2 RV+BaGP). These novel findings suggest that the presence of whole GP extract may diminish the innate antiviral response in an individual sensitized towards that GP. Studies to date have alluded to the role of IgE crosslinking on the FCεR1 receptor found on pDC, resulting in diminished IFN-α response towards influenza A in a mixed age population with allergic asthma (3-35 year old, n=56) (Gill et al., 2010) and diminished IFN-α and IFN-λ1 response RV-16 in children with allergic asthma (Durrani et al., 2012). However, in both these studies, anti-IgE was used to crosslink IgE. In this chapter, whole extract of a cohort relevant allergen, as indicated by skin prick test for the adults (Table 4.1 *P. notatum* SPT) and serum specific IgE levels for the adolescents (Figure 4.5 A) was used and elicited a similarly reduced type 1 interferon and antiviral biomarker (IP-10) response. While allergen mediated IgE-cross linking may be the most likely mechanism to explain the inhibition of antiviral response mediated by whole pollen extract based on literature, the findings of this study suggest the present of other non-IgE dependent interactions.

A reduced level of RV-16 induced IP-10 following co-exposure with whole *P. notatum* extract was observed in the non-allergic adults group (Figure 4.1 A3 RV+BaGP), though it was not statistically significant (p = 0.0580) possibly due to the low number of participants in the non-allergic group (n=8). No significant inhibition of RV-16 induced IP-10 and IFN-α2 by whole *P. notatum* extract was observed in the no rhinitis group of the adolescent participants (Figure 4.7 A3, B3 RV+BaGP) although both observed medians were lower than the uninhibited RV-16 test condition (A3, B3 RV).The lack of

statistical significance could be due to the low participant number in the non-allergic adult group relative to the GP-allergic group (n=15) and that in the adolescent no rhinitis group (n=6) relative to the other groups (n=19 and n=17 respectively). It may be that there is a direct effect of GP extract on innate antiviral responses that is independent of IgE. This observation is nonetheless important as it corroborates a recent study that demonstrated the inhibition of the antiviral response by another grass pollen in non-allergic participants, which suggests that this inhibitory action occurs independent of GP allergy status. That study on 3D models of primary nasal epithelial cells (NEC) of non-allergic participants (n=18) across all age groups showed that exposure to aqueous extract of *P. pratense* pollen resulted in diminished IFN-λ (IL-29) production, a type 3 interferon involved in the innate antiviral response (Gilles et al., 2020). This suggests that the interaction of grass pollen extract with the immune response towards RV-16 could be independent of allergen sensitization and the presence of IgE-crosslinking. The alternate analysis of the adolescent cohort (Section 4.9) revealed a significant correlation between the percentage of inhibition of IP-10 by whole *P. notatum* pollen extract with the inflammatory mediators IL-6, TNF-α and GM-CSF (Figure 4.9.4) but not serum total IgE or specific IgE towards *P. notatum* pollen. This finding suggests that the whole *P. notatum* pollen extract interacts with the IP-10 induction pathway through a direct, non-IgE mediated mechanism that is correlated with the inflammatory response.

There was significantly reduced IP-10 and IFN-α2 levels following co-exposure of RV-16 and whole *P. notatum* extract in the allergic rhinitis and asthma group of the adolescent cohort (Figure 4.7 A1, B1 RV+BaGP) but no significant reduction of these antiviral mediators were observed in co-exposure with Pas n 1 (A1, B1 RV+Pas n 1). There was also significantly increased levels of the innate inflammatory mediators IL-6, GM-CSF and TNF-α by whole *P. notatum* extract in the allergic rhinitis and asthma group, but was not evident in the allergic rhinitis only group (Figure 4.8 C1, D1, E1). The fold induction of the innate inflammatory mediators in the allergic rhinitis and asthma group was higher by whole *P. notatum* pollen extract compared to Pas n 1 (Figure 4.8 C4, D4, E4). The percentage of inhibition of RV-16 induced IP-10 and IFN-α2 by

whole *P. notatum* pollen extract (Figure 4.7 E, F AR+A BaGP) was significantly higher than Pas n 1 in the allergic rhinitis and asthma group (E, F AR+A BaGP), but this effect was not observed for the allergic rhinitis only and no rhinitis groups. These findings suggest that whole *P. notatum* extract has a more pronounced effect on the innate immune response compared to Pas n 1 in the allergic rhinitis and asthma group. Studies have shown differences in immune response between whole GP extract and purified major allergen. Roschmann and colleagues examined gene regulation and cytokine induction in immortalized airway epithelial cells induced by both whole *P. pratense* pollen extract and its purified major allergen, Phl p 1. They found that whole GP extract regulated more genes and induced innate cytokines IL-6 and IL-8 more strongly compared to the purified allergen (Röschmann et al., 2011; Röschmann et al., 2012). This chapter corroborates the differential response between whole *P. notatum* pollen extract and Pas n 1 but suggests that it could be linked to both allergic rhinitis and asthma disease states.

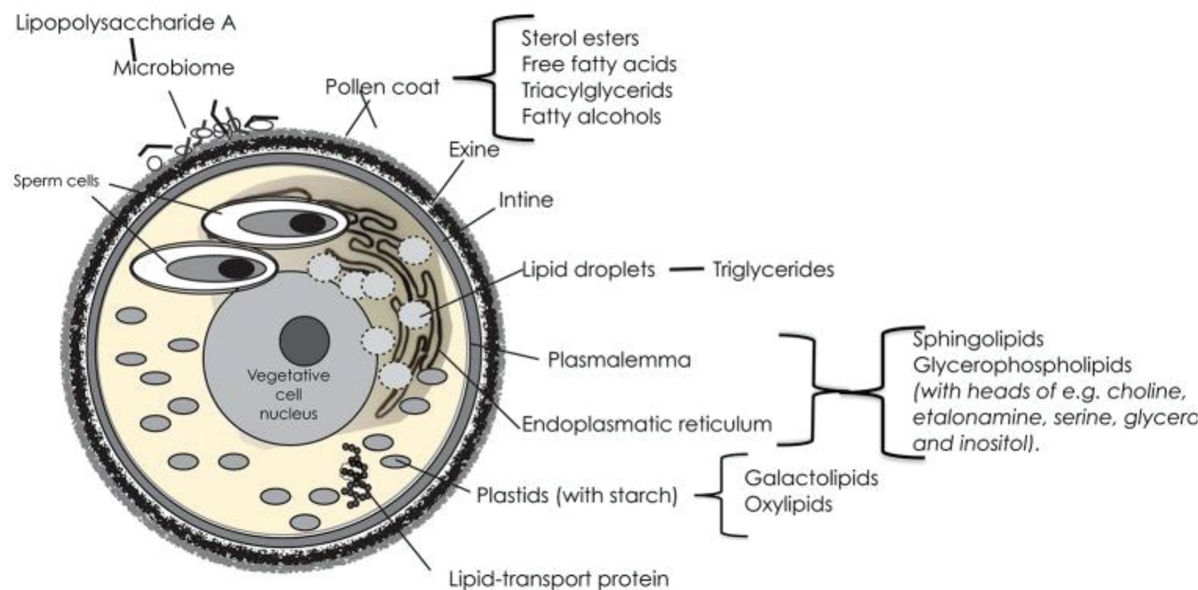


Figure 4.14 Structure and composition of grass pollen. Adapted from (Dahl, 2018)

This difference in immune interactions in PBMC between whole *P. notatum* pollen extract and Pas n 1 could be explained by the difference in allergen source composition. Whole *P. notatum* pollen extract contains more IgE reactive proteins than just the major allergen Pas n 1. Subtropical grass pollen was found to include group 13 allergens and pan-allergens such as profilins and polcalcins, which were found to have serum IgE reactivity in patients with

subtropical grass pollen sensitization (Kailaivasan & Davies, 2018). There are non-IgE reactive mediators within the pollen extract composition that can enhance the IgE-mediated response. A study examining different preparations of *C. dactylon* pollen revealed the presence of novel IgE binding proteins and proteolytic proteins distinct from the major allergen Cyn d 1 on the pollen coat (or extracellular matrix) that can be lost upon defatting and washing with organic solvents (Bashir, Ward, et al., 2013). Further investigation into the pollen coat revealed the presence of lipid mediators, the most abundant of which are saturated fatty acids found in 22 allergenic pollen species which include the subtropical grasses *C. dactylon* and *S. halepense*. These fatty acids found to stimulate dendritic and natural killer cells in a mouse model and induce TNF- α, which could amplify the allergic response towards allergenic proteins (Bashir, Lui, et al., 2013). Another lipid mediator in pollen, E_1-phytoprostanes found *P. pratense*, were also found to modulate dendritic cell function , interact with the airway epithelium and weakly induce inflammatory mediators IL-8, TNF-α and GM-CSF in immortalized bronchial epithelium cells (Gilles et al., 2009). Pollen of grasses including *P. notatum* and *P. pratense* were found to have intrinsic NADPH oxidase activity that interact with the airway epithelium and generates reactive oxygen species (ROS), resulting in the augmentation of antigen-induced allergic airway inflammation (Boldogh et al., 2005). Generation of ROS by pollen was found to modulate dendritic cell function (Csillag et al., 2010; Matsue et al., 2003). Another lipid mediator, phytoprostane E_1, were also found to regulate dendritic cell function to favor Th2 polarization and weakly induce innate inflammatory cytokine GM-CSF and IL-8 in airway epithelial cells (Blume et al., 2015; Gilles et al., 2009).

There appeared to be an asthma driven effect on the immune response towards whole *P. notatum* pollen extract. Significant induction by whole *P. notatum* pollen extract of innate inflammatory mediators IL-6, TNF-α and GM-CSF was observed only in the allergic rhinitis and asthma group (Figure 4.8 C1, D1, E1). Significant inhibition of the innate antiviral response towards RV-16 by whole *P. notatum* pollen extract was observed only in the allergic rhinitis and asthma group (Figure 4.7 E,F; AR+A). Studies have shown differences in innate proinflammatory and antiviral mediator release between asthmatics and healthy

controls. Exposure of RV-stimulated immortalized BECs with supernatant from RV-stimulated PBMC of atopic asthmatics resulted in lower IL-6, IL-8 and RANTES compared to healthy control, suggesting that the atopic environment down-regulates the innate inflammatory response towards viruses (Xatzipsalti et al., 2008). Moskwa and colleagues have shown a heightened IP-10, but lower IFN-λ1 response, towards rhinovirus-1B and parainfluenza virus-3 in BECs of asthmatics (n=10) compared to healthy controls (n=9) (Moskwa et al., 2018). Both these studies show the effect of asthma in modulating the innate immune response towards respiratory viruses. However, the results of this chapter show no significant differences in the induction of IP-10 and IL-6 between the experimental groups in both the adult and adolescent cohorts. Further examination of the asthmatic group in the study by Moskwa revealed a heightened response IFN-λ1 protein and IFN-α and IFN-β mRNA expression in atopic asthmatics compared to non-atopic asthmatics (Moskwa et al., 2018). This study suggests that the innate response towards viruses in asthmatics could be up regulated by atopy, further suggesting that significant differences in the innate antiviral response is dependent upon both allergic rhinitis and asthma disease states. However, this observation could not be conclusively made as no significant differences in induction of innate antiviral mediators was observed between the allergic rhinitis and asthma and allergic rhinitis only experimental groups.

4.5.9 Study limitations

For the adult participant study, the major limitation of the cohort was the high incidence of asthma in all three experimental groups. As studies have shown that asthma can diminish the antiviral response, we are unable to distinguish the effect of asthma on the differential induction of IP-10 by RV-16 from that of allergic rhinitis within the grass-pollen allergic and other allergies groups. There was also a low number of non-allergic and other allergies participants (n=8 each) relative to the grass-pollen allergic group (n=15).

When comparing the adult cohort to the adolescent cohort, a key immunological difference is the primary sensitization, where the adult cohort is primarily sensitized towards *P. notatum* pollen as demonstrated by the high SPT towards pollen extract relative to total SPT (Table 4.1 *P. notatum* SPT, total SPT), while the adolescent cohort was not, as indicated by the low serum specific IgE levels to *P. notatum* pollen (Figure 4.5 A) relative to total IgE (Figure 4.4 B). Despite these immunological differences, we still observed an inhibition of antiviral mediators by whole *P. notatum* pollen extract in the adolescent cohort, suggesting that allergen-specific IgE may not have been essential for the observed effect on response to RV. Analysis of the relationship between total IgE, serum specific IgE towards *P. notatum* pollen or HDM with percentage of inhibition of antiviral mediators by whole *P. notatum* extract also showed no significance. These observations further support that the modulation of the innate antiviral response by whole GP extract occurs is independent of IgE-crosslinking. However, the presence of an IgE-dependent modulation of the innate antiviral response could not be eliminated due to limitations in experimental design. A positive control such as anti-IgE was not included in experimental design to definitively show an effect of IgE-crosslinking on the innate antiviral response and provide comparison for the capacity of whole *P. notatum* pollen extract to crosslink IgE in the PBMC culture. Furthermore, depletion of IgE in the PBMC culture could also be performed to eliminate the presence of IgE and conclusively provide evidence of a non-IgE dependent response.

The adolescent participant recruitment numbers did not fulfil the required sample size numbers indicated by the power calculations. The biggest

deficiencies in participant numbers were observed in the allergic rhinitis and asthma group (n=19 instead of n=41) and no rhinitis group (n=6 instead of n=21). As such, this study was not powered to distinguish the effect of asthma from that of allergic rhinitis within the groups, although there were differences between these two groups observed. This is compounded by the very low number in the no rhinitis group. Recruitment was dependent upon the number of participants presenting to collaborating GP in a week and participant attendance. A major challenge in recruitment was participant fear or unwillingness to have their blood drawn and in two cases, hampered by language barriers.

The adolescent cohort also appears to be primarily sensitized towards HDM, as reflected by the higher serum IgE being much higher than *P. notatum* (Figure 4.5 D vs A). There was also a high incidence of food allergy in the cohort. Most of the participants from the QPIAS recruitment site presented primarily for severe food allergy and only had incidental allergic rhinitis. Initial attempts to recruit participants with food allergy and allergic rhinitis exclusively resulted in very low recruitment numbers in the early phases of the study and thus this criterion was adjusted to make recruitment more practical. Furthermore, the frequency of sensitivity (serum IgE levels above 0.35 kU/L) for HDM were higher compared to *P. notatum pollen* extract. Thus, whole *P. notatum* extract may not be the most suitable allergen in terms of immunological relevance to this cohort.

The adolescent participant cohort is immunologically complex, as reflected in the high variation in the antiviral mediator induction by RV-16 in all three groups. Thus, this study was not able to conclusively determine if the modulation of the innate antiviral response by GP allergens was asthma driven. This variation was not due to the incidence of asthma as high variation in induction of antiviral mediators was also observed in the allergic rhinitis only and no rhinitis groups. This could be due to underlying medical conditions that were not quantitatively measure in this study. This includes severity of food allergy for participants recruited from QPIAS and other underlying medical conditions for participants recruited from ENT that necessitated the turbinate reduction procedure. Furthermore, there was no truly healthy controls for both

cohorts as they were recruited from clinical sites, where they presented for either an allergic disease or asthma.

4.5.10 Conclusions

The results of this chapter show that the induction of the innate antiviral mediator IP-10 was significantly inhibited by whole *P. notatum* pollen in both adults and adolescents with allergic rhinitis. This inhibition of rhinovirus induce IP-10 and IFNa2 did not appear to be solely dependent on serum specific IgE towards *P. notatum* pollen but correlated with the induction of innate pro-inflammatory responses to the pollen extract; IL-6, TNFα, GMCSF and IL-10.

In adolescents, the immune interaction between whole *P. notatum* extract and RV-16 induced antiviral response was more dependent on asthma status than grass pollen allergy status and in participants with asthma and allergic rhinitis pro-inflammatory responses to whole pollen were more marked than to purified natural Pas n 1. Therefore, it appears that whole *P. notatum* pollen extract interacts with the innate antiviral response via a direct, non-IgE mediated effect.

Chapter 5: Thesis Discussion

Allergic rhinitis is a major health concern in Australia, with grass pollen being the most abundant and clinically important outdoor aeroallergen. There is significant biodiversity in allergenic grass species, with climate being a key determinant in a grass species abundance within a biogeographical region. While the phenotypic and genotypic differences between these allergenic grass species have been established, the immunogenic differences between pollens of different subfamilies, and their impact of regional difference in exposure to different pollen on allergic respiratory diseases including AR and asthma are poorly understood.

The findings in Chapter 3 have revealed in adult patients with allergic rhinitis and GP allergy significant differences in serum IgE recognition of purified allergen components of representative temperate (Pooideae) and subtropical (Panicoideae and Chloridoideae) subfamilies, with participants from the subtropical region Brisbane showing higher specificity for the subtropical species *P. notatum* pollen and those from Adelaide showing higher specificity for the temperate species *L. perenne* pollen. These findings corroborate that of previous studies examining biogeographical variation of serum IgE recognition in grass pollen allergic participants according to the predominant grass species in the region (Davies et al., 2012; Davies et al., 2011). The novelty in the design of this study included the larger panel of GP compared to other studies of similar design; 5 GP in total with 2 species each from the Panicoideae and Pooideae with *C. dactylon* representing Chloridoideae. Moreover, whilst previous studies in Europe, USA and Australia had assessed serum IgE cross-reactivity with whole pollen extracts by pollen extract inhibitors, in this study we coated immunoassay plates with purified major allergen (Aalberse, 2007; Andersson & Lidholm, 2003; Johansen et al., 2009; Nony et al., 2015; White & Bernstein, 2003). The detection of serum IgE reactivity towards purified allergen gives a more precise evaluation of for IgE-based epidemiology as allergenic GP extracts have multiple allergen components, including pan allergens and cross-reactive carbohydrate determinants that may confound the findings (Scala et al.,

2010b).This approach with the inclusion of a cross inhibition assay showed differences in avidity of serum specific IgE binding within and between GP subfamilies further highlighted their immunological differences. The multicentre design of the GPAS study also enabled comparison between participants from four different states in Australia; Brisbane (QLD) and Sydney (NSW) in addition to two understudied regions of Perth (WA) and Adelaide (SA), with the findings demonstrating unique sensitization patterns between all regions. Unfortunately, the patterns were less obvious for NSW due to low number of participants in the cross-inhibition assays. To facilitate the cross-inhibition assay of a multiple combinations of serum IgE reactivity towards three purified allergens and five inhibitory GP extracts in participants from four different states, a microscale assay format was developed which enabled minimal sera usage and the multitude of assay conditions to be tested, assisted by semi-robotic liquid handling.

The differences in serum IgE avidity between allergenic GP species observed in the cross-inhibition assays implies diversity of IgE-binding epitopes between the major allergens Lol p 1 of ryegrass, Cyn d 1 of Bermuda grass and Pas n 1 of Bahia GP. Low IgE cross-reactivity between purified allergen Pas n 1 and Lol p1 has been demonstrated in a cohort of participants from a temperate region, with high serum IgE reactivity observed with Lol p 1 (Davies et al., 2011). A study on Pas n 1 revealed a dominant CD4 + T Cell epitope with 66% amino acid identity with that of of Cyn d 1 and Lol p 1, but the Cyn d 1 and Lol p 1 peptides had low cross-reactivity in Pas n 1-specific T cell lines generated from the PBMC of a cohort primarily sensitized to *P. notatum* pollen (Etto, de Boer, et al., 2012). Moreover, only two of the three dominant Pas n 1 T cell epitopes were shared with Cyn d 1 and Lol p 1 (Burton et al., 2002b; N. Eusebius et al., 2002). Another study also shown differences between the major T cell reactive allergens within a GP source, that is Lol p 1 and Lol p 5 from *L. perenne* pollen in *L. perenne* specific T cell lines generated from a sensitized cohort (Burton et al., 2002b). Unique IgE epitopes have also been found between the different allergen groups of *P. pratense* pollen that do not overlap with the major allergen, Phl p 1 (Levin et al., 2014; Westritschnig et al., 2008). Collectively, these suggest that GP from different subfamilies are not sufficiently cross-

reactive with one another, and these differences have implications for both diagnostics and therapeutics.

Chapter 3 findings show a region-specific pattern in IgE recognition, indicating the dominance of one GP allergen source over others in terms of IgE epitope recognition. As current diagnostic methods measure a patients' capacity to identify these allergens through serum specific IgE assays (Hamilton, 2015 #188), the tests' accuracy may be reduced if the locally relevant and immunologically distinct allergen is not present in a GP extract or allergen panel. For example, if a patient from a subtropical region is tested for specific IgE towards a panel of allergenic GP not containing the Panicoideae or Chloridoideae species, there is a possibility of low sensitivity of detection of allergic sensitization or even a false negative test. The differences in IgE-binding epitopes between allergenic GP and its subsequent recognition also has clinical implications. A nasal provocation study using *P. pratense* and *P. notatum* as allergen on two groups with sensitization towards either GP species showed higher symptom scores when exposed to the primarily sensitized GP species and none with the other (Phillips et al., 1989). An allergen chamber study in southern USA examining the effect of *P. pratense* pollen exposure on a group with predominant exposure to subtropical GP species showed slower kinetics of symptom escalation (Ramirez et al., 2015). Precision in the specificity of diagnosis of GP-related allergic rhinitis is required, with utilization of a wider panel of allergens and adoption of component resolved diagnostic sensitization tests that account for wider geographically relevant allergen sources. However, this may result in a diagnostic panel that is too large to be practical or too expensive to produce commercially. Hence, evidence and identification of a regionally relevant allergens is needed to inform the development of relevant diagnostic panels in different regions in Australia and large countries with diverse climates such as USA.

GP exposure in parts of Australia has been found to be less seasonal than climatically homogenous regions such as France, with multiple peaks of pollen concentration throughout the year, suggesting more diverse species of grasses, including both spring flowering temperate species and summer flowering subtropical species (Beggs et al., 2015; Devadas et al., 2018). DNA

metabarcoding of subtropical GP in Brisbane, Australia, a subtropical region, revealed a predominance of *P. notatum* and *C. dactylon* in the aerobiome, with multiple peaks in abundance throughout the year (Campbell et al., 2020). With the cross-inhibition assay of serum-specific IgE binding to purified allergen of the major allergenic GP performed in Chapter 3, a novel insight into the relative differences in immunological recognition and IgE avidity between known allergenic GP in Australia was provided. This enables more accurate identification of the GP a patient is primarily sensitized to by enabling specificity and avidity comparisons between the various GP they could be exposed to daily. Together with new detailed knowledge pollen exposure levels through the establishment of a national standardized network of pollen monitoring sites and innovative forecasting and molecular aerobiome approaches, a more comprehensive understanding of patterns of exposure to GP, and now, specificity of allergic sensitization of patients is available.

A more accurate diagnosis of the cause of GP-related allergic rhinitis will allow for more efficacious and specific treatment choices. The cross-linking of GP-allergen epitopes with specific IgE results in the manifestation of symptoms of the allergic disease state by induction of basophil activation and mast cell degranulation, which results in immediate release of preformed inflammatory mediators such as histamine. Basophil activation tests have been shown to have diagnostic value for GP-related allergic rhinitis in a monosensitized cohort in temperate regions of Europe (Saporta et al., 2001). In a Melbourne, Australia cohort with multiple sensitizations detected to GP from different subfamilies, *L. perenne, C. dactylon* and *P. notatum*, the strongest induction of basophil activation was observed for the most immunologically relevant allergen; ryegrass pollen and Lol p 1, based on serum IgE specificity and avidity of those patients. (Davies et al., 2011). Whilst basophil activations have not been undertaken in Brisbane Australia, based on the cross-inhibition assays performed in Chapter 3, patients from a subtropical region, the most specific and avid serum IgE reactivity were with *P. notatum* pollen extract and one would expect lower dose of exposure would be required to achieve basophil activation relative to temperate GP. Basophil activation tests could be used to predict symptom manifestation and further establish the clinical relevance of serum IgE reactivity

with GP components. While the capacity of serum specific IgE levels to predict the onset of allergic rhinitis has been established elsewhere, the threshold levels of serum IgE concentrations required for symptom manifestation is still a subject of debate (Hatzler et al., 2012). Thus, a basophil activation test preceded by the identification of the most specific and avid serum IgE responsive allergen for a patient, an accurate diagnosis of an allergen associated with the morbidity of allergic rhinitis in a polysensitized cohort. This once again underlines the importance of establishing serum IgE specificity and avidity in a cohort with multiple allergen exposures and sensitization profiles.

Epidemiological associations have been made between asthma and GP exposure. GP exposure has been associated with increased asthma-related emergency department presentations and hospitalizations in both children and adults (Erbas et al., 2018; Osborne et al., 2017). Cumulative exposure to ambient pollen, including grass, weeds and trees, in infancy has also been associated with an increased risk of developing hayfever at 3 months and asthma between 4 to 6 months (Erbas et al., 2013). *P. pratense* GP exposure has also been identified as the cause of the thunderstorm asthma event in Melbourne, where an unprecedented number of emergency department presentations and 10 associated deaths were reported due to preceding increase in GP concentrations in the air (Lee et al., 2017; Thien et al., 2018). With clear clinical associations between the two disease states, the utility of allergen immunotherapy in the management of asthma exacerbations was investigated. Grass pollen immunotherapy is a targeted therapeutic method to treat GP-related allergic rhinitis by inducing clinical and immunological tolerance in the patient, of which efficacy is dependent upon the cross-reactivity between the therapeutic GP utilized and the GP the treated patient is primarily sensitized to (Slovick et al., 2014).

A study comparing two commercially available sublingual GP immunotherapy options, ORALAIR which contains pollen extracts of 5 temperate grasses (Cocksfoot, Meadow-grass, ryegrass, Sweet vernal-grass and Timothy), and Grazax, which consists of only Timothy GP (*P. pratense)*, showed stronger inhibition of IgE binding and therefore, coverage of sensitization profiles, by the 5 grass extract due to its larger IgE repertoire, in a cohort of

patients from Sweden and Spain, two climatically different regions in Europe (Batard et al., 2019). As discussed previously, the diversity of allergenic GP sensitization profile is further heightened in climatically diverse regions such as Australia and the USA, which further reduces the efficacy of single GP immunotherapy options and any Pooideae family derived options due to the prevalence of Panicoideae or Chloridoideae as major allergen sources in the subtropical regions. Therefore, the identification of the most immunological relevant GP-allergens in a region will enable more accurate diagnosis and subsequently more efficacious treatment of GP-related allergic rhinitis, paving the way for precision medicine in care pathways for allergic rhinitis (Hellings et al., 2017).

While clinical associations between GP-related allergic rhinitis and asthma are well established, the immunological association appears to be rather complex. Both are disease states characterized by airway inflammation and have been associated with allergen exposure, which suggests a potential link in IgE. Examination of a subset of the patients affected by Melbourne thunderstorm asthma event (n=85) revealed that 97% of patients with allergic rhinitis who were sensitized towards *L. perenne* pollen but with no previous history of known asthma (Lee et al., 2017). Serum specific IgE initiates a signalling cascade in allergic rhinitis, resulting in the manifestation of airway inflammation similar to that observed in asthma. Serum IgE is also the focus of targeted therapy for severe asthma. Omalizumab, a monoclonal antibody selectively targeting IgE, was shown to decrease respiratory symptoms and subsequently exacerbation-related hospitalizations in patients with asthma (Pelaia et al., 2017). More pertinent to this thesis, Ferrando and colleagues conducted a critical appraisal of randomized control trials on grass pollen-based sublingual immunotherapy administration in children with seasonal rhinoconjuctivitis and have concluded that there is a protective effect on asthma (Ferrando et al., 2018). The most recent study conducted in this area is a 5-year cohort study on children with a history of GP-related rhinoconjunctivitis were found to have reduced risk of developing asthma symptoms and asthma-related medication usage on top of reduced allergic rhinoconjunctivitis symptoms and pharmacotherapy usage when treated with sublingual *P. pratense* pollen immunotherapy (Valovirta et al., 2018).

While grass-pollen based immunotherapy is increasingly accepted for its protective effects on asthma, the currently tested immunotherapy options such as Grazax and ORALAIR are based on Pooideae subfamily of grasses, chiefly *P. pratense*. Hence, their efficacy and potential protective effect on allergic rhinitis as well as asthma may not be similar when administered to children that are primarily exposed to and sensitized towards Panicoideae GP species, such as that in Chapter 4, due to the immunological differences discussed previously. Thus, there is a need for viable subtropical GP based immunotherapy to not only address subtropical GP related allergic rhinitis per se, but to also manage allergic rhinitis-associated asthma.

Allergic rhinitis has significant comorbidity with asthma but the major trigger of asthma exacerbation is respiratory viruses, with 60-70% of asthma exacerbations in Sweden and Spain associated with lower respiratory tract infections (Adeli et al., 2019). Human rhinovirus has been established as the leading viral cause of asthma exacerbation in children, ahead of aeroallergen sensitization (Johnston et al., 1996; Lemanske et al., 2005; Murray et al., 2006). Jackson and colleagues were the first to propose a causal relationship between aeroallergen sensitization and human rhinovirus-induced wheezing in children, with aeroallergen sensitization leading to an increased risk of human rhinovirus-induced wheezing (Jackson et al., 2012). This finding alludes to an immunological interaction between asthma, aeroallergen sensitization and rhinovirus infection. A recent observational prospective cohort study (n=84) investigated the possibility of an interaction between rhinovirus infection and aeroallergen exposure on asthma severity and found that at the time of asthma exacerbation, rhinovirus-positive asthmatic children had higher serum specific IgE levels to house dust mite and total IgE compared to RV-negative asthmatic children (Kantor et al., 2016). Another study with a case-crossover design on a larger cohort of children in Australia (n=644) showed that a combination of GP exposure and human rhinovirus infection increased asthma exacerbation in boys (Erbas et al., 2015). Collectively these studies suggest a possible IgE-dependent interaction between RV infection and aeroallergen sensitization. Thus, there was a need to understand whether GP exposure had an effect on antiviral immunity to rhinovirus and to understand the underlying immune interactions between

aeroallergen and RV, to manage allergic rhinitis in patients with asthma more effectively.

Epidemiological associations have been made between aeroallergen sensitization and RV-induced asthma exacerbation, but the underlying immune interaction is poorly understood. Until recently, there has been a lack of studies distinguishing the effect of asthma from the effect of allergen sensitization on the immune response towards respiratory viruses, as both have the capacity to individually alter this response. In Chapter 4, an observational prospective study was conducted in two cohorts, adult and adolescents with sensitization towards *P. notatum* pollen to investigate the interaction of *P. notatum* pollen and RV-16 PBMC using a novel co-exposure design. A diminished response of the antiviral biomarker IP-10 towards RV-16 was observed in *P. notatum* allergic adult participants' PBMC following exposure to whole *P. notatum* pollen extract. The chapter also showed a diminished IP-10 and IFN-α response in PBMC of adolescents with allergic rhinitis and asthma when RV-16 was co-cultured with whole *P. notatum* pollen extract, corroborating the findings of Gill and Durrani (Durrani et al., 2012; Gill et al., 2010). A potential IgE-dependent interaction between RV infection and aeroallergen sensitization could be explained in retrospect by the studies by Gill and Durrani and colleagues who examined the effect of cross-linking IgE on pDC and its subsequent inhibition of the type 1 interferon response, the primary innate antiviral mediator (Durrani et al., 2012; Gill et al., 2010). A novelty of both the adult and adolescent cohorts studied herein was the use of a cohort relevant GP allergen, *P. notatum* pollen, to allow for allergen cross-linking of IgE instead of anti-IgE. In the adult cohort, GP sensitization by skin prick test was shown. In the adolescent cohort serum IgE was shown but not all participants in the allergic rhinitis and asthma and allergic rhinitis only groups were sensitized to GP unlike the GP-allergic group in the adult cohort.

The initial theory on the inhibition of the innate antiviral response towards RV by whole *P. notatum* pollen was that it was dependent on IgE cross-linking, but this is called into question with several observations in Chapter 4 suggesting it may not be exclusively IgE dependent. A novel observation made in the adult cohort in Chapter 4 was a diminished IP-10 and IFN-α2 response

following co-exposure of RV with whole *P. notatum* pollen extract in the non-allergic group, however there were only a small number of participants in this group of the PAAS cohort. In the adult GAAS cohort, participants who were not GP-allergic showed comparable inhibition of RV- induced IP-10 induction by *P. notatum* pollen extract as those who were GP-allergic, suggesting allergen-specific IgE was not essential for the effect. A similar finding was made recently by Gilles and colleagues, who found a diminished IFN-λ towards human RV or TLR-3 ligand stimulation by *P. pratense* pollen extract in NEC of non-atopic donors (Gilles et al., 2020). Together these observations suggest that the *P. notatum* GP may inhibit the innate antiviral response towards RV in PBMC regardless of the allergy status, and influenced innate immunity via a direct, non-IgE dependent mechanism. However, for the adult GAAS cohort there was a high prevalence of asthma within both experimental groups including the non-grass pollen allergic group, and factors inherent to asthma status could influence the innate antiviral response. Gilles and colleagues noted a lack of information on sensitization or asthma status of their Gothenburg adolescent cohort, acknowledging the lack of capacity to address the influence of allergic sensitization or asthma to immune responses observed in their study (Gilles et al., 2020). Thus, in Chapter 4, a subsequent adolescent cohort was recruited with asthma as a stratifying factor to determine its effect on the innate antiviral response and response to whole *P. notatum* pollen extract co-exposure.

Significant inhibition of the RV induced innate antiviral mediators IP-10 and IFN-α2 was observed in the allergic rhinitis and asthma group as well as the allergic rhinitis only group of the adolescent cohort. No significant inhibition of the IP-10 and IFN-α2 was detected following co-exposure of RV-16 and whole *P. notatum* pollen extract in the no rhinitis group with no sensitization to *P. notatum* pollen, but there were only six participants in this group due to low recruitment of children without allergies in the PAAS study. Notably, the degree of inhibition of RV induction of IP-10 by whole *P. notatum* pollen extract was higher in those with asthma and allergic rhinitis, suggesting an asthma driven effect in the inhibition of innate antiviral mediators induced by RV by whole GP extract. A correlation was found between the percentage of inhibition of IP-10 but not IFN-α2 with the induction of proinflammatory antiviral mediators IL-6,

GM-CSF and TNF-α induced by whole *P. notatum* pollen extract. Significant induction of these mediators was also exclusively observed in the allergic rhinitis and asthma group, but not the allergic rhinitis only and no rhinitis groups. Collectively, these findings suggest a heightened proinflammatory response towards *P. notatum* pollen extract and a role in subsequent inhibition of the innate antiviral response that is driven by asthma disease state. It also suggests that the non-IgE dependent immune interaction between RV and whole *P. notatum* pollen extract is linked to the innate proinflammatory response towards the whole GP extract, and that the asthma disease state heightens this response.

Whilst there appears to be an argument for a non-IgE dependent immunological interaction, the role of an IgE-dependent interaction between aeroallergen and RV exposure needs to be further examined. In the adolescent cohort of chapter 4, no correlation was observed between the induction of antiviral mediators and percentage of inhibition of antiviral mediators by whole *P. notatum* pollen extract with either total IgE or specific IgE towards whole *P. notatum* pollen. No differences between median total IgE and specific IgE towards whole *P. notatum* pollen extract was observed between the allergic rhinitis and asthma group and the allergic rhinitis only group. Clinical associations have been made between total IgE and asthma severity, with higher total IgE inversely associated with forced expired volume FEV_1 scores (Ahmad Al Obaidi et al., 2008; Naqvi et al., 2007). Further evidence on this association is demonstrated by the utility of omalizumab, a monoclonal antibody selectively targeting IgE, was shown to decrease respiratory symptoms and subsequently exacerbation-related hospitalizations in patients with asthma (Pelaia et al., 2017). Kennedy and colleagues highlighted several aspects in the relationship between specific IgE towards aeroallergens and asthma that need to be clarified, including the degree of correlation between specific IgE titer and severity of asthma exacerbation and whether the effect of anti-IgE treatment is indirect by decreasing inflammation or direct by reducing the concentration of IgE, preventing allergen cross-linking on mast cells or basophils (Kennedy et al., 2012).

While the inhibition of the antiviral response by GP extract in PBMC is a novel finding in itself, previous studies have shown impaired antiviral immunity towards rhinoviruses in atopic asthma, characterized by deficient RV-induced IFN-γ and IL-12, which allude to an impairment of a T-helper cell type 1 (Th1) response (Papadopoulos et al., 2002). Examination of gene expression in lymphocytes by RV-A and RV-C found a diminished Th1 response, characterized by IFN-γ, in asthmatics compared to non-asthmatic children for both RV. Interestingly, this study also found a heightened IFN-γ and STAT1 in RV-A compared to RV-C, and a diminished response to IP-10 (or CXCL10) to RV-C only, demonstrating differences in induced Th1 response between RV species, with RV-C being less Th1 driven than RV-A (Anderson et al., 2020). Transcriptomics analysis of upper airway cells revealed two major phenotypes characterized by high or low interferon regulatory factor 7 (IRF-7) transcription, which correspond to high or low levels of Th1 response, characterized by type 1 interferon and IFN-γ responses, in children with acute wheezing. Children with the low IRF-7 phenotype had longer symptom durations, higher odds ratio of admission for asthma exacerbation and shorter time to recurrence compared to the high IRF-7 phenotype, showing the clinical implications of a deficient type 1 interferon response on RV-induced asthma exacerbations (Khoo et al., 2019). Wark recently proposed that children who develop asthma in early life display an immune phenotype characterized by deficient type-1 and type 3 interferon responses and its regulator, IRF-7, which makes them susceptible to RV infection, and that this phenotype is worsened by both the development of type 2 airway inflammation associated with allergic sensitization and positive feedback effect of recurring RV-A and RV-C responses (Wark, 2020).

The type 1 interferon response produced against human rhinovirus in the blood is characterized by IFN-α, with pDC's identified as the primary producer of this cytokine (Xi et al., 2015). pDC depletion in PBMC cultures were found to result in deficient IFN-α response towards RV-16 and deficient activation of RV-induced genes, most prominent of which are that of IRF7, IFN-γ and TNF-α (Xi et al., 2017). These findings suggest an association between pDC and the deficient innate antiviral response towards rhinovirus observed in asthmatics, with previous cellular studies showing that relative deficiency of circulating

pDCs during infancy was a risk factor of asthma later in childhood (Upham et al., 2009). However, in the adolescent cohorts in Chapter 4, no significant differences in the fold induction of IFN-α2 by RV-16 between the allergic rhinitis and asthma group and allergic rhinitis only, showing no asthma driven deficiency in the type 1 interferon response between the groups of participants with allergic rhinitis. No significant differences in fold induction of IFN- α2 by RV-16 between these two groups and the no rhinitis groups were also observed, although the low number of participants in the no rhinitis group prevents ruling out an allergic rhinitis driven effect on the deficient innate antiviral response. There was a large variation in the induction of IFN-α2 observed, which could be explained by the presence of a high IRF7 and low IRF7 phenotype within the cohort, which thus raises the importance of asthma phenotyping prior to examination of the cohort.

While the lack of statistical significance could be due to the low number of participants in the non-allergic adult group (n=8) and the no rhinitis adolescent group (n=6), the observations by Moskwa and Xatzipsalti were made in bronchial epithelial cells, while the results here are demonstrated in PBMC. Xi and colleagues showed that PBMC from asthmatic adult donors (n=9) produced lower IP-10 in response to RV-16 stimulation compared to that of healthy adults (n=7), contrary to the observation made by Moskwa, despite using a different strain of rhinovirus (RV-16 vs rhinovirus-1B) (Xi et al., 2015). Thus, it is possible that the innate immune response towards both allergen and respiratory virus stimulation is different between the airway epithelium and blood. Most of the current literature examining the immune effects of GP exposure, and aeroallergens in general, has focused on examining the effects at the airway epithelial cell level (Gandhi et al., 2013; Hosoki et al., 2015). The airway epithelium, especially nasal and bronchial epithelial cells, are the first site of contact for aeroallergens, and hence the initiation of the immune response towards them at the mucosal level. Hosoki and colleagues have proposed a two-signal hypothesis for the induction of pollen-induced allergic airway inflammation; a stimulation of the innate response at the epithelium by reactive oxygen species resulting in neutrophil recruitment, and a subsequent antigen-induced allergic inflammatory cascade that is IgE-mediated and results in mast

cell degranulation (Hosoki et al., 2015). This summary implies that the immune response towards GP allergens is initiated at the epithelium and that there are direct, non-IgE mediated mechanisms in their induction of an allergic response at the epithelium. However, these studies have examined only the effect of *P. pratense* GP and non-GP aeroallergens such as HDM, Ragweed pollen and Birch pollen, with the effect of subtropical GP yet to be examined. To further compound this, there is currently no studies to the best of my knowledge examining the innate effects of subtropical GP allergens in the blood.

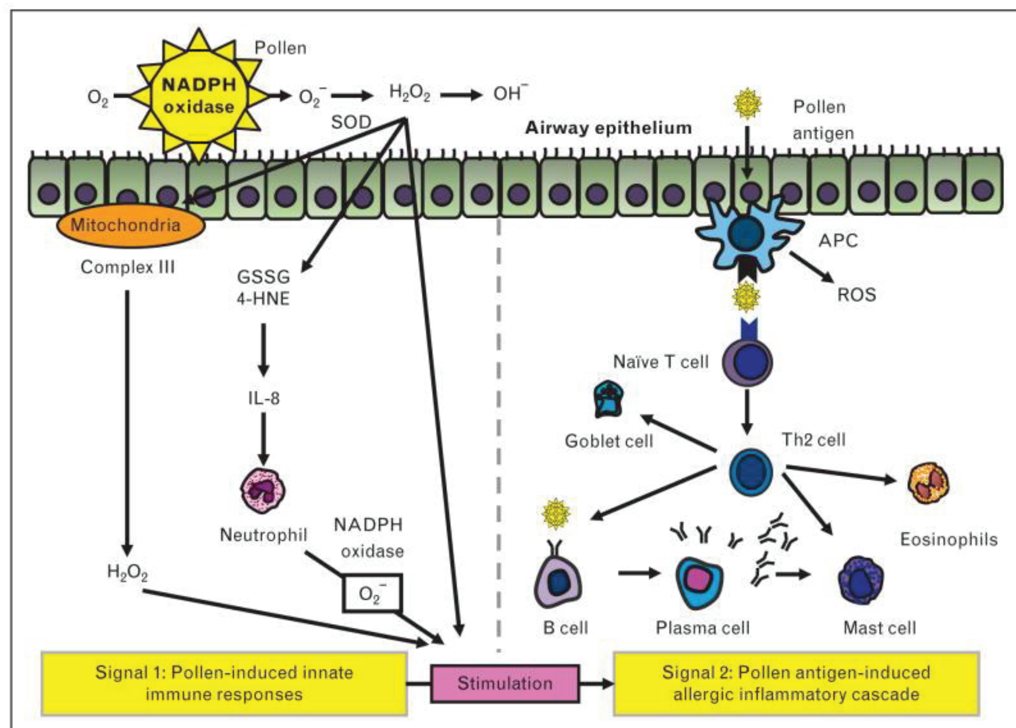


Figure 5.1 Two signal hypothesis on pollen-induced allergic airway inflammation. Adapted from (Hosoki et al., 2015)

The innate immune response against RV is also initiated at the airway epithelium, beginning with the binding and internalization of the virus with the ICAM-1 receptor (Greve et al., 1989). Studies on primary bronchial epithelial cells (BEC) from asthmatic children have shown a deficiency in IFN-α and β production, a deficiency in type 1 interferon production paralleling that observed in PBMC (Baraldo et al., 2012; Edwards et al., 2013). RV infection also induces the release of the alarmin cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) from the epithelium, which induces type 2 inflammation characterized by the release of hallmark Th2 cytokines IL-4, IL-5 and IL-13, with studies confirming this in both airway epithelial cell culture and a mouse

model (Han et al., 2017; Jackson et al., 2014). However, the propagators of this type 2 inflammation not by Th2 cells but instead, a novel group of cells called type 2 innate lymphoid cells (ILC2). Genetic studies on ILC2 revealed its regulation by the transcription factor GATA3, a known regulator in the type 2 signaling pathway and subsequent propagation of allergic airway inflammation (Hoyler et al., 2012). Computational analysis of multimorbidity between asthma, eczema and rhinitis revealed 15 shared immunological pathways including the GATA3 pathway, which shows a genetic link of ILC2 with allergic rhinitis and asthma (Aguilar et al., 2017). ILC2 has been implicated in the pathogenesis of asthma due to their capacity to produce large amounts of IL-5 and IL-13 in response to both allergens, such as HDM (Klein Wolterink et al., 2012) and *Alternaria* spores (Doherty et al., 2013) and also to influenza virus (Chang et al., 2011). More pertinent to this thesis, ILC2 represent a propagator of allergic airway inflammation independent of an IgE-interaction and facilitates crosstalk between the epithelium and blood in the propagation of airway inflammation. Th2 cytokines IL-4 and IL-13 were found to constrain IFN-β and IFN-λ1 response towards RV-16 in BEC (Marco Contoli et al., 2015) and conversely, IFN-α production by pDC was found to constrain the Th2 cytokines IL-5 and IL-13 in blood (Pritchard et al., 2012), further demonstrating the extent of crosstalk between the epithelium and blood. While PBMC serves as an accessible indicator in blood for innate immunity towards RV due to the prominent role of pDC, there are further upstream regulators such as the alarmin cytokines that can affect the innate immune response towards RV that are unable to be accounted. Conversely, if a study is conducted on AEC, the effect of circulating Th2 cytokines and spIgE in the serum cannot be accounted. Thus, we must question if the current design of individually looking at blood or epithelium cell comprehensive in explaining the complex immune interactions between the allergic airway inflammation and the innate antiviral response. Perhaps more comprehensive results can be achieved with matched AEC and PBMC cultures, allowing for better understanding of the innate immune response towards GP allergens and RV (Figure 5.2) along with the associated effects of the allergic rhinitis and asthma disease states on it.

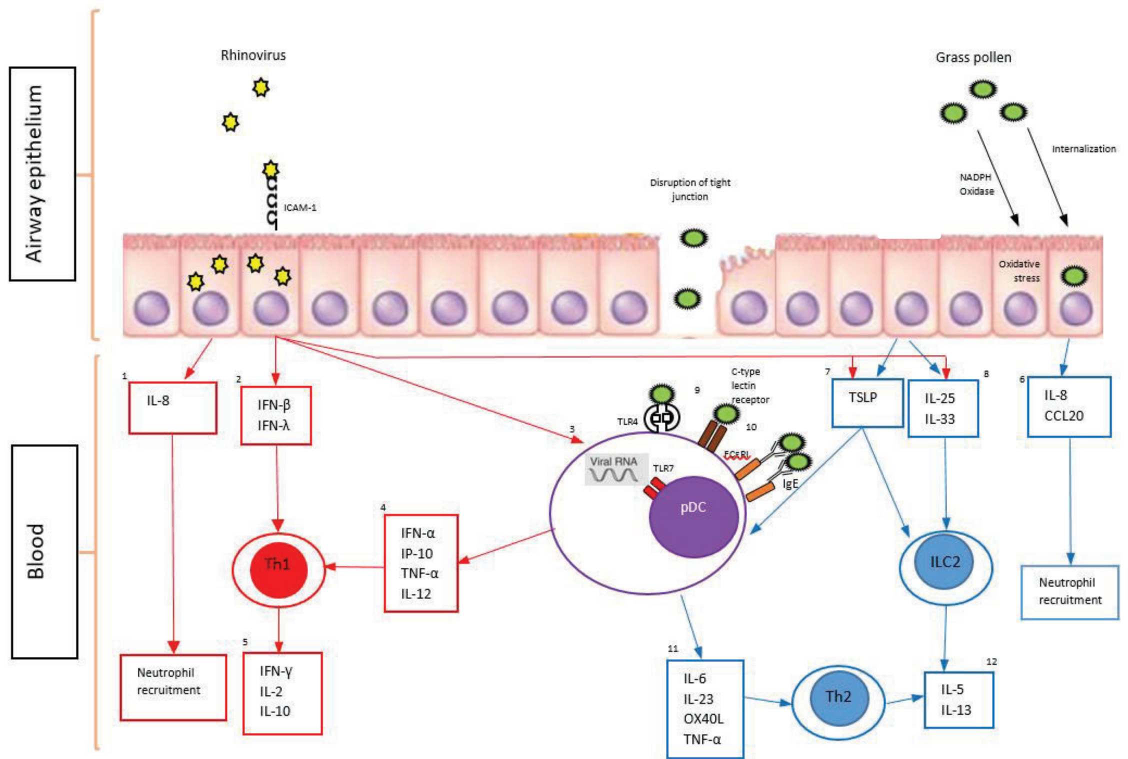


Figure 5.2 Potential innate immune interactions of human rhinovirus and grass pollen allergen across the airway epithelium and blood

Chapter 6: Thesis Conclusions

This thesis aimed to investigate the impact of a clinically relevant GP allergen, *Paspalum notatum* pollen on grass pollen sensitization profiles in AR in Australia and on the immune response towards human rhinovirus in adults with GP allergy and adolescents with AR and asthma.

In Chapter 3, the findings showed biogeographical variation in spIgE reactivity towards GP from different subfamilies across climatically diverse regions in Australia, with more specific and avid IgE reactivity towards the pollen of the most abundant grass species in the region. The differences in specificity and avidity of IgE reactivity indicated differences in immunological recognition between GP from different subfamilies, with GP from the same subfamilies only showing similarities in recognition. This chapter highlights the need to identify the most relevant GP allergen in a patient cohort to ensure more accurate diagnosis and targeted, efficacious treatment.

In Chapter 4, inhibition of the antiviral response mediated by a cohort relevant GP allergen, *P. notatum*, was observed independent of GP allergy status in the adult cohort. A similar inhibition of the antiviral response was observed only in the AR and asthma group of the adolescent cohort, indicating an asthma driven effect on the inhibition. The inhibition of the antiviral response was driven by both IgE-dependent and non-IgE dependent mechanisms.

Collectively, the findings in this thesis highlight the emerging importance of subtropical GP allergens such as *P. notatum* and its potential impact on AR and asthma disease burden in Australia. This thesis also provides evidence to inform the need for consideration of the management of GP-related AR in the management of asthma.

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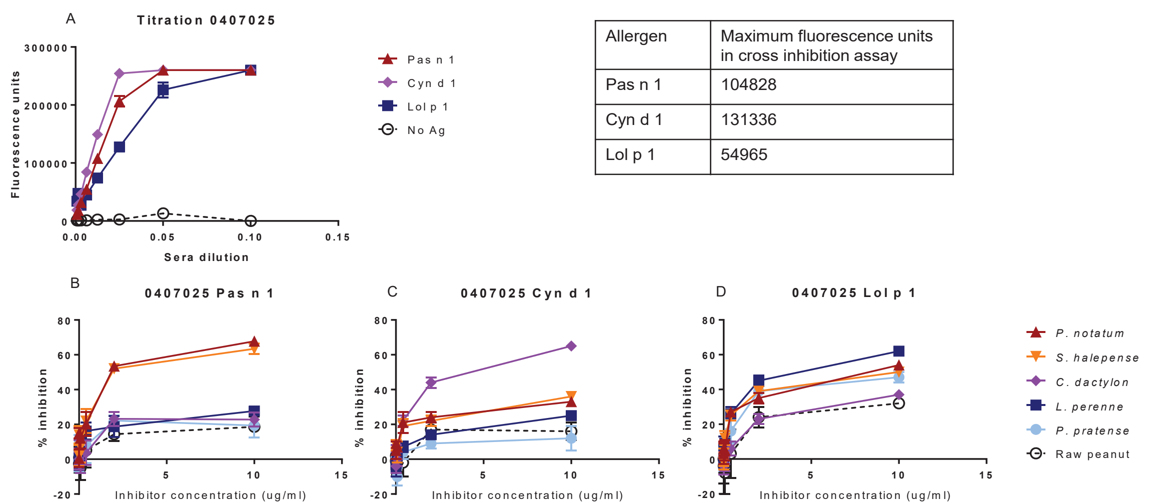
Appendices

Appendix A Serum titration of an exemplary patient from Queensland using the DELFIA-TRF assay format (Perkin Elmer, USA) expressed as fluorescence units with standard deviation. Cross-inhibition of serum IgE reactivity with Pas n 1 (B), Cyn d 1 (C) and Lol p 1 (D) by extracts of *P.notatum, S.halepense, C.dactylon, L.perenne, P. pratense* and raw peanut using same assay format expressed as percentage of inhibition over uninhibited IgE reactivity with standard deviation.



Allergen	Maximum fluorescence units in cross inhibition assay
Pas n 1	104828
Cyn d 1	131336
Lol p 1	54965

Appendix B Questionnaire used in the recruitment of adolescent PAAS cohort

Ver. 1.4, 1.8.2018

Children's Health Queensland Hospital and Health Service

Pollen Allergy and Asthma Study Questionnaire

PAAS Study ID: ____________	Date of Birth: ____________
Study Doctor code: ____________	Postcode: ____________
Recruitment Site: Clinic ☐ ENT ☐	Date of recruitment: ____________

To be completed by patient and/or parent

1. Gender Male ☐ Female ☐ **2. Weight** ______ **kg** **3. Height** ______ **cm**

4. What is your main Ethnic Origin? Caucasian ☐ Indigenous ☐ Asian ☐ Other ☐

If other, please specify : ____________________

	City, State ,Country	*How long did you live there?*
5. Where do you live now?	____________ , ____________ , ____________	___ *years* ___ *months*
6. Where were you born?	____________ , ____________ , ____________	___ *years* ___ *months*
7. Where else have you previously lived?	____________ , ____________ , ____________	___ *years* ___ *months*

8. How many hours do you spend outside each week?	0-5 ☐	6-10 ☐	11-20 ☐	more than 20 ☐

9. Have you ever had/been diagnosed with food allergy? **No** ☐ **Yes** ☐				
10. Have you had these symptoms after eating certain food?	Swelling of face, lips or eyes ☐	Hives or welts on skin ☐	Abdominal pain ☐	Vomiting ☐
11. Have you had these symptoms after eating certain food when NOT having a cold or flu?	Difficult or noisy breathing ☐	Swelling of tongue ☐	Swelling of throat ☐	Difficulty talking ☐

12. Please specify which foods that cause these symptoms or that which you are allergic to.

____

13. Have you ever had/been diagnosed with hay fever/allergic rhinitis? **No** ☐ **Yes** ☐				
14. Have you had these symptoms when NOT having a cold or flu?	**Blocked nose**	**Runny nose**	**Itchy, watery eyes**	**Sneeze**
i. in the last one week?	☐	☐	☐	☐
ii. in the last one year?	☐	☐	☐	☐

15. Has the symptoms you ticked above persisted for;	**Yes**	**No**
i. less than 4 days per week	☐	☐
ii. more than 4 days per week	☐	☐
iii. less than 4 weeks at a time	☐	☐
iv. more than 4 weeks at a time	☐	☐

16. Have the symptoms you ticked above impaired the following activities?	Sleep ☐	Daily activities ☐	School or work ☐	Sport ☐

17. When did you experience hay fever? (circle those appropriate)

Jan Feb Mar Apr May Jun Jul Aug Sept Oct Nov Dec

Page 1 of 6

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18. Have you ever had allergen specific immunotherapy? No ☐ Yes ☐

If yes, for which allergen ______________________

**If patient has received allergen specific immunotherapy, they are excluded from this study*

20. Have you ever had/been diagnosed with asthma? No ☐ Yes ☐				
Have you had these symptoms when NOT having a cold or flu?	**Wheeze**	**Shortness of breath**	**Chest tightness**	**Cough**
21. in the last one week?	☐	☐	☐	☐
22. in the last one year?	☐	☐	☐	☐

	Yes	**No**
23. Have you been admitted to the hospital for asthma?	☐	☐
24. Has your chest sounded wheezy after exercise?	☐	☐
25. Have you ever had dry cough at night without having a cold or chest infection?	☐	☐
26. In the past 12 months, has your wheezing been severe enough to limit your speech to one or two words at a time between breaths?	☐	☐

27. How many attacks of wheezing have you had in the past 12 months?
None ☐ 0-3 ☐ 4-12 ☐ More than 12 ☐

28. On average, how often has your wheezing been severe enough to disturb your sleep in the last 12 months?
Never ☐ Less than one night per week ☐ One or more nights per week ☐

29. Asthma Control

Points	**1**	**2**	**3**	**4**	**5**
In the past 4 weeks, how much of the time did your asthma keep you from getting as much done at work, school or at home?	All of the time ☐	Most of the time ☐	Some of the time ☐	A little of the time ☐	None of the time ☐
During the past 4 weeks, how often have you had shortness of breath?	More than once a day ☐	Once a day ☐	3-6 times a week ☐	Once or twice a week ☐	Not at all ☐
During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?	4 or more nights a week ☐	2-3 nights a week ☐	Once a week ☐	Less than once a week ☐	Not at all ☐
During the past 4 weeks, how often have you used your blue puffer or reliever medication (such as *Ventolin, Asmol, Airomir, ApoSalbutamol* or *Bricanyl*)?	3 or more times per day ☐	1 or 2 times per day ☐	2 or 3 times per day ☐	Once a week or less ☐	Not at all ☐
How would you rate your asthma control during the past 4 weeks?	Not controlled at all ☐	Poorly controlled ☐	Somewhat controlled ☐	Well controlled ☐	Completely controlled ☐
TOTAL SCORE					
Score breakdown	20 – 25 : Asthma is well controlled 19 or less : Asthma not well controlled.				

30. If you have hay fever and asthma, was your asthma more severe when hay fever was experienced?
No ☐ Yes ☐

General health questions

	Yes	No
31. Have you had a cold recently? 32. If yes, i. in the last week? ii. in the last month?	☐ ☐ ☐	☐ ☐ ☐
33. Do you currently smoke?	☐	☐
34. Have you ever previously smoked?	☐	☐
35. Do any of your parents/guardian smoke?	☐	☐

Thank you for your time

To be completed by Healthcare Professional (Nurse or Doctor)

Diagnosis

Asthma	No ☐ Yes ☐	Mild / Moderate ☐ Severe ☐ Severe is classified as having any of these observations; • Use of accessory muscles of neck or intercostal muscles or 'tracheal tug' during inspiration or subcostal recession ('abdominal breathing') • Unable to complete sentences in one breath due to dyspnoea • Obvious respiratory distress • Oxygen saturation 90–94% (if available) Consider medication use and patient history. If none present, classify as mild/moderate.
Rhinitis	No ☐ Yes ☐	Mild/Moderate ☐ Severe ☐ If one or more impairment of activity indicated in question 16, classify as severe.
		Persistent ☐ Intermittent ☐ If symptoms present for (refer question 12-15, page 1); less than 4 days/4 weeks at a time – Intermittent more than 4 days/4 weeks at a time – Persistent

Does the patient have medication for their allergy and/or asthma?				
Type of medication	Usage		Frequency	
	No	Yes	Daily	Occasional
a. ***Relievers:*** eg Ventolin /Asmol / Epaq, Bricanyl;	☐	☐	☐	☐
b. ***Preventers:*** eg. Seretide, Flixotide, Pulmicort, Qvar, Symbicort, Alvesco;	☐	☐	☐	☐
c. ***Oral steroids:*** eg Prednisone, Prednisolone;	☐	☐	☐	☐
d. ***Nasal steroids;*** eg Rhinocort, Avamys, Nasonex;	☐	☐	☐	☐
e. ***Antihistamines;*** eg Claratyne, Zyrtec, Telfast;	☐	☐	☐	☐
f. ***Nasal saline wash, spray***	☐	☐	☐	☐

Is the patient on any other medication(s)? No ☐ Yes ☐

If yes, please specify:____

Appendix C Primary research article publication containing results from Chapter 3 in Clinical and Translational Immunology

Clinical & Translational Immunology

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ORIGINAL ARTICLE

Biogeographical variation in specific IgE recognition of temperate and subtropical grass pollen allergens in allergic rhinitis patients

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Abstract

Objective. Globally, grass pollens (GP) are major aeroallergen triggers of allergic rhinitis (AR) and asthma. However, patterns of allergic sensitisation to pollen of temperate (Pooideae: *Lolium perenne*) and subtropical (Chloridoideae: *Cynodon dactylon* and Panicoideae: *Paspalum notatum*) subfamilies in diverse climates remain unclear. This study aims to evaluate the level of allergic sensitisation and IgE specificity for major GP allergens representing the three subfamilies in biogeographically distinct regions. **Methods.** Participants (GP-allergic with AR, 330; non-atopic, 29; other allergies, 54) were recruited in subtropical: Queensland, and temperate: New South Wales, Western and South Australia, regions. Clinical history, skin prick test (SPT), total and specific IgE to GP and purified allergens (ImmunoCAP) were evaluated. Cross-inhibition of sIgE with Pas n 1, Cyn d 1 and Lol p 1 by GP extracts was investigated. **Results.** Queensland participants showed higher sensitisation to *P. notatum* and *C. dactylon* than *L. perenne* GP. sIgE was higher to Pas n 1 and Cyn d 1, and sIgE to Pas n 1 and Cyn d 1 was inhibited more by Panicoideae and Chloridoideae, respectively, than Pooideae GP. Conversely, participants from temperate regions showed highest sensitisation levels to *L. perenne* GP and Lol p 1, and sIgE to Lol p 1 was inhibited more by Pooideae than other GP. **Conclusion.** Levels and patterns of sensitisation to subtropical and temperate GP in AR patients depended on biogeography. Knowledge of the specificity of sensitisation to local allergens is important for optimal

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Appendix D Review article publication on the molecular allergology of subtropical GP allergens in Molecular Immunology

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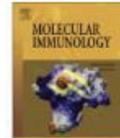
Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



The molecular allergology of subtropical grass pollen

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ABSTRACT

Grass pollens are amongst the most important aeroallergen sources world-wide triggering allergic rhinoconjunctivitis and asthma in sensitised patients. Much of what we know about the allergen components of grasses is informed by research on pollen of temperate (Pooideae) species that are abundant in the temperate climate zones. However, climate changes are altering the biogeographical distribution as well as timing and allergenicity of grass pollens. This provides an impetus for better understanding of the contribution of subtropical subfamilies of grasses to pollen allergy globally. Pollen of Chloridoideae (e.g. *Cynodon dactylon*; Bermuda grass) and Panicoideae (e.g. *Paspalum notatum*; Bahia grass or *Sorghum halepense*; Johnson grass) subfamilies are clinically important in subtropical zones of Australia, Asia, India, Africa, and America. These grasses differ ecologically and phylogenetically from temperate grasses and, importantly their allergen composition is qualitatively different. For example, subtropical grass pollens appear to lack the major group 5 grass pollen allergen family. In this review we summarize current knowledge of the epidemiology and immunology of subtropical Chloridoideae and Pancoideae pollen allergens, describe the biochemical characteristics of known isoforms and variants as well as properties and structures of subtropical pollen allergen components. Whilst only one subtropical allergen component; Cyn d 1 of Bermuda grass pollen, is available commercially for diagnostic use, in a natural purified form, a number of allergens of Panicoideae grass pollen; Zea m 1, Zea m 3 and Zea m 13 of maize, Pas n 1 and Pas n 13 of Bahia, as well as Sor h 1, Sor h 2, Sor h 13 and Sor h 23 of Johnson grass, have been discovered. Research effort is directed towards making available subtropical grass pollen allergen components as innovative treatment and diagnostic options that more specifically address the needs of patients from warmer regions of the globe.

1. Biogeography, epidemiology and immunology of subtropical grass pollen

There is an inverse biogeographical distribution of temperate and subtropical grasses with the subtropical species being more abundant closer to the equator (Esch, 2004). The size of the world's population living in subtropical climates is increasing globally and the subtropical climate zones are widening (Gupta, 2002; Seidel et al., 2008). In southern United States of America (USA), such as Florida, Texas, Louisiana and Mississippi, the population increased by 18.3%–52.3 million between 2000 and 2010 (US Census Bureau). The biomass of subtropical grasses (Morgan et al., 2011) and their range is predicted to expand with climate change (Gupta, 2002), increasing the exposure to subtropical GP allergens and intensifying the burden of allergic respiratory diseases (Beggs, 2009; Ziska and Beggs, 2011). The epidemiology of subtropical grass pollens and their contribution to allergic rhinoconjunctivitis and asthma in subtropical regions has previously been reviewed (Davies, 2014). Here a comparison between temperate and subtropical grass pollen biogeography, epidemiology and immunology is summarized.

In 1972, Hensel and Griffith (1972) examined sensitisation frequencies in the 429 patients from Louisiana, a subtropical region in USA; Bahia (*Paspalum notatum)* GP was the most frequently recognized GP but the patterns of sensitization included SPT positivity to Bahia, Dallis (*Paspalum dilatatum*), Johnson (*Sorghum halepense*), Bermuda (*Cynodon dactylon*) and Timothy (*Phleum pratense)* GP. Application of the Prauss Kausner test (Cohen and Zelaya-Quesada, 2002) with serum of five Bahia GP-allergic patients to non-allergic recipients, showed higher SPT reactivity to Bahia than other species of GP (Hensel and Griffith, 1972).

A survey of sensitisation of 345 children of military personnel with allergic diseases in Lackland, Texas, Bahia GP showed the highest frequency (38%) of positive SPT of a panel of 51 common aeroallergens (Calabria and Dice, 2007). Interestingly, the frequency of sensitivity to

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