

**Clinical and immune effects of
probiotic and peanut oral
immunotherapy for treatment of
peanut allergy**

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of Doctor of Philosophy

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Dedicated to my sons Ryan and Jonathan

Abstract

Food allergen oral immunotherapy (OIT) is emerging as a potential treatment for food allergy. Probiotic and Peanut Oral Immunotherapy (PPOIT) showed effectiveness inducing non-responsiveness to peanut in the immediate post-treatment period. This doctoral thesis investigates the long-term clinical and immunologic effects of PPOIT in children with peanut allergy and the efficacy and tolerability of a modified PPOIT dose escalation schedule designed to improve treatment feasibility.

Declaration

I, Kuang-Chih Hsiao, certify that:

- The thesis comprises only my original work towards the PhD except where indicated in the Preface,
- Due acknowledgement has been made in the text to all other material used,
- The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Date: 1 September 2020

Signed: *Kuang-Chih Hsiao*

Preface

As a clinician, I am honoured and privileged to be involved in the care of children with food allergies. I am humbled by the impact on my patients and their families of having to live with food allergies and this initially motivated and continues to inspire my pursuit of deeper understanding about potential food allergy treatments. Joining Professor Mimi Tang's research group at a time when new data was emerging regarding a novel approach to treating food allergy gave me an unique opportunity to witness and contribute to the exciting development of this candidate therapy. Completing this doctoral thesis has been an incredibly interesting and amazing journey and I hope through my writing I can share in the excitement of new knowledge discovery with my readers.

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List of abbreviations

AE	Adverse events
CI	Confidence interval
CPE	Crude peanut extract
DBPCFC	Double-blind placebo-controlled food challenge
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
FAQLQ-PF	Food allergy quality of life questionnaire-parent form
GEE	Generalised estimating equation
GM	Geometric mean
IFN- γ	Interferon gamma
IL	Interleukin
IQR	Inter-quartile range
<i>L. rhamnosus</i>	<i>Lactobacillus rhamnosus</i>
LTSU	Long-term sustained unresponsiveness
MCRI	Murdoch Children's Research Institute
OIT	Oral immunotherapy
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PPOIT	Probiotic and peanut oral immunotherapy
RCH	Royal Children's Hospital
RCT	Randomised controlled trial
SD	Standard deviation
sIgA	Specific-IgA
sIgE	Specific-IgE
sIgG4	Specific-IgG4
SLIT	Sublingual immunotherapy
SPT	Skin prick test
SU	Sustained unresponsiveness
TGF- β	Transforming growth factor beta
Th	T-helper
Treg	T-regulatory

Chapter 1. Review of the literature

1.1. Chapter introduction

The literature review chapter is divided in three sections.

The first section is a peer-reviewed and published literature review article describing novel treatments for established food allergies with a focus on oral immunotherapy (OIT). The doctoral candidate aimed to first determine the state of the evidence for OIT around the time of commencement of the doctoral research. This section discusses the concepts of desensitisation and sustained unresponsiveness as possible clinical outcomes following treatment.

In the second section, the doctoral candidate summarises more recent developments in the field of peanut OIT since the publication of the literature review article.

The third section is a literature review of immune mechanisms mediating clinical effects of food oral immunotherapy (OIT). This section describes the effect of OIT on immune compartments and describes immune changes associated with post-treatment remission of allergic response including desensitisation and sustained unresponsiveness.

1.2. Novel treatments for established food allergies (the accepted manuscript)

This section is the accepted manuscript version of the peer-reviewed publication[1], first-authored by the doctoral candidate. The final publication is available at <https://link.springer.com/article/10.1007/s40124-016-0116-1>.

1.2.1. Abstract

Current management of food allergic children centres on allergen avoidance and emergency management of allergic reactions due to accidental ingestion. These measures minimise harm to patients but the need for constant vigilance and dietary restriction may have a major long term negative impact on quality of life. Research has focused on searching for a curative treatment that can modify the natural history of disease. Both non antigen specific and antigen specific immune modulation approaches have been explored. Oral immunotherapy (OIT) has drawn greatest interest as a potential treatment for food allergy. OIT is effective at inducing desensitisation, however its ability to induce long-lasting tolerance or sustained unresponsiveness remains to be confirmed. There is growing evidence that addition of immune modifying adjuvants may enhance efficacy of OIT in inducing long-lasting sustained unresponsiveness. This article reviews recently published literature on this rapidly evolving field.

Key words: food allergy, desensitisation, tolerance, sustained unresponsiveness, oral immunotherapy, adjuvants, quality of life

1.2.2. Introduction: Food allergy prevalence and current management

Globally there has been a significant increase in the prevalence of food allergy over the past two decades. Epidemiologic studies suggest that factors contributing to this epidemic may include timing and route of allergen exposure, sunlight exposure, vitamin D levels and microbial diversity and composition. While allergies to egg, milk, wheat and soy generally resolve during childhood, the age at resolution appears to be

increasing with many cases resolving in adolescence. Furthermore, peanut, tree nut, shellfish and fish allergies tend to persist throughout life. The current standard of care for children with food allergy includes allergen avoidance and education of patient and parent or carer in the recognition and emergency management of allergic reactions. Although these measures are designed to minimise harm to a food allergic child, they have a major negative impact on the child's and their family's quality of life due to the constant vigilance required to avoid foods, impact on participation in social events and increased fear and anxiety of having a potentially life-threatening allergic reaction. There is a need for disease modifying therapy. This article reviews the literature on novel approaches to treatment of IgE mediated food allergy in children published in recent years. Mechanisms of oral tolerance and immune changes associated with oral immunotherapy, covered in a recently published article [2], will not be reviewed here.

1.2.3. The search for an effective treatment

There are two outcomes of interest when evaluating the clinical effectiveness of potential food allergy treatments - 'desensitisation' and 'tolerance'. Desensitisation describes the ability to ingest a food without reaction while maintaining regular exposure to that food (for example continuing on oral immunotherapy). Successful desensitisation in the absence of tolerance is mediated by changes in effector cells such as mast cells and basophils, without modulation of underlying pathogenic immune mechanisms. 'Tolerance' on the other hand is the ability to ingest a food without reaction even after discontinuation of exposure to the food for a period of time. Tolerance is believed to reflect sustained reprogramming of the immune response to allergen, involving T regulatory cells (Treg) and/or allergen-specific anergy and clonal deletion. Desensitisation can be evaluated clinically by administering a food challenge while a subject is still receiving oral immunotherapy or eating regular doses of the food. In contrast, tolerance is confirmed by performing a food challenge after oral immunotherapy or intake of the food has been stopped for an extended period of weeks to months. Currently, there is no consensus on the exact duration of secondary elimination that is required to demonstrate true long-lasting tolerance. For this reason, some experts have introduced the term 'sustained unresponsiveness' (SU) in place of

tolerance to acknowledge that the ability to tolerate a food after discontinuation of OIT may not be long-lasting and may not represent a permanent state of immune tolerance.

1.2.4. Non-antigen specific immune modulation

One approach to treatment of food allergy is to induce desensitisation or tolerance in a non-antigen specific way.

Monoclonal antibodies (mAb) targeting IgE induce suppression of basophil FcεRI expression which may confer clinical protection against a reaction following allergen exposure. Early studies of anti-IgE mAb (either HU-901 or omalizumab) for treatment of peanut allergy described enhanced tolerability of peanut in peanut allergic individuals. A recent pilot study [3] treated 14 peanut allergic subjects (aged 18 to 50 years) with omalizumab for 6 months and demonstrated a greater than 50 fold increase in the median reaction eliciting dose of peanut double blind placebo controlled food challenge (DBPCFC) (80mg versus (vs) 5080mg, $p=0.005$). Mechanistic studies showed suppression of basophil histamine release within 8 weeks of treatment and later onset suppression of mast cell reactivity by 6 months. These findings suggest that anti-IgE treatment can improve tolerability of allergen and is associated with suppression of effector cell function. However, it remains unclear whether such treatment modulates adaptive immune responses or can induce long-lasting tolerance.

FAHF-2 is a 9 herb Chinese herbal formula, which in animal model studies and phase 1 trials was found to be safe and well tolerated. In a multi-centre DBPC phase 2 randomised clinical trial [4], 68 subjects (aged 12-45 years) with challenge confirmed allergies to one or more foods including peanut, tree nut, sesame, fish and/or shellfish, were randomised to receive FAHF-2 ($n=46$) or placebo ($n=22$) for 6 months without concurrent exposure to the offending allergen. FAHF-2 was not superior compared to placebo at improving tolerance to food allergens. Surprisingly, the placebo treated subjects had a high rate (45.5%) of treatment success (the study's primary outcome), defined as being able to consume 2 g of allergen protein on double blind placebo controlled food challenge (DBPCFC) or a greater than 4-fold increase in allergen dose required to induce a reaction when compared to the baseline reaction inducing dose. Even using more stringent criteria of clinical response, i.e. a 10-20-fold increase in the

amount of allergen protein tolerated, the placebo-treated group attained a high rate of success (13.6%) within a short period of time. The authors could not explain the high rate of treatment success amongst placebo participants given the generally accepted rates of spontaneous resolution of allergy to peanut (0-15%), tree nut (up to 10%), fish (1%) and shellfish (2%). This highlights the importance of including a placebo group in clinical trials evaluating food allergy treatments. Interestingly, despite a lack of clinical effect *in vivo*, *in vitro* culture of participants' peripheral blood mononuclear cells (PBMCs; obtained at study entry) with food allergen showed significant differences in interleukin (IL)-5 (lower), IL-10 (higher) and CD4+CD25+Foxp3+ T-regulatory (T-reg) cell numbers (higher) in the presence of FAHF-2 compared with media control. This suggests that co-exposure to antigen and FAHF-2 may be required to induce antigen specific switch from allergic to tolerogenic responses, and/or that the concentrations of FAHF-2 achieved *in vivo* were insufficient to reprogramme the immune response to allergen.

1.2.5. Antigen specific immune modulation

Noon and Freeman first described allergen specific immunotherapy over 100 years ago. Since then immunomodulation of antigen specific responses through the gradual exposure to offending allergen by subcutaneous injection has become standard of care for patients with severe allergic rhinitis or asthma and for insect venom anaphylaxis. Attempts at food allergen specific subcutaneous immunotherapy were associated with safety concerns. Subsequent studies of sublingually delivered food immunotherapy did not confer adequate levels of protection. Therefore, oral immunotherapy (OIT) has remained the major focus of ongoing study in the search for an effective food allergy treatment.

OIT involves the oral administration of increasing doses of food allergen starting at very low doses and building up to a higher maintenance dose. Modern OIT regimens generally comprise a rapid induction phase, a gradual build-up phase and a maintenance phase. During the rapid induction phase, the dose is rapidly increased from fractions of milligrams to tens of milligrams over several hours. In the build-up phase, the patient continues to take the last tolerated dose on a daily basis, with dose increases performed periodically (usually every 2 weeks) over a number of months

until the target maintenance dose is reached. After reaching the maintenance dose, the same dose is maintained on a daily basis for months to years.

1.2.6. OIT using whole food protein

1.2.7. Inducing desensitisation

Early studies of OIT focused on evaluating the effectiveness of OIT at inducing desensitisation. Results of these earlier studies have been summarised in recent meta-analyses and systematic reviews [5-7].

A meta-analysis of five cow's milk randomised controlled trials (RCT) published up to October 2012 involving a total of 196 patients (106 active OIT treatment and 90 controls) reported successful desensitisation, defined as being able to tolerate a 200ml serve of cow's milk, in 62% of children in the OIT group compared to 8% of the control group (Yeung, Kloda et al. 2012). More recent studies report similar desensitisation rates of 61-88% [8] [9].

A Cochrane review of double blind placebo controlled (DBPC) RCT of egg OIT published up to December 2013 included four studies involving 167 egg allergic children (100 active OIT treatment and 67 controls) [6]. 39% of children in the OIT group were successfully desensitised, defined as being able to tolerate a full serving of egg (10-13.6 g of egg protein or 10mL raw egg white) compared to 11.9% of the control group, with a pooled relative risk ratio (RR) of 3.39 (95% confidence interval (CI) 1.74-6.62). Partial desensitisation (being able to tolerate 1 to 7.5 g of egg protein) was achieved in 79% of the OIT group, resulting in a pooled RR of 5.73 (95% CI 3.13 to 10.50). However, the range of treatment duration in the included studies was wide (6 to 22 months) and the meta-analysis did not adjust for the potential effect of treatment duration on outcome. Reporting of adverse events (AE) was highly heterogeneous. 69% of children reported mild to severe AE during OIT treatment with pooled RR of 6.06 (95% CI 3.11 to 11.83). Overall, 5 out of 100 (5%) OIT treated participants required adrenaline.

A meta-analysis of all peanut OIT and sublingual immunotherapy (SLIT) DBPC RCT published up to March 2013 included 1 OIT study (19 active treatment and 9 placebo treatment, children 2-11 years) and 2 SLIT studies (31 active treatment and 27 placebo

treatment, children and adults) [7]. Random effects model analysis of pooled results found that OIT or SLIT had a positive effect on peanut allergy, as defined by the ability to tolerate an increased amount of peanut protein from the start of therapy, with 86% (43/50) in the active treatment group achieving the primary outcome compared to 8.3% (3/36) in the control group (odds ratio [OR], 38.44; 95% CI, 6.01-245.81). There was no difference in the number of patients needing adrenaline between groups (OR, 0.51; 95% CI, 0.03-10.20).

A more recent RCT evaluating the effect of low dose peanut OIT at a target maintenance dose of 0.8 g/day of peanut protein for six months included 99 peanut allergic children aged 7 to 16 years of age [10]. 62% (24/39) of the active treatment group compared to none (0/46) in the placebo treated group achieved the primary outcome of passing a desensitisation DBPCFC to a cumulative dose of 1.4 g of peanut protein. The median increase in peanut threshold after OIT was 1345 mg (range 45-1400; $p < 0.001$) equating to a 25.5 fold increase (range 1.82-280; $p < 0.001$). Limited mechanistic studies performed after 6 months of treatment demonstrated reduced peanut skin prick test wheal size and increased peanut sIgE but no change in basophil reactivity.

Overall, reported rates of desensitisation from cow's milk, egg and peanut OIT studies range from 40-90% in actively-treated subjects, compared to 0-12% of control or placebo-treated subjects, indicating that OIT can induce desensitisation. However, it is important to highlight that there is significant heterogeneity in treatment protocols including duration of treatment, study design and outcome measures, which may limit comparability of reported outcomes.

1.2.8. Long-term outcomes in subjects achieving desensitisation

It is important to understand long term outcomes following OIT when considering the risk vs benefit of this treatment approach. Keet et al presented long-term follow-up data on 32 children from 2 early cow's milk OIT studies [11]. In both studies, desensitisation was the primary outcome and participants were advised to continue regular cow's milk intake following completion of the studies. After a median of 4.5 years in the first study and 3.2 years in the second study, 7 of 32 (22%) participants were limiting their cow's milk intake due to symptoms and only 25% of children had no symptoms with long-

term cow's milk intake. Moreover, 22% (6/27) of participants who continued regular cow's milk intake reported at least one episode of anaphylaxis and one subject who maintained regular cow's milk intake reported using intramuscular adrenaline at least twice a month for reactions to cow's milk. Similarly two other long term follow-up studies reported that 58-81% of cow's milk desensitised children were able to maintain regular cow's milk intake up to seven years after completion of treatment, however cow's milk induced symptoms were reported by 18-50% of participants [12 13].

These findings suggest that desensitisation may not be an optimal outcome for patients with food allergy in the longer term as allergic reactions are common and can occur unexpectedly. Further studies evaluating quality of life measures, as well as the frequency and severity of allergic reactions in desensitised individuals compared with those maintaining allergen avoidance are required to clarify the benefits and safety of desensitisation as an outcome in food allergic patients.

1.2.9.Sustained unresponsiveness

The ability to tolerate a food allergen after OIT has been discontinued for a period of time, referred to as sustained unresponsiveness (SU), has been assessed in a small number of studies.

An early egg OIT RCT [14] reported that 11 of 40 (27.5%) egg OIT (receiving 2 g/day of egg protein) treated children achieved SU (secondary elimination period of 4 to 6 weeks) after 22 months of treatment compared with 0% (0/15) of placebo treated participants. It is important to note that placebo participants were unblinded after 10 months and assessment for SU was only performed if their egg sIgE fell below 2 kU/L; hence, only 1 of 15 placebo participants underwent the second oral food challenge to assess for SU at 22 months, whereas SU food challenges was completed for all active (OIT treated) participants who had not withdrawn from the study by that time point. This study design introduces bias and findings should be interpreted with caution. In a follow up study, OIT was continued in OIT treated participants who failed to achieve SU at 22 months until such time as their egg sIgE fell below 2 kU/L. SU was achieved in 20 of 40 subjects (50%) after up to 4 years of treatment, however once again this finding should be interpreted with caution since there was no parallel placebo treated

comparison group to control for natural resolution of egg allergy [15]. Two other studies in which participants received active egg OIT for shorter periods (one egg or approximately 6 g of egg protein every 48 hours for 3 months [16]; 4 g/day for 4 months followed by 6 months ad libitum egg containing diet [17]) reported lower rates (31-37%) of SU. These findings suggest that duration of treatment and/or cumulative OIT dose (as a product of maintenance dose and the duration on maintenance dose) may affect the likelihood of achieving SU.

An open uncontrolled study of peanut OIT [18] involving 39 peanut allergic children (age 1-16 years) (maximum maintenance dose of 4 g peanut protein/day), reported that 12 of 24 children evaluated at trial end achieved SU and passed a 5 g peanut protein DBPCFC following 4 weeks of secondary peanut elimination [19]. However, it should be noted that the criteria for stopping a patient's treatment and administering the SU challenge was a reduction in peanut sIgE to below a certain threshold which was changed (from <2 kU/L to <15 kU/L) half way through the study, resulting in significant variability in treatment duration (mean 4.0 years, standard deviation 1.8 years). Furthermore, the study lacked a placebo group to control for the natural rate of resolution, which is relevant given the extended follow-up period. A recent peanut OIT study involving younger children (age 9-36 months) employed a similar study design, whereby treatment was continued and SU assessed only upon achievement of pre-specified benchmarks - at least 12 months in the maintenance phase; peanut sIgE ≤ 15 kUA/L; SPT ≤ 8 mm; and no severe peanut-related symptoms in the previous 6 months. After a median duration of treatment of 2.4 years (IQR 2.1-3.9 years) at maintenance doses of 300mg (n=20) or 3000mg (n=17), 78% (29/37) of participants passed a 5 g peanut protein DBPCFC following 4 weeks of secondary peanut elimination [20]. Several caveats should be noted when interpreting these findings. Firstly, the study did not include a 'no-treatment' or placebo control group, but included a retrospective cohort of age-matched peanut allergic patients receiving standard care (n=154) followed for an average of 3.6 years (95%CI 3.3-3.8 years). The rate of natural resolution of peanut allergy amongst controls was estimated to be 4%, however the accuracy of this estimate is uncertain given the likely bias in both the selection of the control group and the decision to perform a food challenge to assess for tolerance. Indeed, the rate of resolution of peanut allergy over a similar time frame was reported

to be 22% (over ~3 years) in the HealthNuts study, which uniformly evaluated all participants for resolution of challenge proven peanut allergy at age 4 years; and 23% (over 4.6 years) in a study by Fleischer et al which challenged patients to assess for tolerance if their peanut sIgE had dropped to <5 kUA/L [21 22]. Secondly, baseline peanut sIgE levels amongst OIT treated participants tended to be lower than values in the control group, which may influence outcomes as lower peanut sIgE is associated with greater likelihood of natural resolution of peanut allergy [21]. Moreover the investigators observed that OIT treatment failures had significantly higher baseline peanut sIgE than treatment successes. To address these caveats, Vickery et al performed a worst-case analysis where they reclassified OIT treated participants who had baseline peanut sIgE <5kUA/L as treatment failures and recalculated the rate of resolution of peanut allergy in the controls based on sIgE levels, and found a much smaller difference in estimated rate of resolution of peanut allergy between groups (57% in OIT participants v.s. 31% in controls; (RR 1.8, 95%CI 1.3-2.6, p=0.007). Hence, for both the peanut OIT studies described above, significant variability in treatment duration and the lack of appropriate controls introduce bias and make reported findings difficult to interpret.

An open pilot peanut OIT study involving 23 patients (aged 5-45 years) who received peanut OIT at a maintenance dose of 4 g/day and 20 age matched control patients (aged 6-20 years) who maintained avoidance of peanut for 24 months reported SU (13 weeks after cessation of OIT) in 35% (7 of 20, excluding 3 who were not able to complete 24 months of treatment) of participants in the active treatment group compared with 0% in the placebo group. After a further 3 months of peanut avoidance (6 months after cessation of peanut OIT), only 15% (3 of 20) OIT treated participants maintained SU [23]. Immune studies revealed upregulation of antigen specific T-regulatory cells (Treg), enhanced in-vitro suppressor function and epigenetic changes in T cells of subjects achieving SU. However, no difference in peanut-specific antibody levels or basophil response were found.

Taken together, these studies suggest that OIT may have a limited ability to induce SU in around 30%-50% of patients with egg or peanut allergy and may be more effective if commenced in children under the age of 3 years.

1.2.10. Long term outcomes in subjects achieving SU

Only one study has evaluated long term clinical outcomes in participants who achieved SU at least one year after discontinuation of treatment.

Jones et al, administered two follow-up questionnaires to assess long-term effects of egg OIT. The first questionnaire was administered after the last subject completed OIT and study challenges and the second questionnaire was administered around one year later. The range of follow-up period following discontinuation of study treatment at the time of administration of the questionnaires, although not described in the study, is expected to be wide (estimated to be 1-5 years after stopping OIT for the second questionnaire) and introduces potential reporting bias. Acknowledging this limitation, at the time of the first questionnaire a significantly higher proportion of actively treated subjects were ingesting any egg as compared to placebo treated subjects (82% vs 36%, $p=0.07$). One year later, there was no difference in the rate of ingestion of any egg between groups (85% vs 67%, $p=0.22$). It is notable that 4 of 18 (22.2%) OIT subjects who achieved SU reported symptoms on egg ingestion in the 12 month period between the first and second follow-up, despite maintaining regular baked and unbaked egg ingestion, indicating re-sensitisation to egg [15].

These recent studies confirm that OIT can induce desensitisation in a majority of patients, however the ability to induce tolerance (SU) remains uncertain and appears to be limited in most reports. Furthermore, Syed et al's study demonstrates that SU may be lost following longer duration of secondary elimination. This suggests that ongoing allergen intake may be required for maintenance of a tolerance state, consistent with recent findings in murine models [24]; nevertheless continued allergen intake may not necessarily provide absolute protection against resensitisation [15].

1.2.11. Combining OIT with immune modifying adjuvants or anti-allergy therapies

1.2.12. Combined administration of probiotic and OIT

Probiotics are live micro-organisms that confer a health benefit to the host and may act by directly interacting with mucosal immune cells and/or indirectly through

modulation of the intestinal microbiota. Tang et al recently completed a randomised placebo-controlled trial [25] evaluating a combined probiotic (*Lactobacillus rhamnosus*) and peanut oral immunotherapy (PPOIT) in 62 peanut allergic children who were randomised to receive either PPOIT or placebo for 18 months. SU (assessed by DBPCFC performed 2-5 weeks after treatment cessation) was achieved in 82.1% (23/28) of PPOIT and 3.6% (1/28) of placebo treated children. Immunomodulatory changes were observed in the active treatment group with reduction of peanut SPT and sIgE and increase in peanut sIgG4 suggesting modulation of the peanut specific allergic response.

This rate of SU is notably higher than previously reported with OIT and suggests that combined administration of the probiotic immune modifying adjuvant may enhance effectiveness of OIT in inducing SU. A further multicenter RCT comparing PPOIT vs peanut OIT vs placebo is underway to clarify whether addition of a probiotic confers greater benefit than OIT alone. It should also be noted that the duration of secondary peanut elimination before assessment of SU in the study by Tang et al was relatively short (2-5 weeks) so it is possible that the rate of SU would be lower if assessed a longer time after stopping PPOIT. The multicenter RCT that is underway will assess for SU after 12 weeks peanut elimination to evaluate the effectiveness of PPOIT to induce longer-term SU compared with placebo or OIT alone.

1.2.13. Omalizumab and OIT

Omalizumab is a monoclonal antibody against IgE. It is expected that administration of omalizumab with OIT should improve the tolerability of OIT. A pilot study involving 13 peanut allergic children reported that 12 of 13 children pretreated with omalizumab for 12 weeks reached the maximum peanut OIT maintenance dose of 4 g/day after 8 weeks. Clinical effectiveness of OIT was not compromised with 12 subjects tolerating 8 g of peanut protein on either DBPCFC or open challenge after a further 12 weeks of peanut OIT without omalizumab [26]. Similarly, in a multi-centre DBPC RCT [27] of omalizumab combined with cow's milk OIT (28 subjects) vs placebo with cow's milk OIT (29 subjects) in milk allergic patients aged 7-35 years of age, tolerability of OIT was improved by the use of omalizumab. Subjects who received omalizumab reported that 91.5% doses taken during the escalation phase were symptom-free compared to 73.9% in the placebo group ($p < 0.0001$). Furthermore, frequency of needing adrenaline

treatment was higher in the placebo treated group vs omalizumab treated group (18 doses in 9 subjects vs 2 doses in 2 subjects) ($p=0.052$). Rates of desensitisation (omalizumab with milk OIT 85.71% (24/27) vs placebo with milk OIT 71.43% (20/28)) and SU after a secondary elimination period of 8 weeks (omalizumab with milk OIT 48.1% (13/27) and placebo with milk OIT 35.7% (10/28)) were comparable. These findings suggest that omalizumab may be used to enhance the safety and tolerability of OIT, without compromising the ability to induce desensitisation or SU.

1.2.14. Ketotifen and OIT

Ketotifen is a benzocycloheptathiophene with histamine receptor antagonist and mast cell stabilising properties. Coadministration of Ketotifen with OIT would be expected to reduce allergic reactions to OIT. In a small study involving 6 peanut allergic children (median age 10 years), ketotifen premedication from 2 weeks prior to and continued until 4 weeks after commencement of peanut OIT improved the tolerability and reduced the rate of gastrointestinal symptoms in subjects receiving OIT (reaction rate of 40% of all doses taken in the ketotifen group vs 76% of all doses in the placebo) [28].

1.2.15. OIT using heat treated food proteins

Heating induces conformational changes to allergenic epitopes responsible for IgE binding, and therefore may improve the tolerability of an allergenic protein in subjects who would otherwise react to natural and unheated forms of the protein. Linear epitopes, unaltered by the heating process, retain their immunomodulatory effects, most probably through induction of allergen-specific IgG and T cell responses. Therefore, OIT using extensively heated forms of a protein may confer clinical effects with improved tolerability profile. Observational studies describe resolution of egg allergy in children who can tolerate and incorporate baked egg in their diets, although the absence of a control group prevents assessment of effectiveness above and beyond natural resolution of disease. A recent pilot study of cow's milk allergic children who had failed to complete the parent unheated cow's milk OIT study, 6 (out of 14) subjects who were placed on baked milk OIT for 12 months achieved a significant increase in the median reaction eliciting dose during an unheated milk challenge (6 vs 360mg, $p=0.005$) [29]. However, the majority of subjects could not tolerate the target

maintenance dose of 1.3 g/d of baked milk protein nor could they complete the study per protocol, suggesting limited tolerability of extensively heated proteins in allergic subjects.

Boiling of nuts may similarly result in loss of allergenic proteins—particularly Ara h 2, 6, and 7—from peanut seeds, possibly through leaching of allergens into cooking water or conformational change. A small observational study involving 4 peanut allergic children showed varying ability to tolerate boiled peanut (which contained 2S albumins) suggesting that boiled peanut OIT may be better tolerated than native peanut OIT [30]. Larger controlled studies are underway (BOPI-1: NCT02149719) to confirm these findings and to evaluate effectiveness of these approaches for induction of SU.

1.2.16. OIT using multiple foods

Food allergic patients often have more than one food allergy. Hence, the ability to perform OIT with several foods concurrently would be more desirable than sequential single food immunotherapy treatments, which may take many years to complete. A single centre open pilot study compared tolerability of single-food and modified multi-food OIT schedules in 15 monoallergic (defined as sensitisation and positive reaction to a cumulative dose of 182mg peanut protein on DBPCFC) and 25 polyallergic (defined as having peanut allergy and at least one additional allergy to a treenut, sesame, milk or egg, with sensitisation and positive reaction to a cumulative dose of 182mg on DBPCFC) participants. Participants receiving single food OIT underwent rapid desensitisation on day one followed by fortnightly dose escalations until the maintenance dose of 4 g/day was reached. Participants receiving multi food OIT received the same total amount of allergenic protein for each allergen as per the standard schedule. Dose escalation phase continued until a daily maintenance dose of 4 g/day of each allergen (up to 20 g cumulative dose for 5 allergens) was reached [31]. There was no difference in reaction rates between multi-allergen v.s. single allergen desensitisation groups (median of 3.3% and 3.7% in multi and single OIT group, respectively; $p = 0.31$). Adrenaline was used twice in each group (8% of multi-allergen treated group; and 13% of single allergen treated group). Clinical efficacy (induction of desensitisation or SU) was not assessed.

1.2.17. Sublingual immunotherapy (SLIT)

Early food allergen SLIT trials reported limited clinical efficacy. A more recent multicentre study randomised 40 peanut allergic subjects, aged 12 to 37 years (median, 15 years), 1:1 to daily peanut or placebo SLIT. After 44 weeks of SLIT, 14 (70%) of 20 subjects receiving peanut SLIT were able to consume 5 g peanut protein or at least 10-fold more peanut powder than their baseline threshold compared with 3 (15%) of 20 subjects receiving placebo ($p < 0.001$) [32]. However, most patients discontinued therapy by the end of year 3, and only 10.8% (4/37) SLIT-treated subjects achieved SU after secondary elimination of peanut for 8 weeks [33].

A head to head peanut SLIT vs OIT study [34] involving 21 children (aged 7-13 years) with challenge confirmed peanut allergy randomised subjects to receive either SLIT (3.7 mg/d maintenance) with placebo OIT or OIT (2 g/d maintenance) with placebo SLIT for 12 months. A 5 g peanut challenge was then administered to evaluate for desensitisation. 1 out of 7 (14.3%) of active OIT treated subjects passed the challenge vs none (0%) of the 9 active SLIT treated subjects. At this point subjects were unblinded. 3 active OIT subjects continued on active OIT (without SLIT), 3 active OIT subjects continued on active OIT with add-on active SLIT and 9 active SLIT subjects continued on active SLIT with add-on active OIT for 6 months. 3 out of 3 active OIT subjects passed the second desensitisation challenge and 2 of them (66.7%) passed the SU challenge after peanut avoidance for four weeks. 1 out of 3 active OIT with add on SLIT subjects passed the second desensitisation but this subject failed the SU challenge. 5 out of 7 active SLIT with add on OIT subjects passed the second desensitisation challenge and only 1 (14.3%) of them passed the SU challenge. These findings suggest that OIT may be more effective at inducing both desensitisation and SU than SLIT. However, the size of the study and its study design potentially dilutes larger effect differences that may have been observed if active therapy with either active OIT or active SLIT was continued for a longer duration.

1.2.18. Epicutaneous immunotherapy (EPIT)

There is evidence suggesting that epicutaneous exposure to allergens elicits immune responses. Early animal models evaluating the delivery of allergens topically and

epicutaneously through intact skin conferred beneficial immunomodulatory effects leading to more recent studies of epicutaneous immunotherapy in humans.

A multicenter DBPC phase II-b trial [35] involving 221 challenge proven peanut allergic subjects (aged 6 – 55 years), randomised subjects to receive Viaskin Peanut (VP), at different doses (50mg, 100mg, 250mg peanut protein) or placebo for one year. The best response was seen in the group that received VP250, with 50% of actively treated subjects responding (defined as an increase in post treatment cumulative reactive dose ≥ 10 fold of basal reactive dose or post treatment cumulative reactive dose reaching ≥ 1000 mg peanut protein) compared to 25% in the placebo group, $p=0.0108$. Effects were greatest in children with limited benefit in adolescents and adults. These preliminary findings suggest that EPIT may be effective at inducing desensitisation and improve subjects' ability to tolerate low doses of peanut following treatment. A follow study to assess induction of SU after an additional 12 months therapy is ongoing.

1.2.19. Conclusion

There is a need for disease modifying treatment options for food allergy. Amongst the various novel approaches being explored in the research setting, allergen specific immunotherapy delivered orally offers the most promising option. There is emerging evidence to suggest that co-administration of OIT with immune modifying adjuvants such as probiotics may further enhance efficacy and tolerability. Without deeper understanding about long term and sustained effects, adverse effects and impact on quality of life, it is still premature to offer novel treatments such as OIT in the routine clinical setting. Elucidating underlying immunologic mechanisms driving the clinical effects of allergen immunotherapy may help identify biomarkers to optimise selection of patients who are most likely to benefit from such treatment.

1.3. Summary of more recent advances in the field of peanut oral immunotherapy (OIT)

More studies confirming the effectiveness of peanut OIT at inducing desensitisation have emerged since the publication of the doctoral candidate's literature review article as presented in the previous section. In one placebo controlled peanut OIT study, 74% (23/31) of OIT treated participants achieved desensitisation (defined as tolerating 300 mg of peanut protein) after 16 months of OIT treatment as compared to 16% (5/31) of placebo treated participants[36]. A large Phase-3 randomised controlled trial of proprietary treatment product, AR101, the treatment schedule of which includes 24 weeks at target maintenance dose of 300 mg/d of peanut protein, confirmed the ability of peanut OIT to induce desensitisation in 67.2% (250/372) of OIT treated participants as compared to 4.0% (5/124) of placebo treated participants, supporting findings from an earlier Phase-2 study involving the same product[37]. Whilst most peanut OIT studies have investigated the effect of treatment on younger patients with peanut allergy, a small randomised controlled trial involving older children and teenagers aged 12 to 18 years showed that peanut OIT was effective at inducing desensitisation in 81% (17/21) participants as compared to 11% (1/9) of those who received placebo treatment[38].

Very few studies have evaluated the durability of desensitisation and sustained unresponsiveness (SU) after discontinuation or dose-reduction of OIT treatment. In a recent study[39], although SU was attained by 84% of peanut OIT treated subjects, after cross-over to placebo treatment, the proportion passing subsequent peanut challenges dropped to 35% by the end of the first year, confirming earlier findings[40] that SU may not be durable once patients discontinued treatment.

There has been recent discussion whether 'remission' from food allergic reactions might better describe the temporary state of non-responsiveness to culprit allergens post-OIT-treatment [41]. This concept acknowledges the variable durability of clinical response and recognises that patients' peanut allergy may relapse off-treatment after an extended period of time. There remains no consensus on the definition of tolerance following OIT treatment but it has been suggested that remaining non-responsive, as

demonstrated by an ability to pass peanut challenges, years after cessation of OIT treatment and ingesting peanut ad libitum, that is, not depended on continued and regimented allergen exposure, might most closely resemble tolerance[42].

It is widely recognised that OIT is associated with more side effects than other allergen immunotherapy. A landmark meta-analysis of 12 peanut OIT trials involving 1041 participants found that OIT treatment was associated with heightened risk of anaphylaxis (relative risk, RR, 3.12; 95% confidence interval, 95% CI, 1.76, 5.55), adrenaline use (RR 2.21; 95% CI 1.27,3.83) and serious adverse events (RR 1.92; 95% CI 1.00,3.66)[43]. The study confirms that despite OIT treatments' aim to protect participants from peanut allergic reactions by inducing desensitisation and/or SU, treated participants experienced more allergic and anaphylactic reactions, highlighting the need to further improve the safety and tolerability of food allergen immunotherapy.

Gastrointestinal side effects are commonly observed during OIT. Whilst these symptoms are generally mild, some symptoms may be severe and may lead to OIT treatment discontinuation. Symptoms of eosinophilic oesophagitis (EoE) resemble many of the recognised gastrointestinal side effects of OIT. Earlier studies reported biopsy-confirmed EoE in 2.7 to 4.7% of patients undergoing food OIT[44 45]. In a recent study reviewing the rates of immunotherapy discontinuation and biopsy-confirmed EoE in 110 OIT studies, the overall rate of OIT treatment discontinuation was around 14%. Reasons for discontinuation were provided in 82 studies and the combined data suggested that gastrointestinal symptoms led to discontinuation in around 4.7% of patients. In this study, the authors defined EoE symptoms as general gastrointestinal symptoms, including abdominal pain, nausea, vomiting, anorexia or gastro-oesophageal reflux. The overall rates for possibly EoE-related symptoms were 34% for general GI symptoms, 32% for abdominal pain and 12% for vomiting. In 18 studies (involving 663 patients), biopsies were conducted to confirm the diagnosis of EoE. The rate of biopsy confirmed EoE was approximately 5.3%[46]. In another review which included 12 recently published food OIT studies involving 620 patients, the authors reported 0.3% of total patients or 8.3% of patients with gastrointestinal symptoms during OIT had biopsy-confirmed EoE[47]. There is emerging data to suggest that in some patients their EoE might not remit despite stopping OIT treatment[48]. More research is needed to

address the knowledge gap of OIT-related and patient-related factors predisposing the development of EoE in patients undergoing food OIT. Excitingly, there may be a role for, combining with food OIT, immune adjuvants effective at redirecting the Th2 immune response and treating EoE such as dupilumab to decrease the risk of EoE and oesophageal inflammation in susceptible patients.

1.4. Immune mechanisms mediating clinical effects of food oral immunotherapy (OIT)

As discussed in the earlier sections, food OIT is effective at inducing states of remission from allergic reactions to culprit food allergens, including desensitisation and sustained unresponsiveness (SU). Elucidating underlying mechanisms that underpin the effect of OIT and the immunologic changes that accompany the desirable clinical effects helps to identify potential therapeutic targets and opportunities to refine food allergy treatment. In addition, such understanding may also lead to the discovery of suitable biomarkers to optimise selection of patients for treatment and monitor their treatment response.

OIT induces a number of immune changes comparable to those observed in other immunotherapy models (for example pollen immunotherapy and venom immunotherapy)[49]. This section focuses on clinically relevant mechanisms of action of OIT observed in human peanut OIT studies. The doctoral candidate will initially describe the effect of OIT on immune compartments throughout treatment (from pre-treatment to post-treatment phases) before moving on to describe immunologic changes that accompany OIT treatment associated clinical outcomes including desensitisation and SU.

1.4.1. The effect of OIT on end effector cells

The role of end effector cells including circulating basophils and tissue mast cells mediating pathogenic allergic responses to food antigens in individuals with food allergy is well-recognised[50]. On commencement of food OIT, exposure to escalating antigen doses is associated with suppression of antigen-specific basophil response[18 32 51 52]. These early effects may be mediated by the internalisation of receptors observed in other desensitisation models[53]. Whilst suppression of basophil reactivity is evident during treatment, this effect is lost soon after treatment cessation and does not appear to be predictive of or associated with more durable clinical remission states such as SU[52].

1.4.2.The effects of OIT on antibody production and B cell responses

1.4.3.Allergen specific-IgG, -IgG4 and -IgA

OIT treatment is often accompanied by an early and rapid rise in allergen specific-IgG4 (sIgG4) and specific-IgA (sIgA) [18 20 54-56], possibly in response to interleukin (IL)-10 and transforming growth factor (TGF)- β production by T-regulatory (Treg) cells, regulatory B cells and/or T-helper (Th) 3 cells. Mucosal sIgA may play a role in gut luminal antigen binding and block antigen transport by gut epithelial cells[49]. Antigen sIgG4 production is increased in response to OIT treatment. IgG and IgG4 antibodies with antigen specificity may block the access of antigens to effector cell surface-bound antibodies and mediate transient basophil and mast cell hyporesponsiveness in OIT-treated subjects. In-vitro studies recently demonstrated that peanut sIgG4 containing plasma obtained from peanut OIT-treated subjects inhibited peanut specific basophil reactivity, whereas depletion of peanut sIgG4 restored peanut induced mast cell reactivity[57]. Circulating IgG4 bind to the inhibitory Fc receptor, Fc γ RIIB, on mast cells to actively suppress effector cell function[58]. In a recent peanut OIT and sublingual immunotherapy (SLIT) comparison trial[34], peanut sIgG4 production was higher in subjects treated with higher dose OIT as compared with lower dose SLIT treatment. Subsequent addition of OIT treatment to subjects who initially received SLIT led to an increased production in peanut sIgG4 to levels comparable to the primary OIT-treated group. This suggests that the magnitude of sIgG4 induction may be immunotherapy treatment dose- and/or route- dependent.

Mucosal sIgA may play a role in gut luminal antigen binding and block antigen transport by gut epithelial cells[49]. It was recently shown in a mouse study that the induction of peanut sIgA through daily gastrointestinal peanut exposure required T cells and CD40 ligand but was T follicular helper (Tfh) cell, germinal center, and T follicular regulatory (Tfr) cell-independent, contrasting the requirement of Tfh for IgG1 and IgE production. These findings suggest an alternative paradigm of divergent cellular mechanism of IgA versus IgE and IgG1 production to food antigens[59].

1.4.4. Allergen specific-IgE

Allergen specific-IgE (sIgE) mediates immediate-onset type-1 allergic reactions in susceptible individuals. Recent in-vitro studies suggest that high affinity sIgE antibodies and their characteristics including clonality, epitope-specificity and post-translational modifications contribute to individual to individual variations in clinical response to culprit allergens[60]. In subjects treated with food immunotherapy, levels of circulating allergen sIgE initially increase in response to treatment. Early uncontrolled peanut OIT observational studies using extended treatment durations of over 24 months showed gradual decline in peanut sIgE over time[61 62]. Whilst levels of sIgE initially increase in response to both SLIT and OIT treatment, OIT-treated subjects have lower levels of sIgE at end of treatment as compared to SLIT-treated subjects suggesting dose- and/or route-dependent response[34]. A recent peanut OIT study reported significantly reduced levels of circulating peanut, Ara h1, Ara h2 and Ara h3 sIgE, and reduced ratios of peanut sIgE versus total IgE in response to treatment[63].

1.4.5. Memory B cells

Food immunotherapy has been shown to modulate B cell repertoire resulting in changes in the characteristics and function of IgE-switched memory B cells[60]. In an early study[62], Vickery's group reported that around a third of peanut OIT-treated subjects had an increase in peanut specific antibody repertoire and concurrent contraction of total peanut sIgE. This suggests that new B cell clones may have emerged and these clones produced novel epitope sIgE in response to treatment. Variability in B cell response may underlie between individual differences in their clinical response to OIT.

In another controlled peanut OIT study, peanut component allergen (Ara h2) specific class-switched memory B cells expanded transiently, peaking at week 7 of treatment and declined rapidly after that. Ara h2 sIgE also increased, peaking around 7 weeks, and slowly declined towards baseline by end of treatment[64]. Similar observations were made in another peanut OIT study[65] where there was also a treatment associated 3-fold increase from baseline in Ara h1 and Ara h2 specific B cells. These studies provide evidence that OIT induced allergen specific B cell and sIgE responses that followed

similar kinetics of initial increase followed by a slow decrease over time whilst on treatment. Long-term B cell and antibody responses years after discontinuation of food immunotherapy treatment still need to be rigorously investigated.

1.4.6. The effects of OIT on T cells and other cellular responses

1.4.7. T-regulatory (Treg) cells

Treg cells are capable of producing anti-inflammatory cytokines such as IL-10 and are thought to contribute to the maintenance of peripheral allergen tolerance in non-allergic individuals[66]. However their precise role in allergic individuals and these individuals' response to OIT remain unclear. The small number of OIT studies investigating circulating Treg numbers report mixed effects, with some studies reporting increases[18 54] and one study reporting no change[19] in association with treatment. Whilst OIT treatment induced antigen-specific Treg cells have suppressive function[40], the durability of such function may not be long-lasting[67].

1.4.8. Pathogenic T cells

Pathogenic T cell response is initiated by epithelially-derived soluble mediators including thymic stromal lymphopoietin (TSLP), IL-25 and IL-33[68 69]. T cell activation contributes to activation of eosinophils and allergen sIgE production through skewed T-helper(Th)-2 response pathways.

On commencement of food immunotherapy, low dose exposure might not only reinforce pathogenic Th-2 cell response but also generate a cytokine milieu including upregulation of IL-4 that impairs the establishment of regulatory mechanisms[69].

Persistent allergen exposure, and possibly through exposure to higher allergen doses, appear to be accompanied by a decrease in Th-2 cell numbers and activity (as measured by cytokine production) and/or changes in their phenotype to IL-10 producing CD4+ T cells or inducible Treg cells[49].

1.4.9. Cytokine production

Excessive IL-4 signaling hampers the induction of food allergen specific Treg cells and their ability to suppress mast cell activation[58]. Inactivation of Th-2 response pathways appears to protect against food allergy[70]. However, findings from OIT studies examining the effects of treatment on cytokine production by peripheral blood mononuclear cells (PBMC) are mixed [18 54 55].

1.4.10. Other cellular responses

In a recent study longitudinally monitoring peanut-component(Ara h2)-specific CD4+ T cells (using MHC-peptide Dextramers to stimulate followed by RNAseq assay) from a small number of HLA-matched patients undergoing peanut OIT (n = 30), there was transient upregulation of TGF- β regulatory activity during OIT treatment[71]. The authors found a cluster of markers with high expression of STAT family members which showed a possible transient increase at week 24 in those with treatment success [defined by the authors as patients who either continued to take 300 mg/d of peanut (n=3), or abstained from peanut for 13 weeks before passing their week 117 challenge(n=3)] relative to those with treatment failure [defined by the authors as those who achieved 4 g end-of-treatment challenge at week 104, but failed week 117 challenge either on or off ongoing peanut ingestion between weeks 104 and 117, (n=16)]. Interestingly, OIT treatment, irrespective of treatment outcome, was associated with the disappearance over time of a cluster, with combined expression of GATA3, IL-4, IL-13 and BCL6, resembling the recently described T-follicular-helper(Tfh)-13 cells[72].

1.4.11. Immunologic changes accompanying clinical outcomes

1.4.12. Desensitisation

As discussed earlier, food immunotherapy is accompanied by an increase in allergen sIgG4. These antibodies may mediate transient basophil and mast cell hyporesponsiveness. In a peanut SLIT study[33], allergen sIgG4 was observed to be higher in successfully desensitised individuals. However, many of these desensitised individuals failed to demonstrate 8-week sustained unresponsiveness (SU) when

challenged one year later, suggesting that the observed rise in peanut sIgG4 does not predict nor mediate persistence of clinical remission post- treatment.

Removal of allergen sIgE does not appear to be necessary for the achievement of desensitisation in early stages of OIT treatment. However, clinical trials[73 74] incorporating adjuncts such as omalizumab, through the removal of IgE, allow more rapid escalation of allergen doses without increased clinical reactions.

Food immunotherapy induced basophil suppression is transient and reverses after withdrawal of treatment[52]. In an early study, peanut OIT induced basophil hyporesponsiveness which was thought to contribute to the clinical state of desensitisation[51]. Although most subjects in this cohort achieved desensitisation, there was no evaluation of SU or attempt to correlate basophil hyporesponsiveness with SU. In another study, basophil reactivity was unable to differentiate OIT induced immune-tolerance versus non-tolerance[40]. Importantly, a recent study reported that peanut OIT treated subjects who failed to respond (defined by the authors as those who became reactive to 4 g of peanut at 13 weeks post-treatment) had higher levels of peanut induced basophil activation than treatment responders (that is, those who had SU)[63]. However, the study was limited by not being able to associate the subjects' basophil activation with their clinical outcomes beyond 3 months post-treatment. In addition, the proportion of subjects having SU declined substantially by the end of the first year post-treatment[39]. Therefore, it remains uncertain whether suppression of basophil activation post-treatment is predictive of durable SU.

1.4.13. Sustained unresponsiveness (SU)

Although the induction of allergen sIgG4 antibodies is one of the most consistently observed immune changes accompanying allergen immunotherapy, levels of sIgG4 often fail to correlate with clinical response[75-77]. There is still no robust evidence that allergen sIgG4 facilitates the acquisition of SU or tolerance after OIT.

In an uncontrolled peanut OIT study[19], peanut sIgG4 increased during treatment but sIgG4 levels at end of treatment failed to discriminate those who attained SU versus those that did not. Similar observations were made in another study where absolute

levels and trajectories of peanut sIgG4 during treatment and at 3 months post treatment did not differ between SU outcome groups[40]. Taken together, these findings suggest that the induction of allergen sIgG4 might not be critical for the induction of remission states such as SU.

In contrast, the suppression of allergen sIgE production may play a role mediating immunotherapy induced SU. Treatment responders of a peanut OIT study[19] had significantly lower peanut sIgE pre-treatment and post-treatment suggesting that low levels of sIgE at end of study was required to support the clinical state of SU. In another study[40], whilst both immune tolerant (IT) and non-immune tolerant (NT) individuals showed downward trends in levels of peanut sIgE from 9 to 12 months of treatment, the sIgE trajectories diverged post-treatment. In the non-responders, peanut sIgE rebounded within 6 months of treatment cessation, in contrast to the ongoing decline in those who attained persistent SU. More studies investigating the longitudinal effects of OIT on allergen sIgE is necessary to provide insight into possible role of IgE suppression in mediating long-term SU (LTSU) and tolerance years after discontinuation of food allergen immunotherapy treatment.

Very few studies have evaluated whether circulating Treg cells can discriminate OIT treatment responders versus non-responders. In one peanut OIT study[40], treatment responders had increased circulating Treg cells. Treg cells from treatment responders also had enhanced suppressive function and had an epigenetic signature characterised by hypomethylation of CpG sites of the FOXP3 gene.

1.4.14. OIT and tissue level immune response

There is still very limited understanding regarding the possible role of gastrointestinal tract stromal cells and secondary lymphoid organs in mediating the effects of OIT. Most human OIT mechanistic studies have examined the peripheral blood given logistic and ethical challenges in association with repeated gastrointestinal tissue sampling from human subjects. There is emerging evidence to suggest that antigen presenting cells (APC) and dendritic cells (DC) may play a role in mediating the effects of OIT[40 78], through the antigens' interaction with cells including epithelial cells, innate lymphoid cells and local microbial factors.

1.4.15. OIT and gut microbiome

Intestinal microbiota play a role in maintaining the balance between immune tolerance and inflammation in the gut[79]. The induction and expansion of mucosal Treg is promoted by intestinal macrophages in response to microbial signals through IL-1 β and downstream local type-3 innate lymphoid cells (ILC-3)[80]. These cells promote secretion of retinoic acid and IL-10 by macrophages and DC. There is also evidence supporting links between B-cell phenotype, gut microbiota composition and allergic sensitization to dietary antigens. Furthermore, microbial antigens or metabolic products, such as toll-like-receptor agonists or short chain fatty acids, can modulate epigenetic, transcriptomic, and metabolic pathways resulting in changes in the production and repertoire of secreted immunoglobulins[81]. On the other hand, there is recent data suggesting that OIT may indirectly affect microbial composition of the gut through the Immunomodulatory effects of OIT on the host immune system[82]. In the presence of a cytokine milieu favouring tolerance, tissue-resident and circulating immune cells may be more responsive to the effects of OIT. Therefore, combining OIT with immune modifying adjuvants such as probiotics may enhance the effects of OIT. However, studies are needed to confirm whether measures aimed at manipulating gut microbial composition favourably enhance patients' clinical and immune response to OIT.

1.4.16. Conclusion

OIT is associated with both early and later changes to allergen specific end effector cell, antibody and cellular responses. Immunologic changes that accompany clinical states of desensitisation may differ to those that accompany more sustained clinical remission states such as SU and tolerance. Desirable changes include sustained anergy and/or deletion of upstream pathogenic memory T cells and B cells in order to maintain durable response.

1.5. Chapter summary

An effective disease modifying treatment for food allergy is needed. Oral immunotherapy (OIT) is emerging as a treatment option and has been shown to induce transient (such as desensitisation) and more persistent (such as SU) remission from allergic reactions in food allergic individuals. There remain many unanswered questions relating to the clinical and immune effects of OIT. Durability of clinical remission in the years after discontinuation of OIT treatment has not been studied extensively. It remains unclear whether short-term immunologic changes involving antibody and cellular responses are maintained long-term. Furthermore, immune responses which accompany the beneficial effects of OIT years post-treatment need to be elucidated.

In this thesis, the doctoral candidate examines the clinical and immunologic effects of a combined probiotic and peanut oral immunotherapy (PPOIT) treatment in 2 clinical trials, (a) a follow-on study of the PPOIT-001 trial, a randomised controlled trial of PPOIT in children with peanut allergy, and (b) the PPOIT-002 trial, a single-arm open-label trial of PPOIT using an accelerated dose escalation schedule, to gain better understanding of (1) the long-term clinical and immunologic effects of PPOIT, (2) participants' antibody and cellular responses to PPOIT treatment, and (3) the clinical effects, feasibility and tolerability of PPOIT using a modified treatment schedule.

Whilst the conception and design of PPOIT treatment was based on a theoretical advantage conferred by manipulating gut microbiome through combining a probiotic (*Lactobacillus rhamnosus* ATCC 53103; with demonstrated immunomodulatory effects in vitro and in vivo[83]) with peanut OIT, the study designs of PPOIT-001 and PPOIT-002 trials, specifically the absence of an OIT alone treatment arm, precluded the ability to investigate any additional clinical and immunologic effects of the combined therapy over and beyond the effects of peanut OIT alone. Whilst not a part of this doctoral thesis, a major multi-center 3-arm randomised trial (PPOIT-003 Trial, ACTRN12616000322437) leveraging findings reported in this thesis and investigating PPOIT, peanut OIT and placebo, is underway to elucidate additional effects of adding a probiotic to peanut OIT.

In the next chapter, the doctoral candidate describes the rationale, aims and hypotheses for the Part 1 of the thesis, a follow-on study of the PPOIT-001 trial.

Chapter 2. Methods: Follow-on study of the PPOIT-001 trial, a randomised controlled trial of a combined probiotic and peanut oral immunotherapy (PPOIT) in children with peanut allergy

2.1. Chapter introduction

As described in Chapter 1, peanut oral immunotherapy is effective at inducing sustained unresponsiveness in 30-60% of treated individuals with peanut allergy. There is no published data on longer term clinical outcome at one year or more after cessation of OIT treatment. Peanut specific-IgG4 (sIgG4) antibody levels generally rise in association with treatment. In contrast, findings regarding other immune markers including peanut specific-IgE (sIgE), T-regulatory (Treg) cells and cytokine responses are mixed. Part 1 of this thesis addresses some of these important knowledge gaps in relation to OIT.

Chapter 2 describes the rationale, aims and hypotheses for conducting for Part 1 of the thesis, a follow-on study of the PPOIT-001 trial and elaborates on the methodological details of the trial.

2.2. Rationale

Food allergy affects 5-8% of children and 2% of adults, and peanut allergy affects 1.5% of children[84-86]. Reactions to peanut may be severe and peanut allergy is responsible for 30% of deaths from food induced anaphylaxis[87]. Unlike other food allergies, peanut allergy is usually lifelong[88]. Current management of food allergy requires strict avoidance of the food; however, 50% of peanut allergic patients are accidentally exposed to peanut within 2 years[89]. Therefore, children with peanut allergy are at continued risk of severe life-threatening allergic reactions throughout their lifetime.

OIT is being pursued as a potential food allergy treatment to induce desensitisation (defined as being able to tolerate the allergen whilst on treatment)[5 14 55 90-93] and/or sustained unresponsiveness (SU; defined as being able to tolerate the allergen after stopping treatment for weeks to months)[40 94]. Studies show that OIT can induce desensitisation in around two-thirds and SU in around one-third of study participants[1]. There is little data on long-term effects of OIT following treatment cessation. One study reported no long-term difference in rates of egg ingestion between OIT-treated and placebo-treated subjects[15]. A second study of milk OIT reported that 75% of desensitised participants experienced symptoms with milk ingestion (25% had no symptoms with milk ingestion), 38% had frequent and predictable symptoms and 19% experienced at least 1 anaphylaxis reaction in the preceding year[11]. There is no published data on long-term outcomes following peanut OIT. A combined probiotic and peanut OIT (PPOIT) was shown to successfully induce desensitisation and sustained unresponsiveness (SU, previously referred to as tolerance) in the PPOIT-001 trial, a double blind placebo controlled RCT[95]. Just over 80% of the participants who received PPOIT attained 2 week SU compared with only 4% of those who received placebo. The trial provided an important opportunity to study the long-term clinical outcomes and longitudinal immunologic effects of PPOIT in children with peanut allergy.

2.3. Aims

The primary aims of the follow-on study of the PPOIT-001 trial were:

- a) To examine the effects of combined treatment with probiotic and peanut OIT (PPOIT) as compared to placebo treatment on conferring (i) long-term ability to continue peanut ingestion, and (ii) '8-week sustained unresponsiveness (SU)' which was defined as the ability to tolerate 4 g of peanut protein in an oral peanut challenge, at 4 years after discontinuation of PPOIT-001 study treatment and at least 8 weeks after discontinuation of all peanut ingestion, in children with peanut allergy;
- b) To examine the longitudinal effects of combined treatment with probiotic and peanut OIT (PPOIT) as compared to placebo treatment on serum or plasma levels of peanut and peanut component specific-IgE (sIgE) and specific-IgG4 (sIgG4) in children with peanut allergy;
- c) To examine the longitudinal effects of combined treatment with probiotic and peanut OIT (PPOIT) as compared to placebo treatment on salivary peanut specific-IgA (sIgA) in children with peanut allergy;
- d) To examine the effects of combined treatment with probiotic and peanut OIT (PPOIT) as compared to placebo treatment on peripheral blood T regulatory (Treg) cell and T-helper (Th)1, Th2 and Treg cytokine production.

2.4. Methods

This section explains the methods used to conduct the original PPOIT-001 randomised trial and the follow-on study conducted at 4 years post-treatment.

2.4.1. Design

This was a single centre, randomised, double blind, placebo-controlled trial of a combined probiotic and peanut OIT (PPOIT) for 18 months in 62 children with peanut allergy.

2.4.2. Participants

2.4.2.1. Inclusion Criteria

Children were eligible for the trial if:

- o They were aged between 1 year and 10 years of age,
- o They were >10kg (weight considered safe for the prescription and administration of an EpiPen)
- o There was a confirmed diagnosis of peanut allergy (as against sensitisation) as defined by:
 - a positive food challenge to peanut within 2 years of enrolment AND a positive skin prick test (SPT) or sIgE to peanut, or
 - a positive food challenge or history of reaction to peanut EVER AND a positive SPT $\geq 8\text{mm}$ or a sIgE $\geq 15\text{kUA/L}$ to peanut

A food challenge to peanut was offered to the potential participant at screening if there was a positive SPT or sIgE but the clinical history of reaction to peanut was unclear.

2.4.2.2. Exclusion Criteria

Children were not eligible for the trial if they had a history of:

- o Severe anaphylaxis (as defined by persistent hypotension , collapse, loss of consciousness or persistent hypoxia)
- o FEV1 <85% at rest or FEV1/FVC $\leq$ 85% at rest or ongoing chronic persistent asthma (as per Australian Asthma Foundation guidelines)
- o Known wheat allergy (the placebo may contain traces of wheat)
- o Use of beta-blockers
- o Inflammatory intestinal conditions, indwelling catheters, gastrostomies, immune-compromised states, post-cardiac and/or gastrointestinal tract surgery, critically-ill and those requiring prolonged hospitalisation or other conditions that may increase the risks of probiotic associated sepsis
- o Already taking Probiotics

Provided that a participant met eligibility criteria and was not excluded on the basis of meeting an exclusion criterion, children with concomitant atopic disease including eczema were included in the trial. Whilst immune-compromised states was an exclusion criteria for entry into the parent PPOIT-001 trial, after completion of PPOIT study treatment, participants were not excluded from the long-term follow-up study even if they were subsequently commenced on immunosuppressive therapies for other atopic conditions such as eczema.

2.4.2.3. Withdrawal from trial

Participants were able to withdraw from the study at any time by their own volition.

A patient was considered to have completed this study upon completion of 60 weeks (+ 4 weeks to allow for flexibility in scheduling visits) of dosing during the active phase of the investigation.

Premature termination was defined as completing 59 or fewer weeks of active dosing. Every effort was made by the investigator to keep patients in the study.

If severe allergic symptoms develop involving the cardiovascular system (reduced BP, loss of consciousness), treatment was discontinued and the patient withdrawn from the study.

The dose was reduced for mild to moderate anaphylaxis defined as “all forms of anaphylaxis with the exception of anaphylaxis where there is cardiovascular involvement”.

A patient was allowed to withdraw from the study prematurely. The following reasons were provided as examples:

1. Lack of efficacy of study treatment
2. Lack of efficacy plus adverse event
3. Serious adverse event
4. Lack of patient compliance
5. Patient/Caregivers decision to withdraw
6. Patient lost to follow-up
7. Protocol Violation (specify)
8. Concurrent disease (specify)
9. Other reason (specify)

The reason for termination was recorded on the patient completion/withdrawal section of the CRF.

Every effort was made to follow-up patients who withdrew from the study with treatment-related adverse experiences in order to determine the final outcome.

If severe abdominal symptoms developed (severe diarrhoea, severe abdominal cramps), and persisted despite holding of the treatment dose, parents were offered the option of having the patient withdrawn from the study.

Note: The term 'severe' was assigned if an experience was incapacitating and not responsive to simple pain reliever medication (eg panadol, ibuprofen).

2.4.3. Study visits

Study visits and assessments were scheduled as follows:

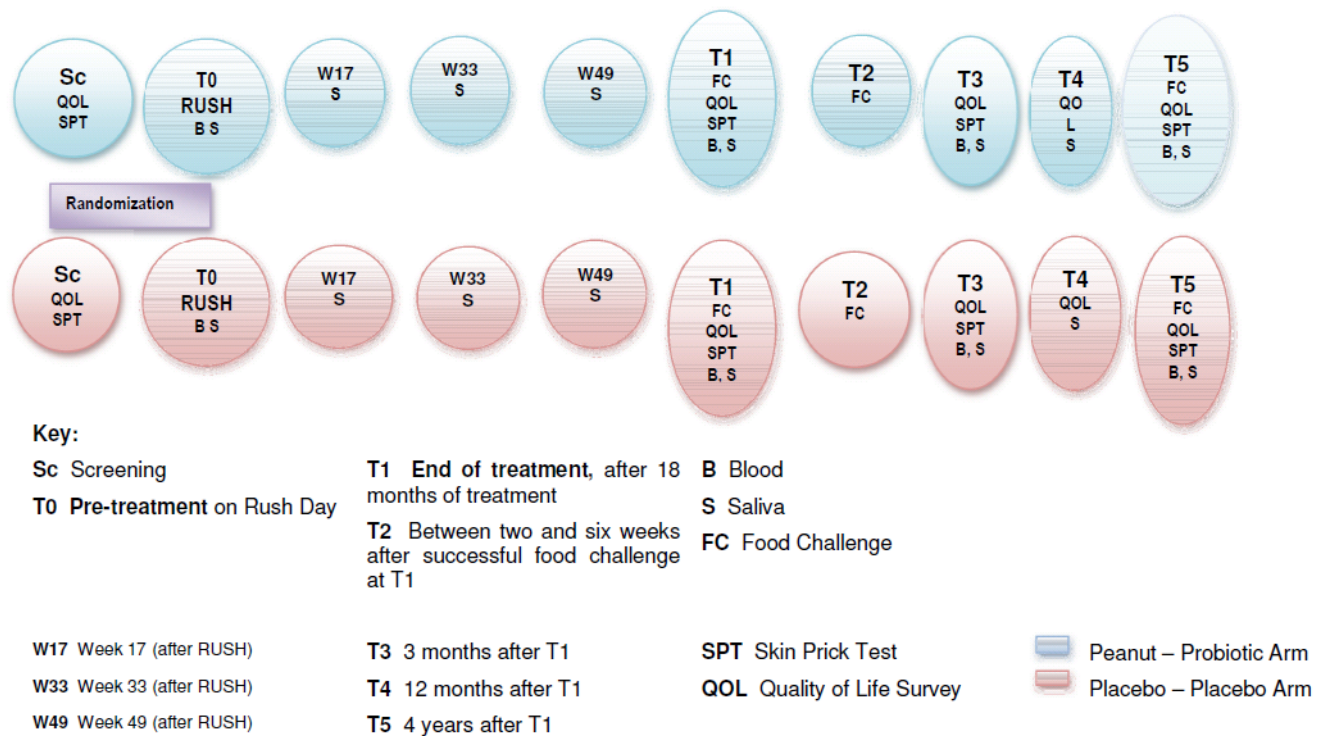
- Screening visit

Eligibility for the trial was determined by screening assessments, medical history, provision of written informed consent and fulfilment of eligibility criteria. Eligibility criteria included confirmation of diagnosis of peanut allergy (as against sensitisation) as defined by either (a) a positive food challenge to peanut within 2 years of enrolment AND a positive skin prick test (SPT) or sIgE to peanut, or (b) a positive food challenge or history of reaction to peanut EVER AND a positive SPT $\geq 8\text{mm}$ or a sIgE $\geq 15\text{ kUA/L}$ to peanut. A food challenge to peanut was offered to the potential participant at screening if there was a positive SPT or sIgE but the clinical history of reaction to peanut was unclear.

- T0/rush day visit;
- T1 – 18 months after T0/rush day (the day after the last day of treatment) visit – DBPCFC;
- T2 – 2-6 weeks after T1 visit – DBPCFC (with strict peanut elimination between T1 and T2);
- T3 – 3 months after T1 visit;
- T4 – 12 months after T1 visit;
- T5 – 4 years after T1 visit - DBPCFC (with strict peanut elimination for at least 8 weeks before DBPCFC)

An overview of assessments is included as Figure 2-1.

Figure 2-1. Overview of assessments



2.4.4. Interventions

2.4.4.1. Study treatment administered

Peanut Flour

Peanut Flour (50% peanut protein) was purchased from a US company (Golden Peanut Company, LTD). Peanut flour is preferred to crushed peanuts as it is easier to disguise. This was the same product used in the peanut OIT studies conducted in the US.

The typical analysis of the peanut powder was as follows:

Protein (N x 5.46)	50%+/- 2%
Fat	12% +/-2%
Moisture	<3.5%
Crude Fibre	10% +/-2%
Ash	4%+/- 2%
Carbohydrate	30%+/-2%
Density	21-23 lbs/cubic feet

Probiotic (*Lactobacillus rhamnosus* GG; ATCC 53103)

The probiotic used was *Lactobacillus rhamnosus* GG. *Lactobacillus rhamnosus* ATCC 53103 is indistinguishable from *Lactobacillus rhamnosus* GG (the name LGG is licenced) as assessed by all current modern taxonomy identification techniques and is considered to be equivalent to LGG in immunological effects in vitro and in vivo.

Probiotic mix or placebo (maltodextrin) was pre-packaged in 200 gram (g) tins by Nestle Nutrition Institute under strict Food Manufacturing Regulations. Each 200 g tin contained 180 g of the probiotic mix or placebo, with probiotic at 4×10^9 CFU/g. The daily dose of 2×10^{10} CFU was measured by parents or caregivers using an 11.3 ml scoop which delivers 5g of the mix with 1 scoop. The accuracy of delivering 5 g using this scoop was independently validated – repeated scoop measurements performed 30

times (surface, middle, bottom; 3 tins) resulted in a mean weight of 4.96 g (standard deviation, SD, 0.08 g, range 4.78 g - 5.11 g), representing a dose range of 1.92×10^{10} - 2.04×10^{10} CFU of probiotic. One 200 g tin would provide sufficient probiotic or placebo for 1 month, and the appropriate number of tins sufficient for the interval until next hospital visit was provided by Pharmacy together with the peanut or placebo sachets. Participants were instructed to mix the probiotic or placebo in water, soy milk or cow's milk, at a temperature NOT exceeding 45 °C. The probiotic maltodextrin matrix has been validated to have a shelf life of more than 3 years at 4 °C and more than 30 days when left at room temperature. Colony counts of probiotic were assessed 6 monthly during the study to confirm viability.

Maltodextrin (Placebo)

Maltodextrin is glucose polymer (malto-oligosaccharide), is a source of carbohydrate often used in oral dietary supplements and tube feeding.

2.4.4.2. Day 1 modified rush phase

- o In the modified rush phase, participants received increasing doses of peanut or placebo oral immunotherapy every 30 minutes to reach a final dose of 12 milligram (mg) of peanut protein (a dose reached by all subjects in the pilot study undertaken at Duke University Medical Center, USA).
- o Peanut protein or placebo were sprinkled onto food (apple sauce/yoghurt).
- o Participants received a single dose of 2×10^{10} CFU LGG or placebo (1 scoop dissolved into water) prior to initiation of the modified rush phase.
- o Participants were monitored for 2 hours after the last dose during modified rush.
- o The study doctor and study nurse were present at all times during the modified rush phase
- o The modified rush phase was performed in the allergy department or the Australian Paediatric Pharmacology Research Unit (APPRU).

Table 2-1. Dosing schedule for day 1 rush induction

Dose	Dosage Peanut Protein	Cumulative Peanut Protein
1	0.1 mg	0.1 mg
2	0.2 mg	0.3 mg
3	0.4 mg	0.7 mg
4	0.8 mg	1.5 mg
5	1.5 mg	3.0 mg
6	3.0 mg	6.0 mg
7	6.0 mg	12 mg
8	12 mg	24 mg

2.4.4.3. Buildup phase (32 weeks)

- o In the buildup phase, the daily dose of peanut OIT or placebo was increased every 2 weeks until a maintenance dose of 2 g was reached (expected to take 8 months).
- o Peanut protein or placebo was provided and sprinkled onto food (apple sauce/yoghurt).
- o Participants also took a fixed dose of 2×10^{10} CFU LGG or placebo (1 scoop dissolved into water, soy or cow's milk, at a temperature NOT exceeding 45 °C) once daily prior to the OIT treatment.
- o Participants were monitored for 2 hours after the treatments were administered.
- o Participants in the control arm followed the same protocol receiving placebo oral immunotherapy (maltodextrin)

o The buildup phase immunotherapy protocol is shown in Table 2-2. Dosing schedule for Buildup Phase (32 weeks) in the PPOIT-001 trial .

Table 2-2. Dosing schedule for Buildup Phase (32 weeks) in the PPOIT-001 trial

Dose	Dosage Peanut Protein	Dose	Dosage Peanut Protein
9	25 mg	17	330 mg
10	50 mg	18	425 mg
11	75 mg	19	550 mg
12	100 mg	20	715 mg
13	125 mg	21	925 mg
14	150 mg	22	1.2 g
15	200 mg	23	1.55 g
16	260 mg	24	2.0 g

2.4.4.4. Maintenance phase

During the maintenance phase participants took a 2 g dose of oral immunotherapy (peanut or placebo) and 2×10^{10} CFU LGG or placebo daily at home until completion of the OIT protocol at 18 months. If the participant did not complete a minimum of 6 months on maintenance dosing at 18 months, the total duration of treatment was extended to ensure a minimum of 6 months maintenance dosing.

2.4.4.5. Dose adjustment rules

If mild allergic symptoms without cardiovascular involvement developed following a treatment dose (urticaria, angioedema, vomiting, diarrhoea, abdominal pain), the dose was repeated at least once before increasing.

If moderately severe allergic symptoms developed without cardiovascular involvement (stridor, wheeze, difficulty breathing), the subsequent dose was reduced to the previous dose amount before increasing.

If severe allergic symptoms of reduced BP or loss of consciousness develop, study treatment was discontinued.

2.4.4.6. Collection of study treatment from Pharmacy

Peanut protein or placebo was made up in RCH Pharmacy and supplied in sachets until 330 mg of the study product, in small medicine containers up to 1.55 g of the study product then in tubs from 2.00 g. Upon successful completion of the Day 1 modified rush phase, participants collected a 2 week supply of treatment sachets containing peanut or placebo from Pharmacy. 20 doses were provided for each 2 week period, so that they were able to continue with their OIT in the event that they missed their appointment with RCH or a dose was accidentally spilt. Unused sachets were returned at their next visit.

The dose to be administered for each 2 week period was determined by study staff performing the updosing procedure and based upon the established criteria as outlined above. When participants reached the 2 g maintenance dose, they collected peanut or placebo sachets sufficient to complete one month of OIT maintenance from RCH Pharmacy.

Participants returned unused doses and used treatment packaging to Pharmacy at each visit so that they may audit compliance

Clinical review with study staff was performed at each hospital visit that is 2 weekly during buildup phase to monitor progress and compliance, and address any issues that arose.

During the maintenance phase diary, compliance and progress was reviewed every 4 weeks.

2.4.4.7. Medications/treatments permitted and not permitted before and during trial

Participants using beta blockers were excluded from the trial as this treatment can impair responsiveness to adrenaline in the event of severe allergic reactions.

Participants were permitted to use any other medications or treatments they were prescribed.

Participants had to continue to strictly avoid all foods containing peanut.

Participants had to continue to strictly avoid probiotic supplements and foods containing probiotic, for example some yoghurt preparations.

2.4.5. Clinical outcomes

The clinical outcomes of the study were (i) double blind placebo controlled food challenges (DBPCFC) at T1, T2 and T5 where participants were challenged up to a maximum dose of 8 g of peanut flour (4 g of peanut protein) (ii) skin prick test at T1, T2 and T5, (iii) a quality of life survey at T1, T3 (3 months post treatment), T4 (1 year post treatment) and T5, (iv) a peanut ingestion questionnaire at T5.

Oral DBPCFC were performed at T1 (the day immediately after the final dose of study treatment), at T2 (2 to 6 weeks after T1) and at T5 (4 years after T1).

If there was a challenge failure at T1, the participant would not proceed to a T2 challenge. The threshold dose at which a reaction occurred and the symptoms of the reaction were recorded.

Participants who failed the T1 challenge were advised to avoid peanut. These participants were assessed as remaining peanut allergic.

Participants who passed the T1 challenge but then failed the T2 challenge were advised to avoid peanut. These participants were assessed as having attained desensitisation.

Participants who passed the T2 challenge were advised to ingest peanut ad libitum. These participants were assessed as having attained sustained unresponsiveness.

All participants were eligible for T5 challenge. Participants had to avoid all peanut ingestion for at least 8 weeks prior to undergoing the T5 challenge. Participants who failed the T5 challenge were advised to avoid peanut. Participants who passed the T5 challenge were advised to ingest peanut ad libitum and were assessed as having persistent or long-term sustained unresponsiveness.

2.4.5.1. Procedure for double blind placebo controlled food challenge (DBPCFC)

T1 (end of treatment), T2 (2-6 weeks post-treatment) and T5 (4 years post-treatment) food challenges were performed in the Royal Children's Hospital Allergy department or APPRU. Nutritional services prepared the doses for the food challenges. There were two sets of containers - one labeled A and the other labeled B. One was the placebo (maltodextrin) and the other was the peanut protein each mixed in an appropriate vehicle food. The doctor and nurse were blinded as to which letter designated which food. The cumulative amount of peanut or placebo powder given was 8 grams weight (4 g of peanut protein).

Food Challenge A began with giving doses from container A as follows:

<u>Dose</u>	<u>Dose (peanut protein)</u>	<u>Weight</u>	<u>Cumulative Dose</u>
1	62.5 mg	125 mg	62.5 mg
2	125 mg	250 mg	187.5 mg
3	250 mg	500 mg	437.5 mg
4	500 mg	1000 mg	937.5 mg
5	1000 mg	2000 mg	1,937.5 mg
6	1000 mg	2000 mg	2,937.5 mg
7	1062.5 mg	2125 mg	4 g

The doses were administered at 15 minute intervals, if the subject had not had a reaction to the previous dose. After completion of Food Challenge A, the subject was

observed for 2 hours for signs and symptoms of a reaction. If there had been no evidence of reaction then Food Challenge B was commenced.

Food Challenge B continues with giving doses from container B as follows:

<u>Dose</u>	<u>Dose (peanut protein)</u>	<u>Weight</u>	<u>Cumulative Dose</u>
1	62.5 mg	125 mg	62.5 mg
2	125 mg	250 mg	187.5 mg
3	250 mg	500 mg	437.5 mg
4	500 mg	1000 mg	937.5 mg
5	1000 mg	2000 mg	1,937.5 g
6	1000 mg	2000 mg	2,937.5 g
7	1062.5 mg	2125 mg	4 g

The doses were administered at 15 minute intervals if the subject had not had a reaction to the previous dose. The subject was observed for a minimum of two hours following Food Challenge B.

The patient was discharged home if no adverse reactions were noted.

The study doctor and study nurse were present at all times and an intravenous cannula was inserted in all patients prior to commencement of the peanut challenge.

2.4.6. Collection of biospecimens

o The effects of combined probiotic and peanut OIT (PPOIT) on systemic and mucosal immune responses were examined, focusing on peanut and peanut component (Ara h1, h2, h3, h8, h9) specific IgE (sIgE) and specific IgG4 (sIgG4), peanut specific salivary IgA, peanut induced cytokine responses, and peanut specific T-regulatory (Treg) cells.

- o A 15-20 ml blood sample and saliva were collected from study participants at study entry (T0), end of treatment (T1), 3 months post treatment (T3), and 4 years post treatment (T5).
- o In addition, saliva was collected from study participants at week 17, week 33, week 49 of treatment and at 1 year post treatment (T4).
- o Serum, plasma and saliva were stored at -80°C for later batch measurement of peanut and peanut component specific IgE and IgG4 and peanut specific salivary IgA.
- o Peripheral blood mononuclear cells (PBMC) were separated from heparinised blood by density gradient centrifugation within 8 hours and cryopreserved (20×10^6 cells/ml) at T0, T1, T3, and T5.
- o PPOIT was conducted from 2009 to 2013 with 62 participants initially enrolled. 6 of 62 study subjects did not complete the study therefore their later visit samples were not available. Blood and saliva samples were collected (where possible) during all of the above visits.

2.4.7. Materials and methods for laboratory analyses

The standard operating procedures (SOP) used for following are presented in the Appendices.

2.4.7.1. Peripheral blood mononuclear cell (PBMC) isolation, cryopreservation and batch thawing

PBMC were isolated from heparinised whole blood by density gradient centrifugation (Ficoll-paque; GE Healthcare; Uppsala, Sweden) and centrifuging for 10 minutes at 700g. The plasma layer was separated and stored in 1ml aliquots at -80°C. The PBMC layer was washed then underwent a second density centrifugation for 30 minutes at 400g. PBMC layer was washed three times in RPMI(Invitrogen, NZ) supplemented with 2% HI-fetal calf serum (FCS; Invitrogen; NZ) and then cryopreserved at a concentration of $8-12 \times 10^6$ cells / mL in a 1:1 ratio of RPMI/HI-FCS. The PBMC were placed into -80°C freezer for 24-72 hours and then transferred to liquid nitrogen for cryopreservation.

Later, cryovials of PBMC were batch thawed in 37°C water baths for 3-5 minutes. The PBMC were washed in RPMI/HI-FCS and resuspended in the appropriate media. A repeat cell count and determination of viability using Trypan Blue (Sigma, St Louis, USA) were done at this stage.

2.4.7.2. PBMC culture and stimulation

Batch thawed PBMC were stained with the cell tracking dye Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE; CellTrace™ CFSE Cell Proliferation Kit; Invitrogen; Orgeon, USA) at a concentration of 10 µL / 1 x 10⁶ cells by incubating at 37°C for 5 minutes. After incubation, PBMC were washed with RPMI and 0.1% autologous serum and spun at 600g for 10 minutes before being resuspended in AIM-V medium CTS™ (Life Technologies; NY, USA) with 5% autologous serum to reach a final concentration of 1 x 10⁶ cells / ml for various culture conditions. The cell suspension was divided in 1 ml samples for cultures with 10 µL crude peanut extract (CPE) stock (100 µg/mL; a kind gift from Professor Wesley Burks' research group; Duke University, NC, USA), 10 µL ovalbumin (OVA; 100 µg/mL) stock and 6.3 µL tetanus toxoid (TT; 1 µg/mL) stock. One 1 ml sample of PBMC resting in media alone was set up as control. All cultures were incubated at 37°C in 5% CO₂ and 95% humidity for 6 days. At the end of the culturing period, cell culture supernatants were separated by centrifugation at 600 g for 10 minutes and stored at -80°C for subsequent cytokine quantification. PBMC were stained for analysis of candidate T-regulatory (Treg) cell populations using fluorescence activated cell sorting (FACS) assay.

2.4.7.3. Fluorescence activated cell sorting (FACS) assay

After resting or culturing with antigens (CPE, OVA or TT) for 6 days, PBMC were resuspended in FACS buffer (phosphate buffered saline, PBS, with 2% HI-FCS) and 1% sodium azide (Australian Chemical Reagents; Queensland, Australia) at a concentration of 1 x 10⁶ cells / 100 µL and surface stained using the following monoclonal mouse anti-human antibodies (BD Biosciences; CA, USA): Peridinin Chlorophyll Protein (PerCP)-conjugated anti-CD4 (2.5 µL), PhycoErythrin-Cyanin (PE-Cy) 7-conjugated anti-CD25 (2.5 µL), AlloPhycoCyanin (APC)-conjugated anti-CD45RA (2 µL) and V450-conjugated anti-CD27 (1 µL). After incubating for 20 minutes, the PBMC were centrifuged at 600 g

for 10 minutes followed by permeabilisation, fixing and intracellular staining using PhycoErythrin (PE)-conjugated anti-Foxp3 (BD Biosciences; CA, USA).

All FACS data were acquired on a 10-colour LSR II (BD, San Jose, CA, USA) flow cytometer and analysed using BD FACSDiva Software Version 10.1 (BD Biosciences, CA, USA). Compensation for spectral overlap was done using positive and negative control beads (BD, San Jose, CA, USA). The same compensation settings were used for each FACS analysis.

T-regulatory (Treg) cells were identified by surface staining using CD4-PerCP, CD25-PE-Cy7 and intracellular Foxp3-PE. Proliferative response was evaluated using cell tracking dye CFSE. CFSE forms fluorescent conjugates by reacting with intracellular amines and are well retained in the parent and daughter cells during cycles of cell division. The level of CFSE fluorescence in a cell reflects the number of cell divisions. Proliferating cells can be identified as CFSE^{lo}. Therefore proliferating Treg were enumerated by applying the standardised gating strategy selecting CD4⁺/CD25⁺/Foxp3⁺/CFSE^{lo} lymphocytes.

An example of FACS gating strategy is presented in the Appendices.

2.4.7.4. Supernatant Cytokine multiplex quantification assay

Concentrations of interleukin (IL)-10, IL-4, IL-5, IL-13 and interferon gamma (IFN- γ) were determined in supernatants obtained from 6 day PBMC cultures using multiplex cytokine bead assay on a Luminex 200 analyser (Luminex Corporation, Austin, TX, USA). The Milliplex MAP Human High Sensitivity Magnetic Bead Panel kit (EMD Millipore, St Charles, MO, USA) contains distinctly coloured anti-cytokine beads with matching anti-cytokine reporters. A standard was provided consisting of IL-10 (6000 pg/mL), IL-4 (7500 pg/mL), IL-5 (2000 pg/mL), IL-13 (1000 pg/mL) and IFN- γ (2500 pg/mL) and an eight-point standard curve was prepared using 1:4 serial dilutions. The coupled beads were incubated with supernatant samples, standards and controls overnight at 4°C in accordance with the manufacturer's instructions. After washing, 50 μ l of the reporter molecule Streptavidin-Phycoerythrin conjugate was added to each well together with 50 μ l of detection antibodies. Following 30 minutes incubation at room temperature, wash steps were performed three times with wash buffer via vacuum filtration using a manual vacuum manifold. Then, cytokine levels were measured using the Luminex 200

analyser where the beads specific to each cytokine were identified by one laser while another laser quantified the cytokines based on the reporter molecule. Results were reported in pg/mL and analysed using MasterPlex QT (Hitachi Solutions America, Ltd; USA). All supernatant samples were analysed in duplicate with low and high quality controls included in each run.

2.4.7.5. Salivary peanut specific-IgA (sIgA) enzyme linked immunosorbent assay (ELISA)

The method for salivary peanut s IgA measurement was adapted from a protocol originally developed by Dr Herman Staats (Duke University School of Medicine, Durham, NC, USA) and modified by Dr Michael Kulis and Professor Wesley Burks (Duke University Medical Centre, Durham, NC, USA) and optimised in the Murdoch Childrens Research Institute. Briefly, 96-well microtitre plates (NUNC MaxiSorp, Thermo Scientific; NY, USA) coated with 100 µL/well of a mixture of crude peanut extract (CPE; 11 mg/mL; kindly provided by Professor Wesley Burks and his research group, Duke University Medical Centre, Durham, NC, USA). After incubation for 1 hour at 37°C, CPE coated ELISA plates were washed four times with phosphate buffered saline (PBS) containing 0.05% Tween 20. Saliva samples were diluted 1:2 in PBS containing 0.5% Tween 20 and 2% fetal calf serum (FCS). The standard was diluted in the range of 1:40 to 1:5 by serial dilutions. An in-house plasma sample with a known high peanut-specific IgA was diluted 1:50, 1:100 and 1:200 to yield positive control samples. PBS containing 0.5% Tween 20 and 2% fetal calf serum (FCS) without saliva was used as a negative control. Salivary samples, standards and controls were then added and incubated for 24 hours at 4°C. After incubation, plates were washed four times and incubated for another hour at 37°C with an HRP-conjugated goat anti-human IgA (Southern Biotech; AL, USA) at a 1:5000 dilution. After this incubation period, plates were washed four times in PBS Tween and distilled water separately. A TMB substrate solution of 11 mL (KPL; Gaithersburg, MD, USA) was then added and incubated for 20 minutes. The reaction was stopped by the addition of 1 M of H₃PO₄ at room temperature. The plates were read within 30 minutes using a microplate reader (BioTek; VT, USA) and results analysed using the KCjunior V1.40.3 software package (BioTek, Winooski, VT, USA). Results were reported in ELISA units (EU/mL). Saliva samples (volume permitting) were analysed in duplicate and all standards and controls were analysed in duplicate.

2.4.7.6. Serum and plasma specific-IgE (sIgE) and specific-IgG4 (sIgG4) fluorezyme immunoassay (FEIA)

Levels of whole peanut and peanut component (Ara h1, h2, h3, h8, h9) sIgE and sIgG4 were measured using the ImmunoCAP 250 instrument (Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions. Levels of whole peanut and peanut component sIgE and sIgG4 were measured in plasma samples, with the exception of whole peanut sIgE which was measured in serum samples.

ImmunoCAP is a fluorezyme immunoassay where the whole peanut or peanut component antigen is covalently bound to a solid phase ('the cap'). After incubation with the participant's serum or plasma, the cap was washed to remove unbound non-specific IgE or IgG4 before enzyme labelled antibodies against the specific IgE or IgG4 were added to form a complex. The bound complex was then incubated with a developing agent. After stopping the reaction, fluorescence of the eluate was measured. The fluorescence intensity is directly correlated with the amount of specific IgE or IgG4 present. The consumables required for the ImmunoCAP assay were supplied by the manufacturer (Phadia AB; Uppsala, Sweden) or supplied as in-kind support by distributor (Abacus-ALs). Serum and plasma samples were centrifuged at 3,000 rpm for 5 minutes prior to aliquoting. Manufacturer's and in-house quality controls (specifically, plasma from participants 101 and 406 at time points T1 (end of treatment) and T5 (4 years post-treatment) for which sIgE concentrations were known) were included in each run. For sIgE, data was reported as kUA/L and sIgG4 data was reported as mgA/L.

2.4.8. Evaluation of Health-Related Quality of Life

Health related quality of life assessments were conducted at screening, T1 (end of treatment), 3 months post treatment (T3), 12 months post treatment (T4) and 4 years post treatment (T5) by means of a validated Food Allergy Quality of Life Questionnaire - Parent Form (FAQLQ-PF) which was completed by the parent/caregiver of the participant.

Analysis of Health-Related Quality of Life data was not a primary aim of this doctoral thesis, but the doctoral candidate was involved in the collection, analysis and preparation for publication of PPOIT-001 trial quality of life data[96].

The food allergy quality of life questionnaire-parent form (FAQLQ-PF) is a disease-specific health related quality of life (HRQL) questionnaire with excellent validity and reliability. This psychometric tool was developed to assess quality of life in children with food allergies (aged 0-12 years) and allows parents to report children's HRQL from the child's perspective. FAQLQ-PF is presented in a single form for all age groups (0-3; 4-6; 7-12 years) and is considered "user-friendly". It contains 30 items, and the response scale ranges from 0 (minimal impairment in HRQL) to 6 (maximal impairment in HRQL). The measure has 3 subscales assessing 3 factors found to be essential for assessing the global impact of food allergy on HRQL: general emotional impact, food anxiety and social and dietary limitations. The total score is calculated as the mean of items.

The food allergy independent measure (FAIM) is a food allergy specific measure based on 6 questions included within the FAQLQ-PF, with a response scale from 0 (extremely unlikely) to 6 (extremely likely). The questions assess the parent's perception of the chance of an adverse outcome for the child with a food allergy.

The questions relate to:

1. The chance of accidental exposure;
2. The chance of a severe reaction on food exposure;
3. The chance of dying from food exposure;
4. The chance of a child effectively treating him/herself or receiving effective treatment, following a food allergic reaction;
5. How many foods were avoided because of food allergy (categorized as single, 2, multiple >2);
6. How much food allergy limits the type of activities that the child can take part in.

2.4.9. Statistical methods

2.4.9.1. Statistical analysis plan (SAP)

The trial was analysed using an “intent-to-treat” classification for the intervention. Relative risk estimates for (1) the effect of probiotic and peanut OIT (PPOIT) compared to placebo on categorical measures of peanut desensitisation, 2-6 week sustained unresponsiveness (SU) or long-term sustained unresponsiveness (LTSU) was calculated using multiple logistic regression models with adjustment for relevant confounders.

Desensitisation to Peanut:

The number and percentage of participants who achieved desensitisation to peanut were presented for all participants. Desensitisation was defined as the ability to tolerate 4000 mg of peanut protein (cumulative dose) during the end of treatment DBPCFC following 18 months of treatment (T1). A participant was considered to have desensitisation to peanut if they passed the DBPCFC at the T1 visit.

2-6 week sustained unresponsiveness (SU) following 18 months of treatment:

The number and percentage of participants who achieved SU at the T2 DBPCFC was presented for all participants. SU was defined as the ability to tolerate 4000 mg of peanut protein (cumulative dose) during the DBPCFC conducted 2-6 weeks after treatment cessation (T2). A participant was considered to have an SU to peanut if they passed the DBPCFC at the T2 visit.

Long-term sustained unresponsiveness (LTSU) at 4 years post-treatment:

The number and percentage of participants who achieved LTSU at the T5 DBPCFC was presented for all participants who underwent evaluation. LTSU was defined as the ability to tolerate 4000 mg of peanut protein (cumulative dose) during the DBPCFC conducted 4 years after treatment cessation (T5) and after at least 8 weeks of peanut avoidance in the lead up to the T5 DBPCFC. A participant was considered to have a LTSU to peanut if they passed the DBPCFC at the T5 visit.

- o Multiple linear regression models was used to assess the effect of the intervention on continuous variables such as peanut SPT wheal size.
- o If a child was lost to follow-up, their last available outcome measure was utilised in the intent-to treat analysis.
- o Immunological variables were compared in PPOIT and placebo groups using standard t-test methods after log transformation of values, with data summarised using geometric means for between group comparisons.
- o Immunological variables were correlated to different clinical outcomes by comparison of variables between those who received probiotic with peanut OIT (PPOIT) and placebo using standard t-test after log transformation of values with data summarised using geometric means for between group comparisons.

Given that the doctoral candidate was investigating a number of hypotheses, the candidate did not adjust p-values for multiple comparisons as per Feise and Perneger[97 98].

2.4.9.2. Data Handling and Record Keeping

- o All the data gathered were recorded directly into the Case Report Form (CRF) as source data.
- o Data from the CRFs were entered into a computer database which had been set up with an appropriate range checks. The database was checked against the case report forms for accuracy. Access to the database was restricted by password.
- o Information on the database was anonymised, with participants referred to by their unique study identification number.
- o The Study Coordinator/ study nurses were responsible for the completeness, accuracy, legibility and timeliness of the data reported.
- o A list of the treatment regimen of each randomisation group was stored in a locked cabinet in the Royal Children's Hospital Immunology Laboratory and in the hospital pharmacy.

2.4.10. Ethics and trial registration

2.4.10.1. Independent ethics committee

The protocol and participant informed consent forms for the follow-on study of the PPOIT-001 trial were approved by the Royal Children's Hospital (RCH) Human Research Ethics Committee (HREC) for the Murdoch Children's Research Institute (MCRI) based at the RCH (HREC 27086Q, 4/12/2014). The Investigators did not participate in the Committee's decisions. Approval was received in writing from the HREC prior to initiating the trial. All amendments were submitted to the HREC for review and approval prior to implementation. The PPOIT-001 trial was registered with ACTRN12608000594325, registered 25/11/2008.

2.4.10.2. Ethical Conduct of the Trial

The trial was conducted in accordance with the principles of International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline for Good Clinical Practice (GCP), (E6)(R2) and the Declaration of Helsinki (2013).

2.4.10.3. Study Participant Information and Consent

As this trial involved enrolling paediatric participants 1 year to 10 years of age, it was necessary to obtain the consent of the parent or legal guardian of the paediatric participants. The parent/guardian of all prospective participants was provided with a Parent/Guardian Information Statement and Consent form (PGISCF) approved by the RCH HREC. The Investigators or delegates provided the Information Statement to parents prior to the screening visit to read and review in a timely manner and ask any questions that he/she had in order to fully understand the nature of the trial, and any risks and procedures involved. Full written informed consent was obtained from all parent/guardians during the pre-screening process, and before any trial-specific procedures were performed. Participants were advised that they were able to refuse to participate in, or to withdraw from the trial at any time, and that the medical care they received would not be affected by their decision regarding participation in the trial.

2.5. Chapter summary

PPOIT-001 was a rigorously conducted double-blind, placebo-controlled randomised trial designed to evaluate the clinical effectiveness of PPOIT in children with peanut allergy. The follow-on study was established to investigate the long-term clinical and immunologic outcomes of participants treated with PPOIT as compared to those treated with placebo.

In Chapters 3-5, the doctoral candidate presents the long-term clinical and immunologic outcomes of participants in the PPOIT-001 trial.

Chapter 3. Results: Long-term clinical effects of PPOIT in the PPOIT-001 randomised trial

3.1. Chapter introduction

In the previous chapter, the doctoral candidate outlined the rationale, overall aims and methods of the follow-on study of the PPOIT-001 trial, a randomised controlled trial of a combined probiotic and peanut oral immunotherapy (PPOIT) in children with peanut allergy. In this chapter, the doctoral candidate presents findings on the long-term clinical effects of PPOIT in the form of a published original research article, first-authored by the doctoral candidate. The final publication is available at [https://www.thelancet.com/journals/lanchi/article/PIIS2352-4642\(17\)30041-X/fulltext](https://www.thelancet.com/journals/lanchi/article/PIIS2352-4642(17)30041-X/fulltext).

3.2. The published manuscript

Articles

Long-term clinical and immunological effects of probiotic and peanut oral immunotherapy after treatment cessation: 4-year follow-up of a randomised, double-blind, placebo-controlled trial

Kuang-Chih Hsiao, Anne-Louise Ponsonby, Christine Axelrad, Sigrid Pitkin, Mimi L K Tang, on behalf of the PPOIT Study Team*

Summary

Background Oral immunotherapy has attracted much interest as a potential treatment for food allergy, yet little is known about its long-term effects. We aimed to assess long-term outcomes in participants who completed a randomised, double-blind, placebo-controlled trial of combined probiotic and peanut oral immunotherapy (PPOIT), which was previously shown to induce desensitisation and 2-week sustained unresponsiveness.

Methods All participants who completed the PPOIT randomised trial were eligible to participate in this follow-up study 4 years after treatment cessation. Peanut intake and adverse reactions to peanut in the 4 years after treatment cessation were systematically documented with a structured questionnaire administered by allergy nurses. Additionally, participants were invited to undergo peanut skin prick tests, measurement of peanut sIgE and sIgG4 concentrations, and double-blind placebo-controlled peanut challenge to assess 8-week sustained unresponsiveness.

Findings 48 (86%) of 56 eligible participants were enrolled in the follow-up study. Mean time since stopping treatment was 4.2 years in both PPOIT (SD 0.6) and placebo (SD 0.7) participants. Participants from the PPOIT group were significantly more likely than those from the placebo group to have continued eating peanut (16 [67%] of 24 vs one [4%] of 24; absolute difference 63% [95% CI 42–83], $p=0.001$; number needed to treat 1.6 [95% CI 1.2–2.4]). Four PPOIT-treated participants and six placebo participants reported allergic reactions to peanut after intentional or accidental intake since stopping treatment, but none had anaphylaxis. PPOIT-treated participants had smaller wheals in peanut skin prick test (mean 8.1 mm [SD 7.7] vs 13.3 mm [7.6]; absolute difference –5.2 mm [95% CI –10.3 to 0.0]; age-adjusted and sex-adjusted $p=0.035$) and significantly higher peanut sIgG4:sIgE ratios than placebo participants (geometric mean 67.3 [95% CI 10.3–440.0] vs 5.2 [1.2–21.8]; $p=0.031$). Seven (58%) of 12 participants from the PPOIT group attained 8-week sustained unresponsiveness, compared with one (7%) of 15 participants from the placebo group (absolute difference 52% [95% CI 21–82], $p=0.012$; number needed to treat 1.9 [95% CI 1.2–4.8]).

Interpretation PPOIT provides long-lasting clinical benefit and persistent suppression of the allergic immune response to peanut.

Funding Murdoch Childrens Research Institute and Australian Food Allergy Foundation.

Introduction

The prevalence of food allergy has risen substantially in the past 20 years.^{1–3} Whereas allergies to egg, milk, wheat, and soy generally resolve during childhood, nut and seafood allergies often persist throughout life. Management includes allergen avoidance and education in the emergency management of allergic reactions. The constant vigilance required to avoid allergens substantially impairs quality of life of both food-allergic children and their families.^{4,5} The quality of life of children with food allergies is worse than that of children with diabetes.^{6,7} Accidental exposure to food allergens is common, with an annual incidence of 12–15% in children with peanut allergy.^{8,9} Although most allergic reactions to food can be managed successfully, fatalities still occur, with an incidence of roughly three per million person-years in children aged 0–19 years.¹⁰ A curative treatment could potentially improve quality of life and prevent allergy-related deaths.

There is intense interest in oral immunotherapy as a food allergy treatment to induce desensitisation (defined as being able to tolerate the allergen while on treatment),^{11–17} or sustained unresponsiveness (defined as being able to tolerate the allergen weeks or months after stopping treatment).^{18,19} Results of randomised placebo-controlled trials suggest that oral immunotherapy can induce desensitisation in around two-thirds and sustained unresponsiveness of several weeks' duration in a small subset of children.²⁰ Whether desensitisation or sustained unresponsiveness persists long term after oral immunotherapy remains unknown.²¹ Long-term effects after oral immunotherapy have been reported in participants with egg or milk allergies, for which natural resolution can be expected in a substantial proportion of people. In the only follow-up study²² of a randomised trial of oral immunotherapy, no difference in frequency of egg ingestion was reported between people in the egg



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Research in context

Evidence before this study

We searched PubMed with the medical subject heading terms “food allergy”, “peanut allergy”, “oral immunotherapy”, and “long term” for articles published in English on or before May 12, 2017. We found four publications about long-term outcomes after cessation of oral immunotherapy, one in children with egg allergies and three in children with milk allergies. However, we identified no studies of similar outcomes in patients who received peanut oral immunotherapy. Unlike most allergies to milk or eggs, peanut allergies tend to persist into adulthood, so long-term outcomes after egg or milk oral immunotherapy cannot be generalised to peanut oral immunotherapy. Furthermore, we identified no studies in which challenge-proven sustained unresponsiveness was assessed years after stopping oral immunotherapy.

Added value of this study

Ours is the first report, to our knowledge, of long-term outcomes several years after cessation of peanut oral

immunotherapy and the first study to incorporate challenge-proven sustained unresponsiveness as an outcome measure. Our findings show that combined probiotic and peanut oral immunotherapy provides long-lasting clinical benefit compared with placebo, with two-thirds of treated participants symptom free after peanut ingestion 4 years after completing treatment. The treatment resulted in symptom-free peanut ingestion, food-challenge-proven sustained unresponsiveness, and persistent suppression of the allergic immune response to peanut 4 years after treatment cessation.

Implications of all the available evidence

Long-term clinical benefits after standard oral immunotherapy remain uncertain. Combined administration of probiotic and peanut oral immunotherapy provided long-term clinical benefits, and the findings of this study are a step towards identification of an effective treatment to address the food allergy problem in developed countries.

oral immunotherapy group and those in the placebo group at least 12 months after treatment.

No data for long-term outcomes after peanut oral immunotherapy have been published. The natural history of peanut allergy is different from that of egg or milk allergy, so long-term outcomes for egg or milk oral immunotherapy cannot be extrapolated to peanut oral immunotherapy. We previously reported that combined probiotic and peanut oral immunotherapy (PPOIT) was effective at inducing desensitisation and sustained unresponsiveness after 2 weeks of secondary allergen information in children with peanut allergy (number needed to treat 1.27 [95% CI 1.06–1.59]).²³ We selected the probiotic *Lactobacillus rhamnosus* CGMCC 1.3724 (NCC4007), which is genetically indistinguishable from *L. rhamnosus* ATCC 53103, for use in PPOIT on the basis of its ability to induce regulatory T cells, antigen-specific IgA, and regulatory and T helper 1 cytokine responses.^{24–28} We postulated that administration of this probiotic with peanut oral immunotherapy would support redirection of the peanut-specific allergic response towards tolerance by providing a tolerogenic milieu at the time of antigen uptake and processing by antigen-presenting cells. In this study, we aimed to examine whether the previously reported clinical and immunological benefits of PPOIT were maintained 4 years after treatment.

Methods

Study background

Participants who completed the PPOIT randomised trial (the parent study) were assessed 4 years after treatment cessation (between January, 2015, and April, 2016). The parent study design and outcomes have been reported previously.²³ Briefly, 62 children with peanut allergy were

randomly assigned (1:1) to receive PPOIT (2×10^{10} colony-forming units of *L. rhamnosus* CGMCC 1.3724 and 2 g of peanut protein) or placebo (two formulations of maltodextrin) once daily for 18 months. During the study, all participants and their parents were reminded at each scheduled study visit to avoid taking probiotics and to remain on a peanut elimination diet as stipulated in the study protocol. At 18 months, all participants underwent a double-blind, placebo-controlled food challenge to assess for desensitisation. Participants who passed the desensitisation food challenge completed a second challenge 2–6 weeks after discontinuation of study treatment to assess for sustained unresponsiveness.

Participants who passed the sustained unresponsiveness challenge at the end of the parent study were instructed to continue intake of peanut ad libitum (ie, as part of their normal diet, without specific instructions on ingestion frequency or amount). Participants who did not attain sustained unresponsiveness were instructed to continue with strict peanut avoidance. Participants probably did not continue to take the probiotic at treatment doses because *L. rhamnosus* CGMCC 1.3724 is not known to the general public by that name, and participants were advised that the dose they were taking was equivalent to 20 tubs of yogurt each day. The parent study's primary outcome was the proportion of participants who attained sustained unresponsiveness.

Data collection and procedures

All participants who completed the parent study (n=56) were eligible for this follow-up study; consent was given at enrolment into the follow-up study (ie, 4 years after treatment). A structured peanut intake questionnaire and a validated food allergy quality-of-life questionnaire (Food

Allergy Quality of Life Questionnaire [FAQLQ]-parent form if participants were younger than 13 years, FAQLQ-teenager form if they were aged 13 years or older)²⁹ were administered to participants or their parents by allergy research nurses. The peanut intake questionnaire systematically recorded participants' peanut intake history, including the average amount ingested, ingestion frequency, and adverse reactions to peanuts after accidental or intentional ingestion since stopping study treatment. We also invited participants to give additional, optional consent for peanut skin prick tests, blood sample measurements of peanut specific IgE (sIgE) and specific IgG4 (sIgG4), and double-blind placebo-controlled food challenge (cumulative dose 4 g peanut protein after peanut elimination for 8 weeks; appendix) to assess for sustained unresponsiveness.

Peanut skin prick tests (peanut extract from Hollister-Stier, Spokane, WA, USA; Greer Pick from Greer Laboratories, Lenoir, NC, USA) were done by allergy research nurses. Serum concentrations of peanut sIgE and sIgG4 were measured by ImmunoCAP (Phadia AB, Uppsala, Sweden). The peanut sIgG4:sIgE ratio was calculated by converting peanut sIgG4 concentrations from µg/L to ng/mL and peanut sIgE concentrations from kU/L to ng/mL and then using the formula:³⁰

$$\frac{\text{sIgG4}}{\text{sIgE} \times 2.4}$$

Ethics approval for this study, including the study protocol and an a-priori statistical analysis plan, was obtained from the Royal Children's Hospital Human Research Ethics Committee (HREC 27086Q).

Statistical analysis

Allowing for 30% loss to follow-up, we calculated that a sample size of 19 participants in each intervention arm would provide 90% power to detect a difference between 12% sustained unresponsiveness in the placebo group (3% spontaneously outgrowing peanut allergy per year) and 60% sustained unresponsiveness in the PPOIT group. Clinical outcomes at 4-year follow-up were analysed by intention to treat. The effects of treatment were estimated in terms of absolute differences, numbers needed to treat, and risk ratios (RRs) with 95% CIs. We tested the hypothesis of no difference in the effect of treatment between intervention groups with the test of proportions with Fisher's exact tests (with double the one-tailed exact probability), and with unadjusted and adjusted (by age, sex, or time since treatment cessation) generalised linear models. We addressed missing data (ie, participants lost to follow-up) with two approaches: worst-case-scenario models for peanut ingestion outcomes and weighted inverse probability models for sustained unresponsiveness outcomes. Detailed description of these approaches and the formula for calculating reaction rates per 10 person-years are in the appendix. Peanut skin prick

test wheal sizes were reported as mean (SD), and between-group comparisons were analysed by *t* test. Other immune markers, including peanut sIgE, sIgG4, and the sIgG4:sIgE ratio had skewed distributions, and were summarised as geometric means (95% CI) and assessed by regression models. Study data were gathered and managed with REDCap³¹ electronic data-capture tools hosted at Murdoch Childrens Research Institute and analysed with Stata (version 14.2).

Role of the funding source

The study sponsors had no role in study design; data collection, analysis, or interpretation; or writing of the Article. K-CH, CA, and MLKT had access to all study data and final responsibility for the decision to submit for publication.

See Online for appendix

Results

48 (86%) of 56 eligible participants were enrolled, 24 from the PPOIT group and 24 from the placebo group (figure). Mean time from treatment cessation to entry into this study was 4.2 years (SD 0.6) in the PPOIT group and 4.2 years (SD 0.7) in the placebo group. Baseline characteristics were similar between groups (table 1). Of the four PPOIT-treated participants who declined to participate in the follow-up study (figure), one was peanut desensitised and three had sustained unresponsiveness at the end of the parent study. All four placebo-treated participants who declined to participate were peanut

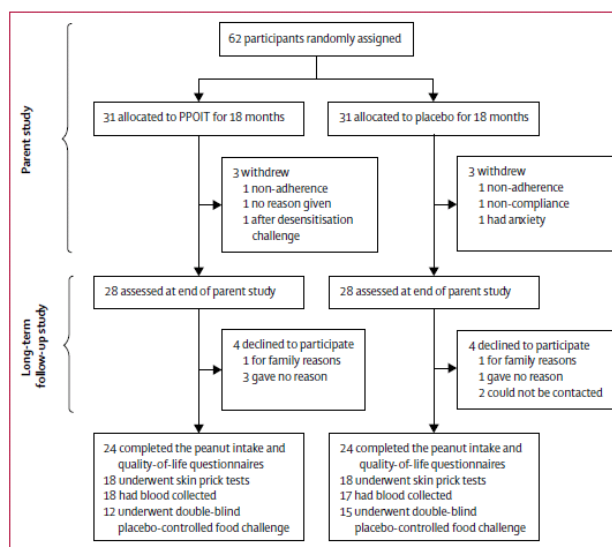


Figure: Participant flow
PPOIT=probiotic and peanut oral immunotherapy.

	PPOIT group (n=24)	Placebo group (n=24)
At entry to parent study		
Male sex	15 (63%)	16 (67%)
Had one or more siblings	22 (92%)	21 (88%)
Physician-diagnosed asthma	13 (54%)	9 (38%)
Physician-diagnosed eczema	19 (79%)	19 (79%)
At entry to 4-year follow-up study		
Age, years	12.1 (2.4)	11.7 (2.9)
Time since commencing study treatment, years	5.8 (0.6)	5.8 (0.7)
Time since completing study treatment, years	4.2 (0.6)	4.2 (0.7)

Data are mean (SD) or n (%). PPOIT=probiotic and peanut oral immunotherapy.

Table 1: Baseline characteristics

	PPOIT group	Placebo group
Peanut ingestion		
Ingesting peanut	16/24 (67%)	1/24 (4%)
Not ingesting peanut	8/24 (33%)	23/24 (96%)
Sensitivity analysis		
Scenario 1*	16/28 (57%)	1/28 (4%)
Scenario 2†	16/28 (57%)	5/28 (18%)
Frequency of ingestion		
Never	8 (33%)	23 (96%)
Less than once a week	5 (21%)	0 (0%)
Once or twice a week	7 (29%)	0 (0%)
Three or more times a week	4 (17%)	1 (4%)
Amount of peanut ingested‡		
None	7 (30%)	23 (96%)
<2 g	4 (17%)	0 (0%)
2 g to <4 g	6 (26%)	1 (4%)
≥4 g	6 (26%)	0 (0%)
≥2 g (ie, moderate-to-large amount)	12 (52%)	1 (4%)
Peanut skin prick test wheal size (mm)		
n	18	18
Mean (SD)	8.1 (7.7)	13.3 (7.6)
Median (IQR)	6.3 (1.0 to 14.0)	14.0 (9.0 to 18.5)

Data are n/N (%) or n (%), unless otherwise specified. PPOIT=probiotic and peanut oral immunotherapy. *All who declined to participate were recorded as not ingesting. †All participants in the PPOIT group who declined to participate were recorded as not ingesting; all placebo participants who declined to participate were recorded as ingesting. ‡n=23 for the PPOIT group because one participant did not provide a response about the amount of peanut they ingested.

Table 2: Peanut ingestion and skin prick test

allergic at the end of the parent study. We noted no between-group differences at entry to the parent study for participants who declined long-term follow-up, but significant differences in skin prick test results ($p=0.001$ at end of study; $p=0.009$ 3 months later). All other demographic features were similar between groups for participants who declined follow-up.

16 (67%) of 24 participants in the PPOIT group were still eating peanut 4 years after stopping study treatment, compared with one (4%) of 24 in the placebo group (absolute difference 63% [95% CI 42–83], $p=0.001$; number needed to treat 1.6 [95% CI 1.2–2.4]; unadjusted RR 16.0 [95% CI 2.3–113.6]; table 2). Adjustment for age, sex, or time since treatment cessation as separate models resulted in RRs of 15.6–16.4 ($p=0.006$). 12 (52%) of 23 PPOIT-treated participants (one person did not provide information about peanut ingestion) were ingesting moderate-to-large amounts of peanut (ie, ≥ 2 g peanut protein) per ingestion episode, and 11 (46%) were ingesting peanut at least once a week (table 2), suggesting ingestion ad libitum. More participants in the PPOIT group were ingesting more than 2 g of peanut protein than in the placebo group (absolute difference 48% [95% CI 26–70], $p=0.001$; unadjusted RR 12.5 [95% CI 1.7–90.6]). The RR was 11.7 (1.5–90.6, $p=0.019$) after adjusting for age, sex, and time since completion of treatment as combined model.

16 (80%) of 20 PPOIT-treated participants who achieved 2-week sustained unresponsiveness at the end of the parent study were regularly ingesting peanuts at 4-year follow-up. Of the other four participants, one ceased all peanut intake shortly after the end of the parent study because they disliked the taste of peanut, two reported symptoms after peanut ingestion within 24 months of treatment cessation, and one maintained regular peanut ingestion for more than 1 year after stopping study treatment but then discontinued peanut intake for around 6 months because of disruptive social circumstances before notifying the study team. The latter three participants subsequently failed formal peanut challenges up to 3 years after stopping treatment and were instructed by the study team to discontinue peanut intake. The one participant in the placebo group who attained sustained unresponsiveness at the end of the parent study continued to ingest peanuts at 4-year follow-up, but did not undergo the 8-week food challenge. The other 23 placebo-treated participants were all peanut allergic at the end of the parent study and had been advised to avoid peanut. None intentionally ingested peanut to test their allergic status.

In the first sensitivity analysis, assuming that all participants who declined to participate in the follow-up study were abstaining from peanuts, the absolute difference between groups was 54% (95% CI 34–73, $p=0.001$), with an unadjusted RR of 16.0 (95% CI 2.2–114.6). The RR was 15.7 (2.1–117.8, $p=0.008$) after adjustment for age, sex, and time since completion of treatment as a combined model. In the second sensitivity analysis, we assumed that all participants from the PPOIT group who declined follow-up had ceased peanut intake and that placebo-treated participants who declined follow-up had acquired the ability to eat peanut. The absolute difference between groups was 39% (95% CI 16–62, $p=0.006$), with an unadjusted RR of 3.2 (1.3 to 7.6) and an adjusted RR of 3.1 (1.3–7.7, $p=0.013$).

	PPOIT group (n=24)	Placebo group (n=24)
Patients who had one or more reactions	4 (17%)	6 (25%)
Reactions per patient		
0	20 (83%)	18 (75%)
1	2 (8%)	3 (13%)
2	1 (4%)	3 (13%)
7	1 (4%)	0 (0%)
Reactions by timepoint		
Total number of reactions	11	9
Within 3 months of stopping treatment	0 (0%)	0 (0%)
Between 3 and 12 months of stopping treatment	2 (18%)	1 (11%)
1–5 years after stopping treatment	9 (82%)	8 (89%)
Reactions by class*		
Anaphylaxis	0 (0%)	0 (0%)
Urticaria	0 (0%)	2 (22%)†
Urticaria plus abdominal pain with or without vomiting	0 (0%)	1 (11%)‡
Urticaria plus transient cough	7 (64%)‡	0 (0%)
Urticaria plus persistent cough	0 (0%)	0 (0%)
Oropharyngeal pruritus	2 (18%)‡	3 (33%)†
Abdominal pain with or without vomiting	2 (18%)†	1 (11%)‡
Abdominal pain plus transient cough plus oropharyngeal pruritus	0 (0%)	1 (11%)‡
Information not available	0 (0%)	1 (11%)‡
Total person-years followed up	99.8	102.0
Estimated number of events per 10 person-years	1.1	0.9

Data are n (%), unless otherwise specified. PPOIT=probiotic and peanut oral immunotherapy. *Two participants in the placebo group had more than one class of reaction. †Two participants. ‡One participant.

Table 3: Reported reactions from completion of study treatment to time of long-term follow-up study

Mean wheal size after skin prick test was similar in both groups before treatment (16.9 mm [SD 6.7] in the PPOIT group vs 16.9 mm [SD 6.9]).²³ At 4-year follow-up, wheals in PPOIT-treated participants (n=18) were smaller than those in placebo-treated participants (n=18; absolute difference -5.2 [95% CI -10.3 to 0.0]; unadjusted p=0.050; age-adjusted and sex-adjusted p=0.035).

20 (83%) of 24 PPOIT-treated participants reported no allergic reactions after peanut ingestion since stopping treatment (table 3). 11 reactions occurred after intentional peanut ingestion in four participants who attained 2-week sustained unresponsiveness in the parent study (table 3). Six placebo-treated participants reported nine allergic reactions after accidental ingestion of peanut (table 3). In both groups, all reactions were minor, none were anaphylaxis, and none necessitated adrenaline treatment (appendix). The number of reactions to peanut was 1.1 per 10 person-years in the PPOIT group and 0.9 per 10 person-years in the placebo group.

	PPOIT group	Placebo group	p value*
Peanut sIgE (kU/L)			
n	18	17	..
Median (IQR)	2.9 (0.7 to 8.8)	9.8 (1.6 to 100.0)	0.057
Geometric mean (95% CI)	2.8 (1.0 to 8.2)	10.7 (3.2 to 36.1)	0.089
Median difference in sIgE since time of entry to parent study (IQR)	-10.9 (-44.9 to -1.0)	-0.2 (-20.3 to 1.6)	0.086
Peanut sIgG4 (mg/L)			
n	15	14	..
Median (IQR)	0.7 (0.1 to 1.6)	0.2 (0.1 to 0.5)	0.150
Geometric mean (95% CI)	0.4 (0.1 to 1.5)	0.2 (0.1 to 0.5)†	0.409
Median difference in sIgG4 since time of entry to parent study (IQR)	0.3 (-0.1 to 1.4)	-0.1 (-0.3 to 0.0)	0.085
Peanut sIgG4:sIgE ratio			
n	15	14	..
Median (IQR)	91.4 (21.4 to 298.1)	2.4 (0.5 to 61.9)	0.018
Geometric mean (95% CI)	67.3 (10.3 to 440.0)	5.2 (1.2 to 21.8)†	0.031
Median difference in ratio since time of entry to parent study (IQR)	35.8 (-0.1 to 296.6)	-1.2 (-3.1 to 6.5)	0.013

PPOIT=probiotic and peanut oral immunotherapy. sIgE=specific IgE. sIgG4=specific IgG4. *The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs). The t test on the log scale was applied for data presented as geometric means (95% CIs). †n=13.

Table 4: Peanut sIgE and sIgG4 concentrations, and sIgG4:sIgE ratio 4 years after completion of study treatment

4 years after treatment cessation, PPOIT-treated participants had numerically lower peanut sIgE concentrations (geometric mean 2.8 kU/L [95% CI 1.0–8.2] vs 10.7 kU/L [95% CI 3.2–36.1]; p=0.089) and higher peanut sIgG4 concentrations (geometric mean 0.4 mg/L [95% CI 0.1–1.5] vs 0.2 mg/L [95% CI 0.1–0.5]; p=0.409) than placebo-treated participants, although these differences were not significant (table 4). Peanut sIgG4:sIgE ratios were significantly higher in PPOIT-treated than in placebo-treated participants (table 4).

27 participants (12 from the PPOIT and 15 from the placebo group) consented to double-blind placebo-controlled food challenge to assess 8-week sustained unresponsiveness. In the placebo group, participants who consented to food challenge were younger than those who declined (p=0.02; appendix), but no other differences—specifically, in peanut ingestion frequency, amount of peanut ingested, peanut skin prick test wheal size, peanut sIgE and sIgG4 concentrations, and status of peanut intake (ingesting or not ingesting) at the time of follow-up—were noted between participants who consented to food challenge and those who did not (appendix). Furthermore, among PPOIT-treated participants, those who achieved 2-week sustained unresponsiveness and maintained regular peanut ingestion afterwards, those who initially achieved sustained unresponsiveness but then discontinued peanut ingestion before the follow-up study, and those who did not achieve 2-week sustained unresponsiveness at the end of the parent study were equally distributed between

	PPOIT group (n=12)	Placebo group (n=15)
8-week sustained unresponsiveness at 4-year follow-up	7 (58%)	1 (7%)*
Achieved sustained unresponsiveness at end of parent study	10	0
Achieved sustained unresponsiveness at end of parent study and maintained it at 4-year follow-up	7	0

Data are n (%) or n. PPOIT=probiotic and peanut oral immunotherapy.
 *One participant was on azathioprine at the time of food challenge; they were not ingesting peanut, had a peanut skin prick test result of 13 mm and a peanut specific IgE concentration of 5.43 kU/L, but they passed the 4 g food challenge.

Table 5: Long-term peanut challenge outcomes in participants who underwent sustained unresponsiveness food challenge after 8 weeks' peanut elimination

the participants who consented to food challenge and the participants who declined.

8-week sustained unresponsiveness as shown by double-blind placebo-controlled food challenge was significantly more common in the PPOIT group than in the placebo group (seven [58%] of 12 vs one [7%] of 15; absolute difference 52% [95% CI 21–82], $p=0.012$; unadjusted RR 8.8 [95% CI 1.2–64.0]; table 5). The RR was 8.7–9.9 ($p=0.023$ – 0.036) after adjustment for age or time since completion of treatment as separate models; the number needed to treat was 1.9 (95% CI 1.2–4.8). Seven (70%) of ten PPOIT-treated participants who had achieved 2-week sustained unresponsiveness at the end of the parent study showed challenge-proven 8-week sustained unresponsiveness 4 years after stopping treatment. The one placebo-treated participant who passed the follow-up food challenge was taking azathioprine for severe eczema (commenced 4 months before the follow-up study) and had a peanut skin prick test wheal size of 13 mm at the time of the challenge. Exclusion of this participant's outcomes strengthened the effect of PPOIT treatment on 8-week sustained unresponsiveness ($p=0.002$). The median cumulative doses tolerated during food challenge were 4000 mg (IQR 2438–4000) in the PPOIT group and 938 mg (188–1938) in the placebo group (Mann-Whitney test $p=0.003$).

The sensitivity analysis to account for non-participation, including backweighting for factors that could relate to the likelihood of failing the food challenge (ie, participant's sex, age at entry to parent study, history of doctor-diagnosed eczema or asthma at entry to parent study, and peanut allergy status [achieved sustained unresponsiveness vs did not achieve sustained unresponsiveness] at the end of the parent study) showed a beneficial effect for PPOIT as measured by challenge-proven 8-week sustained unresponsiveness (adjusted RR 10.0 [95% CI 1.3–78.5], $p=0.029$).

Characteristics of the five PPOIT-treated participants who did not have sustained unresponsiveness after food challenge are in the appendix. Two were peanut allergic

(ie, did not attain 2-week sustained unresponsiveness after the parent study), one attained sustained unresponsiveness at the end of the parent study but became resensitised 26 months after treatment cessation, and two reported regular peanut ingestion without reactions before the follow-up study, but reacted during food challenge at a cumulative dose of 4 g peanut protein. Two participants in the PPOIT group and four in the placebo group developed anaphylaxis during food challenge and required intramuscular adrenaline (appendix). Quality-of-life questionnaires were also completed as part of this study; findings will be reported separately.

Discussion

PPOIT was associated with long-lasting peanut tolerance 4 years after stopping treatment. Two-thirds of PPOIT-treated participants were able to continue regular peanut ingestion, and more than half were ingesting moderate-to-large amounts of peanut on a regular basis, compared with only one (4%) of 24 placebo-treated participants. Allergic reactions from intentional peanut ingestion were uncommon and all reactions were mild, suggesting that those who achieved PPOIT-induced sustained unresponsiveness can safely continue peanut ingestion. However, larger studies and meta-analyses of long-term safety outcomes are needed. 58% of PPOIT-treated participants who completed a double-blind placebo-controlled food challenge 4 years after stopping treatment attained 8-week sustained unresponsiveness, and 70% of participants who had attained sustained unresponsiveness at the end of the parent study maintained this status.

Strengths of our study include the multidimensional assessment of long-term efficacy, the standardised follow-up protocol, the high rate of participation (86%) with equal numbers from PPOIT and placebo groups, and the administration of double-blind, placebo-controlled food challenge after an extended 8-week period of secondary peanut elimination, with similar levels of participation from both groups. Furthermore, the inclusion of placebo-treated participants in this follow-up study allowed detection of effects attributable to the natural resolution of peanut allergy over time. In our study, excluding the participant who was taking a systemic immunosuppressant at the time of food challenge, none of the placebo-treated participants who were allergic to peanuts at the end of the parent study acquired an ability to ingest peanut or passed the food challenge 4 years later.

The major limitation of our study is low participation in the food challenge at 4-year follow-up. However, to our knowledge, this study is the first peanut oral immunotherapy study in which persistence of sustained unresponsiveness by double-blind, placebo-controlled food challenge years after treatment cessation was assessed. Furthermore, prolonged secondary peanut elimination for 8 weeks allowed a high level of confidence in outcome. We noted no differences in

clinical and immunological characteristics (including age at follow-up, peanut ingestion frequency, amount of peanut ingested, sustained unresponsiveness vs allergy status at end of parent study, peanut skin prick test wheal size, and sIgE and sIgG4 concentrations) between those who participated in the challenge and those who did not, suggesting that our findings are probably representative of the total sample of the parent study. Furthermore, we used inverse probability weighting to account for the potential effect of participants lost to follow-up, and a sensitivity analysis accounting for non-response, which still showed a favourable effect of PPOIT treatment 4 years after treatment. Nonetheless, we acknowledge that some caution is needed when generalising our challenge findings to those who declined to participate in the food challenge.

Other limitations include the retrospective collection of data for peanut ingestion and reactions, although this approach has been used in other oral immunotherapy follow-up studies,^{32,33} and the small sample size, which was similar to that in other published oral immunotherapy follow-up studies^{14,15,17–19,22} and reflects the proof-of-concept nature of the parent study. Generalisability of our findings might be partly limited by the single-centre study design. We also acknowledge the limitations of the parent study design, which we have previously discussed.²³ Briefly, the absence of a probiotic only group and an oral immunotherapy only group limit our ability to delineate the individual contributions of the two constituents of PPOIT. We note, however, that PPOIT did not modify skin prick test wheal sizes for other food allergens,²³ suggesting that probiotic alone probably did not produce the noted beneficial effects. Future randomised controlled trials comparing allergen oral immunotherapy, a combination of a probiotic and allergen oral immunotherapy, and placebo are needed to establish whether the probiotic acts synergistically with oral immunotherapy. The parent study also did not include a double-blind, placebo-controlled food challenge at study entry to confirm peanut allergy, and instead relied on objective reaction to peanut and 95% positive predictive value thresholds for sIgE concentrations and skin prick tests, in line with other oral immunotherapy trials at the time. However, randomisation would distribute any misclassifications evenly between groups, and placebo participants had a low frequency of sustained unresponsiveness, suggesting that misclassification of peanut allergy was unlikely.

Participants who achieved 2-week sustained unresponsiveness after 18 months of study treatment were instructed to incorporate peanut into their normal diet *ad libitum*, without predefined ingestion frequency or amounts, which is generally the advice given to patients who have naturally outgrown their food allergy. Consistent with the general view, we do not consider *ad libitum* peanut ingestion to be the same as continuing active peanut oral immunotherapy. The proportion of

PPOIT-treated participants who were ingesting peanuts 4 years after treatment compares favourably with those from other post-treatment follow-up studies of milk and egg oral immunotherapy.^{32,33} 80% of participants who attained 2-week sustained unresponsiveness after PPOIT treatment were ingesting peanut *ad libitum* 4 years after treatment, with 60% tolerating moderate-to-large amounts (>2 g peanut protein). Few studies of sustained unresponsiveness years after cessation of oral immunotherapy are available for comparison, but the proportion of patients achieving sustained unresponsiveness at 4 years is more than double that achieved immediately after oral immunotherapy alone in most studies, suggesting that addition of the probiotic could enhance the tolerance-inducing capacity of oral immunotherapy.

An important finding was that reactions to intentional peanut ingestion were uncommon in those who had attained sustained unresponsiveness at the end of the parent study and who had been instructed to ingest peanut freely. The frequency and severity of reactions in PPOIT-treated participants were similar to those after accidental ingestion in placebo-treated participants, suggesting that PPOIT-induced sustained unresponsiveness offers an equivalent safety profile to peanut avoidance. Furthermore, PPOIT-treated participants who maintained their sustained unresponsiveness status did not report any allergic reactions to peanut in the preceding 4 years. By comparison, 24 (75%) of 32 children who were desensitised after milk oral immunotherapy reported reactions in the preceding 12 months when assessed 4–5 years after treatment cessation, with anaphylaxis in six (25%).³² These findings suggest that sustained unresponsiveness is a preferred outcome for individuals with food allergy. Further studies are needed to establish the safety of continuing peanut ingestion in patients who initially achieve sustained unresponsiveness but subsequently have symptoms after ingestion. Investigation of long-term quality of life in people who achieve sustained unresponsiveness or desensitisation compared with those who do not achieve such outcomes is also important.

Reactions to accidental peanut ingestion in placebo participants were infrequent and reaction severity tended to be mild. This finding contrasts with published reports of retrospective studies,^{34–36} which describe accidental reactions in 50% of children with peanut allergy within 1–2 years, and 75% within 3–5 years, with roughly 50% of these reactions involving the respiratory system. In both the parent study and this follow-up study, we systematically gathered information about reactions and calculated the number and severity of reactions over a fixed period in all participants. This procedure probably increased the accuracy of information gathered but could also have modified participant behaviour, leading to improved vigilance and early management of allergic reactions. During the parent study, allergy nurses reviewed the symptoms and emergency management of

allergic reactions with each participant or their parents, or both, at each study visit (every 2 weeks for at least 8 months, then every month for up to 10 months). This intensive education probably provided trial participants and their caretakers with increased knowledge, confidence, and ability to avoid allergens and recognise and treat allergic symptoms, which could have led to fewer and less severe reactions. This intensive education is the most likely explanation for the low frequency of accidental peanut ingestion and mild reactions reported by participants in the placebo group, and consistent with the common observation that participation in a clinical trial leads to improved outcomes for placebo participants, highlighting the importance of including a placebo group. However, another plausible explanation is that published data for rates and severity of accidental reactions might not be generalisable to the wider population or to our select study population.

We did not establish whether the three PPOIT-treated participants who attained 2-week sustained unresponsiveness at the end of the parent study but did not achieve 8-week sustained unresponsiveness at 4 years had become resensitised or had never attained true sustained unresponsiveness in the first place. Perhaps these participants would not have passed an 8-week sustained unresponsiveness food challenge at the end of the parent study despite passing the 2-week challenge, because the likelihood of passing a sustained unresponsiveness food challenge reduces with increasing periods of secondary allergen elimination.^{19,37} Alternatively, initial sustained unresponsiveness might have been lost during the subsequent 4 years, a possibility that is supported by the finding that reactions to peanut occurred more than 12 months after stopping treatment despite continued peanut intake. Future studies of 8-week sustained unresponsiveness immediately after treatment and again years later will clarify this issue.

Probable reasons for participants to decline to participate in the placebo-controlled double-blind food challenge include potential inconvenience, risk of severe allergic reaction, and perceived risk of losing the ability to ingest peanut. Regular intake is known to maintain a desensitised state. Thus, many PPOIT-treated participants who had attained sustained unresponsiveness at the end of the parent study and were ingesting peanut declined to participate in the follow-up study food challenge (data not shown). Conversely, some participants welcomed the opportunity to undergo a food challenge to clarify whether they had maintained sustained unresponsiveness or were only desensitised. We suggest that sustained unresponsiveness as confirmed by placebo-controlled, double-blind food challenge should be used to monitor long-term clinical effectiveness in oral immunotherapy trials.

Although our results suggest long-lasting modulation of the peanut-specific immune response with reduction in typical allergy markers and increased sIgG4, these

changes have also been reported with desensitisation in the absence of tolerance,³⁸ emphasising the need to identify accurate and reliable biomarkers to distinguish desensitisation from sustained unresponsiveness.

To conclude, our results suggest that PPOIT is effective at inducing long-term sustained unresponsiveness that persists for up to 4 years after completing treatment and is safe. Furthermore, the finding that sustained unresponsiveness was maintained without the need to follow a regular prespecified ingestion schedule provides a compelling argument that PPOIT-induced immune tolerance. Ours is the first study to show prolonged 8-week sustained unresponsiveness several years after treatment has ceased, and suggests the possibility that tolerance is a realistic target for food allergy treatments. In a future study, microbial composition of stool samples will be analysed to examine effects of PPOIT therapy on the gut microbiome. Furthermore, a three-group, multicentre randomised controlled trial (ACTRN12616000322437) of PPOIT versus peanut oral immunotherapy versus placebo is underway to address the important and as-yet unanswered question of whether the addition of a probiotic confers greater benefit than oral immunotherapy alone.

Contributors

K-CH contributed to study conception and design; managed the study; did the clinical procedures, mechanistic work, and statistical analysis; drafted the Article; and contributed to the final version. A-LP contributed to study design and conduct, statistical analysis, data interpretation, and writing of the final report. CA and SP did the clinical procedures, and contributed to and reviewed the final report. MLKT conceived the parent PPOIT randomised trial, developed the follow-up study protocol, was responsible for oversight of study conduct and data analyses, and contributed to writing of the Article as senior author.

PPOIT Study Team

Wesley Burks, Susan Donath, Francesca Orsini, Dean Tey, Marnie Robinson, Ee Lyn Su.

Declaration of interests

K-CH reports grants from Murdoch Childrens Research Institute and the Australian Food Allergy Foundation. A-LP reports grants from Murdoch Childrens Research Institute and the Australian Food Allergy Foundation (previously the Ilhan Food Allergy Foundation), and personal fees from the National Health and Medical Research Council. MLKT reports grants from the Australian Food Allergy Foundation and Murdoch Childrens Research Institute, and personal fees from Nestlé Nutrition Institute, Danone Nutricia, GLG Consulting, Deerfield Consulting, Bayer, Prota Therapeutics, and Wiley. She also has a licensed patent method for inducing tolerance. CA and SP declare no competing interests.

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3.3. Supplementary Material

SUPPLEMENTARY MATERIAL

Title: Long-term clinical and immunological effects of Probiotic and Peanut Oral Immunotherapy (PPOIT) post-treatment cessation: Four-year follow-up of a randomized double-blind placebo-controlled trial

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METHOD

DBPCFC procedure

DBPCFC were performed by allergy research nurses under medical supervision. Two sets of containers (A and B) were prepared by the Royal Children's Hospital (RCH) Clinical Trials Pharmacy. One container (either A or B) was the placebo (maltodextrin, brown food coloring and peanut essence) and the other (B or A, respectively) was the peanut protein (12% defatted peanut flour, 50% peanut protein; Byrd Mill, Ashland, Va). The RCH Clinical Trials Pharmacy randomly assigned placebo or peanut to part A for each participant DBPCFC using a random number table provided by the study statistician. Individual challenge doses were prepared by RCH Nutritional Services. The doctor and nurse administering the challenge remained blinded to the contents of each part of the challenge and to the participant's pre-challenge peanut SPT wheal size. Doses were given at 15-minute intervals according to the challenge-dosing schedule shown in Supplementary table 5, identical to the DBPCFC protocol used in the parent study¹. Participants were observed for 2 hours after the last challenge dose. Pre-determined challenge stopping criteria (Supplementary table 6) were used to ensure consistency in interpretation of reaction symptoms and signs. DBPCFC was classified as 'passed' if both parts of the challenge were passed. DBPCFC was classified as 'failed' if the participant experienced objective symptoms during the peanut but not placebo component of the DBPCFC. DBPCFC was classified as inconclusive if the participant experienced objective symptoms during the placebo challenge – no cases of inconclusive challenge occurred.

Statistical analysis

The number of reactions on peanut ingestion per 10 person-years in each intervention group was estimated using the formula: number of reactions x 10 / (number of participants followed-up x mean number of years since completion of treatment).

We addressed missing data (participants lost-to-follow-up) using two approaches. For the outcome of peanut ingestion, we estimated the RR in two worst-case scenarios. In the first scenario, we assumed all participants who declined follow-up were abstaining from peanut ingestion. In the second scenario, we assumed all PPOIT participants who declined follow-up had ceased peanut intake and all placebo-treated participants who declined follow-up had acquired the ability to eat peanut. For the DBPCFC-based SU outcome, we employed inverse probability models, weighted to correct for attrition after parent study end as follows: the weight for each subject was the inverse of the probability of the subject undergoing the DBPCFC at four year follow-up, among those that completed the parent study (and therefore eligible for long-term follow-up, n=56). These weights to correct for non-response accounted for participant's sex, age at entry to parent study, history of doctor diagnosed eczema at entry to parent study, history of doctor diagnosed asthma at entry to parent study and peanut allergy status (achieved SU vs. did not achieve SU) at end-of-parent-study.

Supplementary Table 1. Details of individual reactions since completion of study treatment

Group	Individual	idno	Months since completion of study treatment	Regular peanut ingestion prior to reaction	Typical amount ingested prior to reaction	Cofactors (Exercise / Intercurrent viral illness / Prolonged avoidance)	Reaction eliciting amount	Summary of symptoms	Current status
Placebo	1	105	16	N	Nil	Nil	Smear	Urticaria	Avoiding peanut
Placebo	2	215	11	N	Nil	Nil	Smear	Oropharyngeal pruritis	Avoiding peanut
Placebo	2	215	45	N	Nil	Nil	Smear	Oropharyngeal pruritis	Avoiding peanut
Placebo	3	221	40	N	Nil	Nil	Smear	Urticaria	Avoiding peanut
Placebo	4	405	56	N	Nil	Nil	Smear	Abdominal pain	Avoiding peanut
Placebo	5	415	46	N	Nil	Nil	Smear	Abdominal pain, occasional cough, oropharyngeal pruritis	Avoiding peanut
Placebo	5	415	55	N	Nil	Nil	Smear	Oropharyngeal pruritis	Avoiding peanut
Placebo	6	429	29	N	Nil	Nil	Smear	*	Avoiding peanut
Placebo	6	429	35	N	Nil	Nil	Smear	Urticaria, abdominal pain, vomit	Avoiding peanut
PPOIT	7	223	5	Y	Large amount (approx 15 peanuts)	Illness	Large amount (approx 15 peanuts)	Hives, transient cough	Still ingesting peanut; large amount (approx 4g or more)
PPOIT	7	223	16	Y	Large amount (approx 15 peanuts)	Illness	Large amount (approx 15 peanuts)	Hives, transient cough	Still ingesting peanut; large amount (approx 4g or more)
PPOIT	7	223	22	Y	Large amount (approx 15 peanuts)	Illness	Large amount (approx 15 peanuts)	Hives, transient cough	Still ingesting peanut; large amount (approx 4g or more)
PPOIT	7	223	28	Y	Large amount (approx 15 peanuts)	Illness	Large amount (approx 15 peanuts)	Hives, transient cough	Still ingesting peanut; large amount (approx 4g or more)
PPOIT	7	223	34	Y	Large amount (approx 15 peanuts)	Illness	Large amount (approx 15 peanuts)	Hives, transient cough	Still ingesting peanut; large amount (approx 4g or more)
PPOIT	7	223	42	Y	Large amount (approx 15 peanuts)	Illness	Large amount (approx 15 peanuts)	Hives, transient cough	Still ingesting peanut; large amount (approx 4g or more)
PPOIT	7	223	45	Y	Large amount (approx 15 peanuts)	Illness	Large amount (approx 15 peanuts)	Hives, transient cough	Still ingesting peanut; large amount (approx 4g or more)
PPOIT	8	401	34	Y	Unsure*	Unsure*	Unsure*	Oropharyngeal pruritis	Still ingesting peanut; moderate amount (up to approx 4g)
PPOIT	8	401	34	Y	Unsure*	Unsure*	Unsure*	Oropharyngeal pruritis	Still ingesting peanut; moderate amount (up to approx 4g)
PPOIT	9	409	21	N	Small amount (< 8 peanuts)	Prolonged avoidance (approx. 4 weeks)	Small amount (satay chicken)	Abdominal pain	Avoiding peanut
PPOIT	10	431	9	N	Small amount (< 8 peanuts)	Prolonged avoidance (approx. 6 weeks)	Small amount (a few peanuts)	Abdominal pain, vomit	Avoiding peanut

Supplementary Table 2. Clinical and immunologic characteristics of subjects who consented to 8-week SU DBPCFC and those who did not

	PPOIT			Placebo		
	Yes (n=12)	No (n=12)		Yes (n=15)	No (n=9)	
Underwent 8-week SU DBPCFC at 4 year follow-up						
Age at time of 4 year follow-up study			p-value*			p-value*
Mean (SD), n	11.1 (2.4), 12	13.0 (2.2), 12	0.072	10.1 (2.2), 15	14.4 (1.5), 9	0.016
Baseline characteristics, n (%)						
% Male	7 (58)	8 (67)	0.674	8 (53)	8 (89)	0.099
% Passed 2-week SU DBPCFC at end of parent study	10 (83)	10 (83)	1.000	0 (0)	1 (11)	N/A
% Ingesting peanut, any frequency	8 (67)	8 (67)	1.000	0 (0)	1 (11)	N/A
% Not ingesting peanut	4 (33)	4 (33)	1.000	15 (100)	8 (89)	N/A
% Ingesting moderate to large amount of peanut	6 (50)	6 (55)#	0.827	0 (0)	1 (11)	N/A
Frequency of peanut ingestion, n (%)						
Nil	4 (33)	4 (33)		15 (100)	8 (89)	
Less than once a week	3 (25)	2 (17)		0 (0)	0 (0)	
1-2 times a week	3 (25)	4 (33)		0 (0)	0 (0)	
3 or more times a week	2 (17)	2 (17)		0 (0)	1 (11)	
Typical amount of peanut ingested, n (%)						
Avoiding peanut	4 (33)	3 (27)		15 (100)	8 (89)	
Small amount (< 2 g)	2 (17)	2 (18)		0 (0)	0 (0)	
Moderate amount (2 g to <4 g)	3 (25)	3 (27)		0 (0)	1 (11)	
Large amount (4 g or more)	3 (25)	3 (27)		0 (0)	0 (0)	
Peanut SPT weal size; Mean (SD), n			p-value**			p-value**
At baseline, mm	15.0 (8.1), 12	18.9 (4.5), 12	0.152	16.7 (7.2), 15	17.2 (6.8), 9	0.876
At completion of study treatment, mm	4.7 (5.2), 12	4.0 (3.5), 12	0.733	13.5 (4.4), 15	13.9 (7.8), 9	0.870
At 3 months after completion of study treatment, mm	4.9 (5.4), 12	3.7 (4.3), 12	0.547	15.1 (6.3), 15	12.3 (5.1), 9	0.273
At 4 year follow-up, mm	6.6 (6.9), 12	11.2 (8.9), 6	0.249	12.9 (6.0), 15	15.2 (15.3), 3	0.656
Immune indices; Median (IQR), n			p-value***			p-value***
Peanut sIgE at baseline, kU/L	9.3 (0.6, 64.4), 12	12.0 (1.8, 131.0), 12	0.583	14.0 (3.1, 51.9), 15	1.9 (0.8, 11.4), 9	0.180
Peanut sIgG4 at baseline, mgA/L	0.4 (0.1, 0.7), 12	0.7 (0.1, 1.1), 12	0.418	0.3 (0.2, 0.8), 15	0.2 (0.2, 0.7), 9	0.612

Peanut sIgE at completion of study treatment, kU/L	2·6 (0·8, 35·7), 12	3·1 (1·1, 33·6), 12	0·863	28·6 (1·9, 72·1), 15	1·4 (0·8, 23·1), 9	0·144
Peanut sIgG4 at completion of study treatment, mgA/L	2·1 (1·3, 28·4), 12	8·1 (2·2, 43·4), 12	0·453	0·2 (0·2, 0·5), 15	0·2 (0·1, 0·6), 9	0·591
Peanut sIgE at 3 months after completion of study treatment, kU/L	3·0 (0·5, 31·9), 12	3·3 (1·4, 30·8), 12	0·863	33·7 (1·8, 155·0), 15	2·0 (1·6, 17·7), 9	0·325
Peanut sIgG4 at 3 months after completion of study treatment, mgA/L	2·4 (1·1, 8·8), 12	3·8 (1·2, 28·0), 12	0·525	0·3 (0·2, 0·6), 15	0·7 (0·4, 1·7), 9	0·189
Peanut sIgE at 4 year follow-up, kU/L	2·4 (0·8, 14·7), 12	8·3 (0·5, 8·8), 6	0·574	20·7 (5·4, 100·0), 14	0·9 (0·0, 100·0), 3	0·251
Peanut sIgG4 at 4 year follow-up, mgA/L	0·8 (0·3, 1·6), 10	0·4 (0·0, 1·1), 5	0·358	0·2 (0·1, 0·5), 13	0·3 (0·3, 0·3), 1	0·710

one of the participants did not provide a response to amount of peanut ingesting

*Logistic regression, **t test, ***Mann-Whitney test

Supplementary Table 3: Characteristics participants who reacted at 8w-SU-DBPCFC despite previous regular ingestion and/or passed 2w-SU-DBPCFC

ID number	228	416	209	101	406
Age at 8-week SU DBPCFC, y	8·5	14·2	11·0	12·5	14·0
Frequency of peanut ingestion	1-2 times a week	Less than once a week	Not ingesting peanut	Not ingesting peanut	Not ingesting peanut
Typical amount of peanut ingestion	Moderate amount (2g to less than 4g)	Moderate amount (2g to less than 4g)	Nil	Nil	Nil
Longest gap without peanut, prior to 8 week SU DBPCFC	Months	Months	N/A	N/A	N/A
Cumulative dose tolerated in DBPCFC (mg)	2937·5	2937·5	187·5	1937·5	1937·5
Reaction eliciting cumulative dose (mg)	4000·0	4000·0	437·5	2937·5	2937·5
DPBCFC reaction signs	Lower respiratory (wheeze), cutaneous, oropharyngeal	Gastrointestinal (vomited), oropharyngeal	Gastrointestinal, cutaneous, oropharyngeal, rhinoconjunctival	Gastrointestinal, cutaneous, rhinoconjunctival	Lower respiratory (wheeze), cutaneous, oropharyngeal
DPBCFC treatment	Oral antihistamine, IM adrenaline (2 doses)	None required	Oral antihistamine	None required	Oral antihistamine, IM adrenaline (2 doses)
Peanut SPT, mm	11·5	7·5	17	12·5	19·5
Peanut sIgE, kU/L	24·7	24·6	100·0	2·1	2·6

Supplementary Table 4. Reactions during 8-week SU DBPCFC

	PPOIT	Placebo
DBPCFC reaction symptoms [as % of all undergoing DBPCFC]		
Any cutaneous, n (%)	4 (33)	8 (53)
Any gastrointestinal, n (%)	3 (25)	12 (80)
Any rhinoconjunctival, n (%)	2 (17)	11 (73)
Any oropharyngeal, n (%)	4 (33)	9 (60)
Any lower respiratory, n (%)	2 (17)	4 (27)
Any cardiovascular, n (%)	0 (0)	0 (0)
DBPCFC related anaphylaxis, n (%)	2 (17)	4 (27)
Patients needing any IM adrenaline, n (%)	2 (17)	4 (27)
Patients needing overnight admission, n (%)	2 (17)	0 (0)

Supplementary Table 5. DBPCFC dosing schedule

Food Challenge A			
1000	Dose 1	62.5mg	
1015	Dose 2	125mg	
1030	Dose 3	250mg	
1045	Dose 4	500mg	
1100	Dose 5	1000mg	
1115	Dose 6	1000mg	
1130	Dose 7	1062.5 mg	Spirometry

Observed for 2 hour, if no allergic symptoms develop continue to food challenge B

Food Challenge B			
1330	Dose 1	62.5mg	
1345	Dose 2	125mg	
1400	Dose 3	250mg	
1415	Dose 4	500mg	
1430	Dose 5	1000mg	
1445	Dose 6	1000mg	
1500	Dose 7	1062.5mg	Spirometry

Observe for 2 hours if no allergic symptoms develop discharge home

Supplementary Table 6: DBPCFC challenge stopping criteria

Standardised cessation criteria for a positive OFC result = ANY of the following objective signs occurring within TWO (2) hours of ingestion:

- 3 or more concurrent non-contact urticaria persisting for at least 5 minutes¹;
- perioral, periorbital¹ or facial angioedema;
- vomiting (excluding gag reflex)¹ and/or diarrhoea;
- persistent cough (i.e. not just intermittent and transient throat clearing), wheeze (either audible (without stethoscope) or on auscultation with stethoscope), change in voice, stridor, difficulty breathing, collapse¹, hypotension (ViCTOR chart)²;
- long bursts of sneezing,³

Standardise criteria to delay the next scheduled dose by 15 minutes:

- persistent throat tightness/pain³ (subjective)
- severe abdominal pain (subjective) and/or notably distressed due to GI symptoms with decreased activity³
- mild subjective cardiovascular response (weak, dizzy) without evidence of hypotension or tachycardia^{2,3}

Objective signs that might not necessitate cessation of challenge or delaying of a dose:

- occasional scratching³
- ³
- a few areas of faint erythema³
- rare bursts (of sneezing), occasional sniffing³
- complaints of nausea or abdominal pain, itchy mouth/throat

Staff required to proceed with a T9 DBPCFC

PPOIT Study Nurse physically present in immediate vicinity of challenge area; and

PPOIT Study Doctor physically present in immediate vicinity of challenge area; and

PPOIT Chief Investigator (CI) or Principal Investigator (PI) physically in Melbourne and contactable by phone

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3.4. Chapter summary

The follow-on study of the PPOIT-001 trial confirmed that the clinical benefits and persistent suppression of peanut allergic immune response associated with PPOIT were maintained at 4 years post-treatment. In the next two chapters (Chapters 4 and 5) the doctoral candidate presents findings on the participants' longitudinal immune responses to PPOIT.

Chapter 4. Results: Immunomodulatory effects of PPOIT on the antibody responses of participants in the PPOIT-001 randomised trial

4.1. Chapter introduction

In the previous chapter (Chapter 3), the doctoral candidate presented long-term clinical outcomes of participants in the PPOIT-001 trial. In this chapter, findings relating to the immunomodulatory effects of PPOIT on participants' antibody responses are presented.

4.2. Abstract

4.2.1. Background

Probiotic and Peanut Oral Immunotherapy (PPOIT) is effective at inducing challenge proven 2-6 week sustained unresponsiveness (SU) at end of treatment and this effect persists to four years post-treatment in the majority of treatment responders, indicating long-term SU (LTSU). The doctoral candidate examined longitudinal effects of PPOIT on peanut specific humoral immune responses and their relationship with LTSU in participants of the PPOIT-001 randomised trial.

4.2.2. Method

Serum or plasma levels of whole peanut and peanut component (Ara h1, h2, h3, h8, h9) specific-IgE (sIgE) and specific IgG4 (sIgG4) were measured by ImmunoCAP in children (n=62) enrolled in the Probiotic and Peanut Oral Immunotherapy (PPOIT) randomised trial (PPOIT-001) at study entry or baseline (T0), after 18 months of treatment (T1), at 3 months post-treatment (T3) and at 4 years post-treatment (T5). Peanut specific salivary IgA was measured by ELISA at T0, at weeks 17, 33, 49 (approximately 4, 8, 12 months respectively) during treatment, after 18 months of treatment (T1), 2-6 weeks post-treatment (T2), 3 months post-treatment (T3) and at 12 months post-treatment (T4). Multivariate regression analyses of log-transformed values were used for point-in-time between group comparisons. Generalised estimating equations (GEE) were used for between group comparisons of longitudinal trends.

4.2.3. Results

PPOIT was associated with changes in peanut and Ara h1, h2, h3 sIgE and sIgG4 over time. sIgE levels were significantly reduced post-treatment (between group comparison at T5, PPOIT v.s. Placebo: sIgE peanut $p=0.089$, Ara h1 $p=0.017$, Ara h2 $p=0.008$, Ara h3 $p=0.042$). sIgG4 levels were significantly increased by end of treatment (between group comparison at T1, PPOIT vs placebo: sIgG4 peanut $p<0.001$, Ara h1 $p=0.022$, Ara h2 $p<0.001$, Ara h3 $p<0.001$) but decreased once treatment was stopped and returned to baseline (that is, study entry) levels by T5 (between group comparison at T5, PPOIT vs placebo: sIgG4 peanut $p=0.409$, Ara h1 $p=0.549$, Ara h2 $p=0.034$, Ara h3 $p=0.234$).

Similarly, salivary peanut sIgA increased during treatment, as early as 4 months of treatment (PPOIT v.s. Placebo $p=0.006$), then reduced post-treatment. In PPOIT-treated participants who attained 2-6 week SU and were instructed to ingest peanut ad-libitum, those who ingested larger amounts of peanut at 4 years post-treatment had lower peanut SPT wheal size (adjusted $p=0.032$) compared to those who were eating a small amount of peanut or not ingesting peanut at all; however the amount of peanut ingestion did not affect peanut sIgE level (adjusted $p=0.462$). Conversely, the amount of peanut ingestion at 4 years post-treatment correlated with peanut sIgG4 levels (adjusted $p=0.015$). To investigate immune changes associated with LTSU at 4 years post-treatment, immune measures were compared in PPOIT-treated participants who did or did not have LTSU at 4 years post-treatment. Participants with PPOIT-induced LTSU had lower SPT wheal size (age-, sex-, ingestion-adjusted $p=0.001$), lower sIgE (age-, sex-, ingestion- adjusted peanut $p=0.003$, Ara h1 $p=0.004$, Ara h2 $p=0.001$, Ara h3 $p=0.023$) and lower sIgG4 (age-, sex-, ingestion-adjusted peanut $p=0.032$, Ara h1 $p=0.002$, Ara h2 $p=0.084$, Ara h3 $p=0.018$) compared with PPOIT-treated participants who did not have LTSU.

4.2.4. Conclusions

PPOIT was associated with broad reduction in peanut specific humoral responses which may mediate clinical effects. Long-term sustained unresponsiveness (LTSU) that persists to 4 years post-treatment was associated with downregulation of the B cell response to peanut.

4.3. Rationale

A novel peanut oral immunotherapy treatment, combining probiotic with peanut oral immunotherapy, (PPOIT) is effective at inducing clinical remission (2-6 week sustained unresponsiveness (SU) at end of treatment[95]; and more importantly, long-term sustained unresponsiveness (LTSU) at 4 years post-treatment[99] as presented in the previous chapter. Gradual exposure to allergen is postulated to suppress the allergic response (desensitisation) through downregulation of effector cells and eventually redirect the allergen-specific immune response from allergy to tolerance. Mechanistic studies during oral immunotherapy (OIT) have demonstrated treatment associated reduction in allergen skin prick test (SPT) wheal size[18 40 94 95 100], which reflects suppression of mast cell activation; however reductions in circulating levels of allergen specific IgE (sIgE) are generally not achieved[18 55 100]. A few studies demonstrate reduced sIgE after years of treatment[19 61], suggesting that the underlying allergic response may be modulated after longer duration of OIT. Allergen specific OIT is consistently associated with upregulation of allergen specific IgG4 (sIgG4)[18 37 55 94 101 102] and salivary IgA[56] during the treatment phase, however the roles of sIgG4 and salivary IgA in tolerance acquisition remain uncertain. Whilst an increase in sIgG4 blocking antibodies has been suggested to be important for the transition to tolerance, evidence for this remains lacking. For example, it is notable that sIgG4 levels increase markedly during allergen immunotherapy even in the absence of tolerance induction[19 40]. This suggests that increases in allergen-specific IgG4 with immunotherapy may reflect allergen exposure rather than transition of the allergen-specific immune response towards tolerance.

The doctoral candidate hypothesised that (1) PPOIT induces upregulation of peanut and peanut component sIgG4 and peanut salivary IgA levels during treatment, (2) PPOIT induces long-term downregulation of peanut and peanut component sIgE levels and (3) long-term sustained unresponsiveness (LTSU) at 4 years post-treatment is associated with reduction in peanut and peanut component sIgE levels.

The specific aims of this study are to:

- Compare the longitudinal changes in peanut and peanut component (Ara h1, h2, h3, h8 and h9) sIgE and sIgG4 levels and peanut SPT wheal size in PPOIT-001 trial participants in the PPOIT (active treatment) and placebo (control treatment) groups;
- Compare the longitudinal changes in peanut salivary IgA levels in PPOIT-001 trial participants in the PPOIT and placebo groups;
- Examine the association between peanut ingestion and peanut SPT wheal size, peanut and peanut component (Ara h1, h2, h3, h8 and h9) sIgE, sIgG4, at 4 years post-treatment (T5) in participants in the PPOIT group;
- Examine the association between LTSU and peanut SPT wheal size, peanut and peanut component (Ara h1, h2, h3, h8 and h9) sIgE, sIgG4, at 4 years post-treatment (T5) in participants in the PPOIT group.

4.4. Abbreviated methods

Detailed methods including laboratory methods for the PPOIT-001 trial are presented in Chapter 2. The following is a summary of select aspects of study methodology relevant to this results chapter.

4.4.1. Participants

The Probiotic and Peanut Oral Immunotherapy (PPOIT-001) trial is a double-blind placebo controlled randomised trial that randomised 62 children aged 1-10 years with peanut allergy, defined as either (i) a clear history of immediate onset allergic reaction following peanut ingestion within the past 24 months in conjunction with positive peanut SPT or sIgE or (ii) clear history of immediate allergic reaction to peanut ever in conjunction with peanut SPT wheal size $\geq 8\text{mm}$ or sIgE level of $\geq 15\text{kU/L}$, to receive daily probiotic adjuvant (2×10^{10} *Lactobacillus rhamnosus* CGMCC 1.3724) and peanut oral immunotherapy (maintenance 2 g/d peanut protein; PPOIT) or placebo and placebo for 18 months.

4.4.2. Evaluation of outcomes

A double-blind placebo-controlled food challenge (DBPCFC) to peanut (cumulative dose 4g peanut protein) was administered to all subjects on the day after the final day of treatment (T1) to assess for peanut desensitisation. Subjects who passed this challenge underwent a second DBPCFC 2-6 weeks later (T2) to assess for sustained unresponsiveness (SU). An end of study visit was completed at 3 months post-treatment (T3). 56 participants completed the parent trial (PPOIT, n = 28; placebo, n = 28) and were eligible for a follow-up study at 4 years post-treatment (T5, long-term follow-up). 48 of 56 (86%) eligible participants enrolled in the follow up study (PPOIT, n = 24; placebo, n = 24) and a subset consented to SPT (PPOIT, n = 18; placebo, n = 18), blood test (PPOIT, n = 18; placebo, n = 17) and/or a DBPCFC following 8-week secondary peanut elimination to assess for long-term sustained unresponsiveness (LTSU) at 4 years post-treatment (PPOIT, n=12; Placebo, n= 15).

4.4.3. Study samples

As per registered PPOIT-001 study protocol, whole blood and saliva samples were collected at study entry, after 18 months of treatment (T1), 3 months post-treatment (T3) and at 4 years post-treatment (T5). Saliva samples were also collected at weeks 17, 33, 49 (approximately 4, 8, 12 months) during treatment, at 2-6 weeks post-treatment (T2) and at 12 months post-treatment (T4) from eligible participants. Only participants who had attained desensitisation at T1 were eligible for follow-up at T2. Blood samples were processed (plasma, serum, peripheral blood mononuclear cells), cryopreserved at -80°C and subsequently batch thawed and analysed by the doctoral candidate and/or by other members of the research group.

4.4.4. Whole peanut and peanut component specific-IgE (sIgE) and specific-IgG4 (sIgG4) measurements

Levels of serum peanut and plasma peanut component (Ara h1, h2, h3, h8 and h9) sIgE and sIgG4 were measured using ImmunoCAP 250 (Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions. Reagents and consumables including whole peanut and Ara h component ImmunoCAPs, were supplied by the manufacturer (Phadia AB; Uppsala, Sweden) or supplied as in-kind support by distributor (Abacus-ALs). Samples were centrifuged at 3000 RPM for 5 minutes prior to aliquoting. Commercial and in-house quality control materials were used. For sIgE, data was reported as kUA/L and sIgG4 data was reported as mgA/L.

4.4.5. Salivary peanut specific-IgA (sIgA) measurement

Salivary samples were analysed for peanut sIgA using an in-house ELISA assay adapted from a protocol originally developed by Dr Herman Staats and modified by Professor Wesley Burks and Dr Michael Kulis[56]. Briefly, ELISAs were carried out in 96-well microtitre plates coated with 100 µL/well of a Crude Peanut Extract (11 mg/mL; kindly provided by Professor Wesley Burks and his research group, Duke University Medical Centre, Durham, NC, USA). Saliva samples were diluted 1:2 in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and 2% fetal calf serum (FCS) and incubated for 24 hours at 4°C. Plates were washed 4 times with PBS-T and incubated (1 hour,

37°C) with horseradish peroxidase (HRP) conjugated goat anti-human IgA (Southern Biotech; AL, USA) at 1:5,000 dilution. After washing four times in both PBS-T and distilled water, separately, tetramethylbenzidine (TMB) substrate (KPL; Gaithersburg, MD, USA) was added at room temperature and the reaction stopped after 20 mins with 1 M phosphoric acid (H₃PO₄). Plates were read within 30 minutes (BioTek plate reader; VT, USA) and results analysed using the KCjunior V1.40.3 software package (BioTek, Winooski, VT, USA). Concentrations were measured in ELISA units (EU/mL).

4.4.6. Statistical methods

Immune indices were examined visually for normality and linearity. Effects of potential confounders including age and sex on immunologic outcome measures were examined using logistic or linear regression models. Age and sex did not have an effect on antibody levels.

As per registered study protocol (ACTRN 12608000594325, 25/11/2008), parametric immunologic variables were analysed using standard t test with data summarised using arithmetic means and standard deviations (SD). Non-parametric immunologic variables were analysed using standard t test on logarithmic scaled values with data summarised using geometric means and 95% confidence intervals (95% CI). Between group analyses for PPOIT versus Placebo were by intention to treat (ITT) where outcome data were available as per the approach adopted in previous publications relating to this study[95 99]. The doctoral candidate performed between outcome group analyses of immunologic variables for PPOIT-treated participants who attained long-term SU (LTSU) versus those that did not.

In addition, for evaluation of longitudinal trends from study entry (T0) to 4 years post-treatment (T5), the doctoral candidate used generalised estimating equation (GEE) models which adjust both parameter estimates and standard errors to allow for clustering within subjects because of repeated measures in longitudinal studies[103 104].

The doctoral candidate categorised peanut and peanut component sIgE and peanut SPT results into positive and negative using standard diagnostic thresholds (negative

defined as: sIgE <0.35kU/L, SPT <3mm above negative control) and presented the proportion of SU and non-SU participants with negative results at 2-6 weeks post-treatment and at 4 years post-treatment.

Given that the doctoral candidate was investigating a number of hypotheses, the candidate did not adjust p-values for multiple comparisons as per Feise and Perneger[97 98].

Data were analysed with Stata release 15 software (StataCorp, College Station, Tex).

4.4.7.Ethics

Ethics approval was obtained from The Royal Children's Hospital Human Research Ethics Committee (HREC 27086). The trial, including the a priori plan to analyse immunologic variables as secondary outcomes, was registered with the Australian New Zealand Clinical Trials Registry (ACTRN 12608000594325, 25/11/2008).

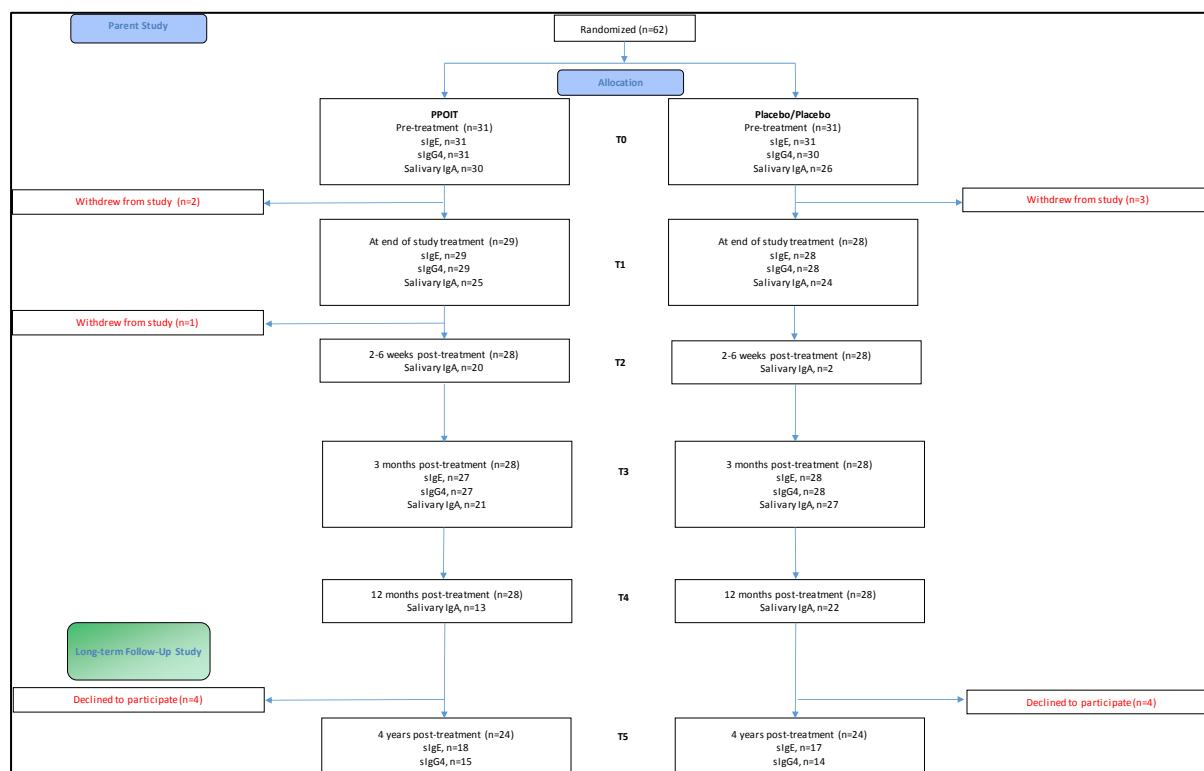
4.5. Results

4.5.1. Participant flow

At T0 baseline (T0), 31 children were randomised to PPOIT and 31 to placebo (Figure 4-1). Two PPOIT-treated children and 3 Placebo-treated children withdrew during treatment leaving 29 PPOIT-treated and 28 Placebo treated children reaching T1. One PPOIT-treated child withdrew between T1 and T2. Of the 56 children who completed the parent study, 48 (PPOIT n=28, Placebo n=28) participated in the long-term follow-up study 4 years post-treatment[99]. One child who was not on immunosuppressive therapy whilst taking PPOIT study treatment but subsequently was commenced on azathioprine by their treating doctors for treatment of eczema participated in this study.

Peanut SPT wheal size and peanut and peanut component sIgE and sIgG4 measurements were available for over 98% of participants from baseline to 3 months post-treatment. Salivary peanut specific IgA measurements were available for over 60% of eligible participants (Table 4-1). At T2, saliva samples were collected only from eligible participants defined as those who had achieved desensitisation at T1. However, this resulted in biased sampling in that biospecimens were only collected from participants who achieved desensitisation. In recognition of potential bias introduced, between group comparison of salivary peanut specific IgA at this time point (T2) was not performed. At the 4 year post-treatment timepoint (T5) blood samples and SPT were available for 73% and 75% of eligible participants respectively.

Figure 4-1. CONSORT diagram of participant flow in the PPOIT randomised controlled trial and the follow-on study of long-term outcome



T0: baseline; T1: end-of-treatment; T2: 2-6 weeks post-treatment to assess for sustained unresponsiveness (SU); T3: 3 months post-treatment; T4: 12 months post-treatment; T5: 4 years post-treatment to assess for long-term sustained unresponsiveness (LTSU).

Salivary peanut slgA was measured at additional time points between T0 and T1: at week 17 (PPOIT=28, Placebo=29), week 33 (PPOIT=29, Placebo=27), week 49 (PPOIT=29, Placebo=26) of treatment and are not shown here.

Table 4-1. Immune markers results available at various time points

		PPOIT	Number Placebo	Total	%
Study entry (T0)	Eligible	31	31	62	
	Peanut SPT	31	31	62	100.0
	Peanut sIgE	31	31	62	100.0
	Peanut sIgG4	31	30	61	98.4
	Ara h2 sIgE	31	30	61	98.4
	Ara h2 sIgG4	31	30	61	98.4
	sIgA	30	26	56	90.3
End of treatment (T1)	Eligible	29	28	57	
	Peanut SPT	29	27	56	98.2
	Peanut sIgE	29	28	57	100.0
	Peanut sIgG4	29	28	57	100.0
	Ara h2 sIgE	29	28	57	100.0
	Ara h2 sIgG4	29	28	57	100.0
	sIgA	25	24	49	86.0
2-6 weeks post-treatment (T2)	Eligible	26	2	28	
	sIgA	20	2	22	78.6
3 months post-treatment (T3)	Eligible	28	28	56	
	Peanut SPT	28	28	56	100.0
	Peanut sIgE	27	28	55	98.2
	Peanut sIgG4	27	28	55	98.2
	Ara h2 sIgE	27	28	55	98.2
	Ara h2 sIgG4	27	28	55	98.2
	sIgA	21	27	48	85.7
12 months post-treatment (T4)	sIgA	13	22	35	62.5
4 years post-treatment (T5)	Eligible	24	24	48	
	Peanut SPT	18	18	36	75.0
	Peanut sIgE	18	17	35	72.9
	Peanut sIgG4	15	14	29	60.4

PPOIT = Probiotic and peanut oral immunotherapy, SPT = skin prick test, sIgE = specific-IgE, sIgG4 = specific-IgG4, sIgA = specific-IgA

4.5.2. Effects of PPOIT treatment on peanut and peanut component sIgE levels

There was a trend towards lower post-treatment peanut (at T1 and T3), Ara h2 (at T1 and T3) and Ara h3 (at T3) sIgE levels in the PPOIT group compared with the placebo group. There were significant reductions in peanut component (Ara h1, h2, h3) sIgE in the PPOIT group at 4 years post-treatment (T5) compared with the placebo group. There were no between group differences for Ara h8 and Ara h9 sIgE levels at any time point. Analysis of longitudinal data revealed that PPOIT treatment was associated with significant downward longitudinal trajectories in peanut, Ara h1, Ara h2 and Ara h3 sIgE levels from baseline to 4 years post-treatment compared with placebo treatment (GEE, age- and sex-adjusted p-values; peanut $p < 0.001$, Ara h1 $p = 0.003$, Ara h2 $p < 0.001$, Ara h3 $p = 0.034$) (Table 4-2).

Table 4-2. Peanut and peanut component (Ara h1, h2, h3, h8, h9) sIgE by treatment group

PEANUT sIgE (kU/L)	Geometric mean (95% CI) of sIgE (kUA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	15.8 (6.2, 40.1)	8.0 (3.5, 18.2)	1.98 (0.59, 6.71)	0.265	0.189
End of treatment, T1	5.4 (2.2, 13.3)	7.8 (3.1, 20.0)	0.69 (0.20, 2.46)	0.566	0.729
3 months post-treatment, T3	4.6 (1.9, 11.5)	13.2 (5.3, 32.5)	0.35 (0.10, 1.23)	0.101	0.158
4 years post-treatment, T5	2.8 (1.0, 8.2)	10.7 (3.2, 36.1)	0.26 (0.06, 1.24)	0.089	0.129
PEANUT sIgE (kU/L)	Median (IQR) of change from study entry in sIgE (kUA/L)				
	T1	-4.4 (-109.4, -1.2)	-0.2 (-3.9, 3.2)		
	T3	-4.5 (-108.1, -0.4)	0.6 (-1.6, 25.6)		
	T5	-10.9 (-44.9, -1.0)	-0.2 (-20.3, 1.6)		
Generalised estimating equation	Coefficient (95% CI)		P-value		
Study entry to 4 years post-treatment	-0.5 (-0.6, -0.3)		<0.001		

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h1 sIgE (kU/L)	Geometric mean (95% CI) of sIgE (kUA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	1.9 (0.5, 6.7)	0.6 (0.2, 2.1)	3.08 (0.55, 17.18)	0.196	0.155
End of treatment, T1	1.0 (0.3, 3.1)	0.8 (0.2, 3.2)	1.24 (0.21, 7.29)	0.809	0.789
3 months post-treatment, T3	0.8 (0.2, 2.7)	0.8 (0.2, 3.3)	1.00 (0.16, 6.07)	1.000	0.923
4 years post-treatment, T5	0.4 (0.1, 1.6)	5.5 (0.9, 33.6)	0.07 (0.01, 0.59)	0.017	0.008

Ara h1 sIgE (kU/L)	Median (IQR) of change from study entry in sIgE (kUA/L)	
T1	-0.1 (-7.4, 0.0)	0.0 (-0.5, 0.0)
T3	-0.2 (-7.3, 0.0)	0.0 (-0.0, 3.6)
T5	-1.2 (-8.6, 0.0)	0.1 (-0.0, 15.0)

Generalised estimating equation	Coefficient (95% CI)	P-value
Study entry to 4 years post-treatment	-0.3 (-0.5, -0.1)	0.003

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h2 sIgE (kU/L)	Geometric mean (95% CI) of sIgE (kUA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	10.3 (4.1, 26.0)	6.2 (2.6, 14.5)	1.68 (0.49, 5.77)	0.405	0.316
End of treatment, T1	3.2 (1.3, 7.7)	6.1 (2.5, 15.2)	0.53 (0.15, 1.80)	0.298	0.376
3 months post-treatment, T3	2.6 (1.0, 6.9)	9.1 (3.8, 21.7)	0.29 (0.08, 1.02)	0.054	0.085
4 years post-treatment, T5	0.9 (0.2, 3.2)	10.7 (2.7, 42.0)	0.08 (0.01, 0.50)	0.008	0.007

Ara h2 sIgE (kU/L)	Median (IQR) of change from study entry in sIgE (kUA/L)	
T1	-4.0 (-32.2, -0.7)	-0.3 (-5.2, 0.2)
T3	-4.5 (-41.8, -0.5)	1.1 (-0.6, 26.7)
T5	-9.5 (-39.4, -1.3)	-4.5 (-14.4, 0.6)

Generalised estimating equation	Coefficient (95% CI)	P-value
Study entry to 4 years post-treatment	-0.6 (-0.7, -0.4)	<0.001

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h3 sIgE (kU/L)	Geometric mean (95% CI) of sIgE (kUA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.4 (0.1, 1.1)	0.4 (0.1, 1.1)	1.00 (0.21, 4.64)	0.997	0.881
End of treatment, T1	0.4 (0.1, 1.1)	0.3 (0.1, 1.0)	1.24 (0.28, 5.54)	0.778	0.794
3 months post-treatment, T3	0.3 (0.1, 0.8)	0.5 (0.1, 1.6)	0.61 (0.13, 2.91)	0.529	0.588
4 years post-treatment, T5	0.2 (0.1, 0.5)	1.2 (0.2, 6.2)	0.15 (0.02, 0.93)	0.042	0.021

Ara h3 sIgE (kU/L)	Median (IQR) of change from study entry in sIgE (kUA/L)	
T1	-0.0 (-0.1, 0.0)	0.0 (-0.0, 0.2)
T3	0.0 (-0.2, 0.0)	0.0 (-0.0, 0.7)
T5	0.0 (-0.2, 0.1)	0.3 (0.0, 6.5)

Generalised estimating equation	Coefficient (95% CI)	P-value
Study entry to 4 years post-treatment	-0.2 (-0.4, -0.0)	0.034

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h8 sIgE (kU/L)	Geometric mean (95% CI) of sIgE (kUA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.0 (0.0, 0.1)	0.0 (0.0, 0.1)	1.30 (0.47, 3.56)	0.599	0.665
End of treatment, T1	0.1 (0.0, 0.2)	0.0 (0.0, 0.1)	1.69 (0.39, 7.20)	0.465	0.453
3 months post-treatment, T3	0.1 (0.0, 0.2)	0.1 (0.0, 0.1)	1.11 (0.28, 4.31)	0.877	0.783
4 years post-treatment, T5	0.1 (0.0, 0.1)	0.1 (0.0, 0.2)	0.77 (0.21, 2.85)	0.684	0.964

Ara h8 sIgE (kU/L)	Median (IQR) of change from study entry in sIgE (kUA/L)	
T1	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
T3	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
T5	0.0 (0.0, 0.0)	0.0 (0.0, 0.1)

Generalised estimating equation	Coefficient (95% CI)	P-value
Study entry to 4 years post-treatment	-0.2 (-0.5, 0.1)	0.288

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h9 sIgE (kU/L)	Geometric mean (95% CI) of sIgE (kUA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.0 (0.0, 0.1)	0.1 (0.0, 0.1)	0.73 (0.26, 2.04)	0.533	0.791
End of treatment, T1	0.1 (0.0, 0.2)	0.1 (0.0, 0.4)	0.81 (0.24, 2.73)	0.725	0.855
3 months post-treatment, T3	0.1 (0.0, 0.2)	0.1 (0.0, 0.2)	1.05 (0.33, 3.31)	0.928	0.703
4 years post-treatment, T5	0.1 (0.0, 0.1)	0.0 (0.0, 0.1)	1.58 (0.55, 4.53)	0.381	0.362

Ara h9 sIgE (kU/L)	Median (IQR) of change from study entry in sIgE (kUA/L)	
T1	0.0 (0.0, 0.1)	0.0 (-0.0, 0.0)
T3	0.0, (0.0, 0.0)	0.0 (0.0, 0.0)
T5	0.0 (-0.0, 0.0)	0.0 (-0.0, 0.1)

Generalised estimating equation	Coefficient (95% CI)	P-value
Study entry to 4 years post-treatment	0.0 (-0.2, 0.3)	0.770

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

4.5.3. Effects of PPOIT treatment on peanut and peanut component sIgG4 levels

PPOIT treatment was associated with increased levels of peanut and peanut component sIgG4. At 18 months [end of treatment (T1)], levels of peanut, Ara h1, h2, h3 sIgG4 were significantly higher in the PPOIT group as compared to placebo group (adjusted $p < 0.001$, $p = 0.011$, $p < 0.001$, $p < 0.001$ respectively). However, sIgG4 levels reduced rapidly after PPOIT treatment cessation and were back at pre-treatment or study entry levels by 4 years post-treatment (T5) (adjusted $p = 0.756$, $p = 0.305$, $p = 0.036$, $p = 0.526$ respectively). Although between group differences in sIgG4 were maintained at 3 months post-treatment (T3), in the PPOIT group, levels of peanut, Ara h1 and h3 sIgG4 had reduced to 50-71% of end of treatment values. Longitudinal repeated measures analyses using generalised estimating equation (GEE) from study entry to 4 years post-treatment demonstrated significant upward trajectories in peanut, Ara h1, h2, h3 sIgG4 levels in the PPOIT group as compared with the placebo group ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$ respectively) (Table 4-3).

Table 4-3. Peanut and peanut component (Ara h1, h2, h3, h8, h9) sIgG4 by treatment group

PEANUT sIgG4 (mgA/L)	Geometric mean (95% CI) of sIgG4 (mgA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.4 (0.3, 0.6)	0.3 (0.2, 0.5)	1.32 (0.70, 2.48)	0.384	0.309
End of treatment, T1	6.4 (3.1, 13.3)	0.3 (0.2, 0.5)	21.51 (9.04, 51.17)	0.000	0.000
3 months post-treatment, T3	4.6 (2.4, 8.8)	0.4 (0.2, 0.7)	10.94 (4.84, 24.75)	0.000	0.000
4 years post-treatment, T5	0.4 (0.1, 1.5)	0.2 (0.1, 0.5)	1.93 (0.39, 9.64)	0.409	0.756
PEANUT sIgG4 (mgA/L)	Median (IQR) of change from study entry in sIgG4 (mgA/L)				
	T1	5.1 (1.1, 39.0)	-0.0 (-0.1, 0.0)		
	T3	3.2 (1.0, 28.5)	0.0 (-0.1, 0.4)		
	T5	0.3 (-0.1, 1.4)	-0.1 (-0.3, 0.0)		
Generalised estimating equation	Coefficient (95% CI)		P-value		
Study entry to 4 years post-treatment	0.9 (0.7, 1.2)		<0.001		

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h1 sIgG4 (mgA/L)	Geometric mean (95% CI) of sIgG4 (mgA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.0 (0.0, 0.1)	0.1 (0.0, 0.1)	0.78 (0.41, 1.46)	0.423	0.599
End of treatment, T1	0.2 (0.1, 0.6)	0.1 (0.0, 0.1)	3.77 (1.22, 11.59)	0.022	0.011
3 months post-treatment, T3	0.1 (0.1, 0.4)	0.1 (0.0, 0.1)	2.15 (0.75, 6.16)	0.149	0.116
4 years post-treatment, T5	0.0 (0.0, 0.1)	0.0 (0.0, 0.1)	0.68 (0.18, 2.52)	0.549	0.305
Ara h1 sIgG4 (mgA/L)	Median (IQR) of change from study entry in sIgG4 (mgA/L)				
	T1	0.1 (0.0, 0.9)	-0.0 (-0.0, 0.0)		
	T3	0.0 (0.0, 0.5)	0.0 (-0.0, 0.0)		
	T5	-0.0 (-0.0, 0.0)	-0.0 (-0.0, 0.0)		
Generalised estimating equation	Coefficient (95% CI)		P-value		
Study entry to 4 years post-treatment	0.6 (0.3, 0.9)		<0.001		

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h2 sIgG4 (mgA/L)	Geometric mean (95% CI) of sIgG4 (mgA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.1 (0.1, 0.2)	0.1 (0.1, 0.2)	0.87 (0.55, 1.37)	0.541	0.617
End of treatment, T1	2.8 (1.0, 7.8)	0.2 (0.1, 0.2)	17.97 (6.09, 53.03)	0.000	0.000
3 months post-treatment, T3	1.7 (0.6, 4.5)	0.2 (0.1, 0.4)	7.51 (2.67, 21.11)	0.000	0.000
4 years post-treatment, T5	0.2 (0.1, 0.6)	0.1 (0.0, 0.1)	3.96 (1.11, 14.04)	0.034	0.036

Ara h2 sIgG4 (mgA/L)	Median (IQR) of change from study entry in sIgG4 (mgA/L)	
T1	2.6 (0.3, 22.1)	-0.0 (-0.1, 0.0)
T3	1.3 (0.4, 13.6)	0.0 (-0.0, 0.1)
T5	0.2 (-0.0, 0.4)	-0.1 (-0.1, -0.1)

Generalised estimating equation	Coefficient (95% CI)	P-value
Study entry to 4 years post-treatment	1.2 (0.9, 1.5)	<0.001

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h3 sIgG4 (mgA/L)	Geometric mean (95% CI) of sIgG4 (mgA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.1 (0.0, 0.1)	0.1 (0.0, 0.1)	0.98 (0.49, 1.96)	0.952	0.772
End of treatment, T1	0.7 (0.3, 1.7)	0.1 (0.0, 0.1)	10.42 (3.89, 27.96)	0.000	0.000
3 months post-treatment, T3	0.5 (0.2, 1.2)	0.1 (0.1, 0.1)	6.21 (2.44, 15.84)	0.000	0.000
4 years post-treatment, T5	0.1 (0.1, 0.3)	0.1 (0.0, 0.2)	1.85 (0.66, 5.19)	0.234	0.526
Ara h3 sIgG4 (mgA/L)	Median (IQR) of change from study entry in sIgG4 (mgA/L)				
	T1	0.6 (0.1, 5.3)	-0.0 (-0.0, 0.0)		
	T3	0.6 (0.1, 3.2)	0.0 (-0.0, 0.0)		
	T5	0.0 (-0.0, 0.1)	0.0 (0.0, 0.0)		
Generalised estimating equation	Coefficient (95% CI)		P-value		
Study entry to 4 years post-treatment	0.9 (0.6, 1.2)		<0.001		

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h8 sIgG4 (mgA/L)	Geometric mean (95% CI) of sIgG4 (mgA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.0 (0.0, 0.0)	0.0 (0.0, 0.1)	0.51 (0.19, 1.39)	0.177	0.202
End of treatment, T1	0.0 (0.0, 0.0)	0.0 (0.0, 0.1)	0.76 (0.29, 2.00)	0.564	0.402
3 months post-treatment, T3	0.0 (0.0, 0.0)	0.0 (0.0, 0.1)	0.68 (0.27, 1.74)	0.409	0.466
4 years post-treatment, T5	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.49 (0.21, 1.14)	0.095	0.176

Ara h8 sIgG4 (mgA/L)	Median (IQR) of change from study entry in sIgG4 (mgA/L)	
T1	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
T3	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
T5	0.0 (0.0, 0.0)	0.0 (-0.0, 0.0)

Generalised estimating equation	Coefficient (95% CI)	P-value
Study entry to 4 years post-treatment	0.1 (-0.2, 0.3)	0.531

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

Ara h9 sIgG4 (mgA/L)	Geometric mean (95% CI) of sIgG4 (mgA/L)		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	0.1 (0.0, 0.1)	0.1 (0.0, 0.1)	1.08 (0.40, 2.89)	0.872	0.787
End of treatment, T1	0.1 (0.0, 0.2)	0.1 (0.0, 0.1)	1.24 (0.44, 3.51)	0.674	0.563
3 months post-treatment, T3	0.1 (0.0, 0.2)	0.1 (0.0, 0.1)	1.61 (0.59, 4.41)	0.347	0.298
4 years post-treatment, T5	0.0, (0.0, 0.1)	0.0 (0.0, 0.0)	1.79 (0.40, 7.93)	0.429	0.731

Ara h9 sIgG4 (mgA/L)	Median (IQR) of change from study entry in sIgG4 (mgA/L)	
T1	0.0 (0.0, 0.1)	0.0 (-0.0, 0.0)
T3	0.0 (0.0, 0.0)	0.0 (-0.0, 0.0)
T5	-0.0 (-0.0, 0.0)	-0.0 (-0.0, 0.0)

Generalised estimating equation	Coefficient (95% CI)	P-value
Study entry to 4 years post-treatment	0.1 (-0.1, 0.3)	0.183

*Adjusted for age and sex; The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs), The t test on the log scale was applied for data presented as geometric means

4.5.4. Effects of PPOIT on peanut SPT wheal size

Participants in the PPOIT group had significantly lower peanut SPT wheal size at end-of-treatment (T1; adjust $p < 0.001$), 3 months post-treatment (T3; adjusted $p < 0.001$) and 4 years post-treatment (T5; adjusted $p = 0.039$) compared with those in the placebo group. Repeated measures analysis using generalised estimating equation from study entry to 4 years post-treatment showed a significant downward trajectory in peanut SPT in the PPOIT group as compared to the placebo group ($p < 0.001$) (Table 4-4).

Table 4-4. Peanut skin prick test (SPT) wheal size by treatment group

Peanut SPT wheal size	Mean (SD) of Peanut SPT (mm), n		Mean difference (95% CI)	Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo			
Study entry, T0	17.3 (6.2), 31	17.6 (6.7), 31	-0.27 (-3.57, 3.02)	0.868	0.919
End of treatment, T1	4.6 (4.1), 29	14.2 (5.6), 27	-9.61 (-12.23, -7.00)	0.000	0.000
3 months post-treatment, T3	4.1 (4.5), 28	14.4 (6.0), 28	-10.29 (-13.12, -7.46)	0.000	0.000
4 years post-treatment, T5	8.1 (7.7), 18	13.3 (7.6), 18	-5.17 (-10.34, 0.01)	0.050	0.039
	Median (IQR) of change from study entry in Peanut SPT (mm)				
Peanut SPT wheal size					
T1	-13.0 (-18.5, -8.0)	-3.0 (-6.5, 3.5)			
T3	-13.0 (-18.0, -9.0)	-3.5 (-6.3, 0.5)			
T5	-9.5 (-14.5, -1.0)	-3.3 (-10.0, 1.0)			
Generalised estimating equation	Coefficient (95% CI)	P-value			
Study entry to 4 years post-treatment	-8.8 (-11.0, -6.7)	<0.001			

*Adjusted for age and sex; The t test was applied for data presented as arithmetic means

4.5.5. Effects of PPOIT on salivary peanut specific-IgA (sIgA) levels during and post PPOIT treatment

Levels of salivary peanut sIgA increased early in the course of PPOIT treatment. These were observed to be significantly higher in the PPOIT group as compared with the placebo group as early as week 17 of treatment [geometric mean (95%CI) PPOIT 43.3 (30.2, 62.0) v.s. Placebo 21.2 (14.8, 30.3); adjusted p=0.014]. This increase in peanut sIgA was sustained at end-of-treatment (T1; adjusted p=0.016). However, sIgA levels declined post-treatment and by 12 months post-treatment (T4), there was no between group difference (adjusted p=0.345) (Table 4-5).

Table 4-5. Salivary peanut specific-IgA (sIgA) by treatment group

Salivary peanut sIgA	Geometric mean (95% CI) of sIgA (EU/mL), n		Ratio of Geometric mean (95% CI)	Unadjusted P- value	Adjusted P- value*
	PPOIT	Placebo			
Study entry, T0	24.7 (17.1, 35.6), 30	27.4 (19.0, 39.6), 26	0.90 (0.54, 1.50)	0.683	0.756
17 wks treatment	43.3 (30.2, 62.0), 28	21.2 (14.8, 30.3), 29	2.04 (1.24, 3.35)	0.006	0.014
33 wks treatment	38.5 (24.6, 60.3), 29	19.3 (13.6, 27.4), 27	1.99 (1.14, 3.50)	0.017	0.020
49 wks treatment	57.1 (34.4, 94.9), 29	22.3 (15.3, 32.3), 26	2.56 (1.37, 4.81)	0.004	0.003
End of treatment, T1	59.1 (35.2, 99.1), 25	25.3 (15.2, 42.1), 24	2.34 (1.15, 4.73)	0.020	0.016
3 mths post-treatment, T3	38.9 (26.4, 57.3), 21	23.7 (17.4, 32.1), 27	1.64 (1.02, 2.64)	0.040	0.105
12 mths post-treatment, T4	39.9 (19.9, 79.8), 13	51.9 (38.9, 69.3), 22	0.77 (0.41, 1.42)	0.389	0.345

*Adjusted for age and sex

4.5.6. Peanut and peanut component sIgG4 levels in PPOIT-treated participants with SU who were still ingesting peanut at 4 years post-treatment compared with those who were no longer ingesting

Given that peanut sIgG4 levels were only increased during the PPOIT treatment phase and returned to pre-treatment or study entry levels once PPOIT treatment was stopped, it was then hypothesised that the increase in peanut and peanut component sIgG4 levels reflects peanut ingestion (allergen exposure) per se rather than reflecting fundamental redirection of immune responses towards tolerance. To investigate this, the doctoral candidate performed post-hoc subgroup analyses examining the relationship between peanut ingestion and sIgG4 levels in PPOIT-treated participants who attained 2-6 week SU at end-of-treatment and were permitted to ingest peanut ad-libitum. As discussed the previous chapter, 80% of PPOIT-treated participants who attained 2-6 week SU and participated in the 4 year follow-up study at T5, were still ingesting peanut.

Amongst participants in the PPOIT group who initially achieved 2-6 week SU (initial treatment responders), peanut sIgG4 levels at 4 years post-treatment were significantly higher in participants who were ingesting peanut at T5 compared with those not-ingesting peanut (age- and sex- adjusted $p=0.026$). Furthermore, peanut sIgG4 levels were also higher in the participants who were ingesting moderate or large amounts (≥ 2 g) compared with those ingesting none or only a small amount (age- and sex- adjusted $p=0.015$). sIgG4 levels were not associated with ingestion frequency, although the small sample size does not allow confident exclusion of an effect (Table 4-6).

Table 4-6. Peanut sIgG4 levels in PPOIT-treated participants who were or were not ingesting peanut at 4 years post-treatment

	Geometric mean (95% CI) of sIgG4 (mgA/L), n		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	Ingesting v.s. Not ingesting				
	Ingesting	Not ingesting			
Peanut	1.480 (0.346, 6.325), 8	0.244 (0.008, 7.572), 4	6.08 (0.47, 78.40)	0.147	0.026
Ara h1	0.023 (0.004, 0.130), 9	0.012 (0.000, 0.531), 3	1.87 (0.08, 45.47)	0.672	0.647
Ara h2	0.398 (0.084, 1.887), 9	0.196 (0.018, 2.142), 3	2.03 (0.13, 32.18)	0.582	0.754
Ara h3	0.148 (0.052, 0.426), 9	0.195 (0.012, 3.054), 4	0.76 (0.11, 5.38)	0.765	0.949
	Ingesting once a week or more v.s. Ingesting less than once a week				
	Once a week or more	Less than once a week			
Peanut	1.814 (0.586, 5.611), 5	0.456 (0.049, 4.252), 7	3.98 (0.31, 51.01)	0.256	0.215
Ara h1	0.018 (0.002, 0.135), 6	0.020 (0.002, 0.253), 6	0.89 (0.05, 14.46)	0.926	0.490
Ara h2	0.325 (0.031, 3.354), 6	0.343 (0.072, 1.625), 6	0.95 (0.08, 10.79)	0.961	0.788
Ara h3	0.144 (0.055, 0.374), 6	0.178 (0.033, 0.961), 7	0.81 (0.13, 4.94)	0.799	0.649
	Ingesting moderate to large amount v.s. Ingesting none or small amount				
	Moderate to large amount	None or small amount			
Peanut	2.992 (0.950, 9.429), 6	0.220 (0.031, 1.554), 6	13.62 (1.91, 97.26)	0.014	0.015
Ara h1	0.025 (0.002, 0.260), 7	0.013 (0.002, 0.073), 5	1.85 (0.11, 30.38)	0.634	0.803

Ara h2	0.498 (0.062, 4.006), 7	0.190 (0.070, 0.515), 5	2.62 (0.24, 28.06)	0.388	0.437
Ara h3	0.231 (0.077, 0.693), 7	0.106 (0.019, 0.608), 6	2.17 (0.38, 12.41)	0.349	0.459

*Age- and sex- adjusted regression (of log-transformed values). The smaller sample sizes are due to a combination of factors including smaller number of participants providing samples for measurement at this timepoint and post-log-transformation exclusion of sIgG4 levels $\leq$ zero in the analytical model.

4.5.7. Peanut and peanut component sIgE levels in PPOIT-treated participants with SU who were still ingesting peanut at 4 years post-treatment compared with those who were no longer ingesting

In contrast to the findings for sIgG4, there was no difference in peanut and Ara h1, h2, h3 sIgE between initial treatment responders who were ingesting and not ingesting peanut at T5. Peanut and peanut component sIgE levels were also similar in PPOIT-treated participants with SU who were or were not ingesting peanut and those who were ingesting larger versus smaller amounts of peanut. This suggests there is no relationship between peanut ingestion amount or frequency and peanut and peanut component sIgE levels, although again the small numbers sample size does not allow confident exclusion of an effect (Table 4-7).

Table 4-7. Peanut sIgE levels in PPOIT-treated participants who were or were not ingesting peanut at 4 years post-treatment

	Geometric mean (95% CI) of sIgE (kUA/L), n		Ratio of Geometric mean (95% CI)	Unadjusted P-value	Adjusted P-value*
	Ingesting v.s. Not ingesting				
	Ingesting	Not ingesting			
Peanut	1.221 (0.260, 5.730), 10	3.349 (0.516, 21.728), 4	0.36 (0.03, 4.57)	0.402	0.344
Ara h1	0.197 (0.018, 2.208), 8	0.208 (0.019, 2.237), 4	0.95 (0.03, 30.88)	0.975	0.999
Ara h2	0.198 (0.033, 1.197), 9	1.927 (0.140, 26.536), 4	0.10 (0.01, 1.82)	0.109	0.153
Ara h3	0.154 (0.035, 0.669), 9	0.095 (0.006, 1.581), 4	1.62 (0.14, 19.27)	0.677	0.533
	Ingesting once a week or more v.s. Ingesting less than once a week				
	Once a week or more	Less than once a week			
Peanut	1.283 (0.203, 8.100), 7	2.068 (0.319, 13.392), 7	0.62 (0.06, 6.42)	0.664	0.182
Ara h1	0.090 (0.006, 1.437), 6	0.450 (0.049, 4.131), 6	0.20 (0.01, 4.33)	0.270	0.236
Ara h2	0.153 (0.017, 1.366), 6	0.911 (0.100, 8.254), 7	0.17 (0.01, 2.65)	0.182	0.079
Ara h3	0.120 (0.025, 0.584), 6	0.145 (0.020, 1.060), 7	0.83 (0.08, 8.30)	0.858	0.444
	Ingesting moderate to large amount v.s. Ingesting none or small amount				
	Moderate to large amount	None or small amount			
Peanut	1.257 (0.162, 9.719), 8	2.302 (0.734, 7.218), 6	0.55 (0.05, 5.72)	0.585	0.462
Ara h1	0.308 (0.012, 8.012), 6	0.131 (0.022, 0.791), 6	2.35 (0.09, 59.26)	0.567	0.705

Ara h2	0.197 (0.018, 2.200), 7	0.909 (0.125, 6.611), 6	0.22 (0.01, 3.67)	0.260	0.225
Ara h3	0.197 (0.029, 1.358), 7	0.084 (0.018, 0.390), 6	2.36 (0.25, 22.16)	0.418	0.438

*Age- and sex- adjusted regression (of log-transformed values). The smaller sample sizes are due to a combination of factors including smaller number of participants providing samples for measurement at this timepoint and post-log-transformation exclusion of sIgE levels $\leq$ zero in the analytical model.

4.5.8. Peanut SPT wheal size in PPOIT-treated participants with SU who were still ingesting peanut at 4 years post-treatment compared with those who were no longer ingesting

In contrast to findings for peanut and peanut component sIgE, amongst initial treatment responders, peanut SPT wheal size at 4 years post-treatment was significantly lower in participants who were ingesting peanut at T5 compared with those not-ingesting peanut (age- and sex- adjusted $p = 0.003$), in the participants who were ingesting moderate or large amounts (≥ 2 g) compared with those ingesting none or only a small amount (age- and sex- adjusted $p = 0.031$) and in the participants who were ingesting once a week or more compared with those ingesting less than once a week (age- and sex- adjusted $p = 0.032$) (Table 4-8).

Table 4-8. Peanut SPT wheal size in PPOIT-treated participants who were or were not ingesting peanut at 4 years post-treatment

Peanut SPT wheal size in PPOIT-treated participants who were or were not ingesting peanut at 4 years post-treatment

Mean (SD) of Peanut SPT (mm), n		Mean difference (95% CI)	Unadjusted P-value	Adjusted P- value*
Ingesting v.s. Not ingesting				
	Ingesting	Not ingesting		
Peanut SPT	2.7 (3.9), 10	15.9 (7.7), 4	-13.23 (-19.77, - 6.68)	0.001 0.003
Ingesting once a week or more v.s. Ingesting less than once a week				
	Once a week or more	Less than once a week		
Peanut SPT	2.1 (4.2), 7	10.7 (8.6), 7	-8.6 (-16.5, -0.7)	0.036 0.031
Ingesting moderate to large amount v.s. Ingesting none or small amount				
	Moderate to large amount	None or small amount		
Peanut SPT	2.8 (4.4), 8	11.3 (9.2), 6	-8.58 (-16.60, - 0.57)	0.038 0.032

*Age- and sex- adjusted regression

4.5.9. Peanut and peanut component sIgE and sIgG4 levels, and peanut SPT wheal size in participants with long-term sustained unresponsiveness (LTSU) at 4 years post-treatment and those without LTSU

Next, the doctoral candidate examined whether peanut SPT, peanut and peanut component sIgE and sIgG4 levels were different in PPOIT-treated participants who attained LTSU and those who did not in order to gain insight into the possible role of peanut specific cutaneous mast cell reactivity (peanut SPT wheal size), peanut sIgE and peanut sIgG4 in mediating these clinical outcomes. As discussed in Chapter 3, DBPCFC after 8-weeks peanut elimination were performed in 12 PPOIT-treated participants at 4 years post-treatment (T5) with 7 of 12 (58%) maintaining LTSU.

LTSU was associated with significantly lower levels of peanut, Ara h1, h2, h3 sIgE and peanut SPT wheal size compared with no LTSU at 4 years post-treatment (age, sex adjusted $p=0.001$, $p=0.002$, $p<0.001$, $p=0.029$, $p<0.001$) (Table 4-9 and Table 4-10). Significance of these associations was maintained after adjusting for post-treatment peanut ingestion (age, sex, peanut ingestion adjusted $p=0.003$, $p=0.004$, $p=0.001$, $p=0.023$, $p=0.001$), suggesting an independent relationship between reduced peanut and peanut component sIgE responses and LTSU in PPOIT treated participants.

Surprisingly, LTSU was also associated with significantly lower levels of peanut and peanut component sIgG4 [age, sex adjusted $p=0.011$ (Ara h1); age, sex, peanut ingestion adjusted $p=0.032$ (peanut), $p=0.002$ (Ara h1), $p=0.018$ (Ara h3)] (Table 4-11).

Taken together, these observations suggest that acquisition of LTSU at 4 years post-treatment is associated with concurrent reduction of both sIgE and sIgG4 levels suggesting long-term downregulation of allergen specific B cell responses.

Table 4-9. Association between long-term sustained unresponsiveness (LTSU) at 4 years post-treatment and peanut and peanut component sIgE levels

PPOIT-treated only At 4 year follow- up	Geometric mean (95% CI) of sIgE (kUA/L), n		Ratio of Geometric mean (95% CI)	Co-efficient*	P-value*	Co-efficient**	P-value**
	SU	No SU					
Peanut	0.6 (0.1, 3.3), 7	12.8 (1.7, 98.3), 5	0.05 (0.01, 0.47)	-0.564	0.001	-0.525	0.003
Ara h1	0.0 (0.0, 0.2), 6	4.1 (0.2, 75.2), 5	0.01 (0.00, 0.12)	-0.342	0.002	-0.319	0.004
Ara h2	0.1 (0.0, 0.3), 7	4.9 (1.0, 23.6), 5	0.02 (0.00, 0.10)	-0.431	0.000	-0.422	0.001
Ara h3	0.1 (0.0, 0.1), 7	0.7 (0.0, 20.3), 5	0.08 (0.01, 0.98)	-0.419	0.029	-0.399	0.023

*Age-, sex- adjusted regression (of log-transformed values); **Age-, sex-, ingestion- adjusted regression (of log-transformed values)

Table 4-10. Association between long-term sustained unresponsiveness (LTSU) at 4 years post-treatment and peanut SPT wheal size

PPOIT-treated only	Mean (SD) of Peanut SPT (mm), n		Mean difference (95% CI)	Co-efficient*	P-value*	Co-efficient*	P-value**
	SU	No SU					
Peanut SPT (mm)	1.6 (1.8), 7	13.6 (4.7), 5	-12.0 (-16.3, -7.6)	-0.064	0.000	-0.084	0.001

*Age-, sex- adjusted regression; **Age-, sex-, ingestion- adjusted regression (of log-transformed values)

Table 4-11. Association between long-term sustained unresponsiveness (LTSU) at 4 years post-treatment and peanut and peanut component sIgG4 levels

PPOIT-treated only At 4 year follow-up	Geometric mean (95% CI) of sIgG4 (mgA/L), n		Ratio of Geometric mean (95% CI)	Co-efficient*	P-value*	Co-efficient*	P-value**
	SU	No SU					
Peanut	0.3 (0.0, 2.6), 6	2.1 (0.1, 43.3), 4	0.14 (0.01, 2.88)	-0.176	0.467	-0.615	0.032
Ara h1	0.0 (0.0, 0.0), 7	0.1 (0.0, 2.2), 5	0.06 (0.01, 0.47)	-0.465	0.011	-0.474	0.002
Ara h2	0.2 (0.0, 1.4), 6	0.7 (0.1, 5.8), 5	0.26 (0.02, 3.18)	-0.272	0.248	-0.290	0.084
Ara h3	0.1 (0.0, 0.2), 7	0.3 (0.0, 1.6), 5	0.27 (0.06, 1.17)	-0.468	0.199	-0.755	0.018

*Age-, sex- adjusted regression (of log-transformed values); **Age-, sex-, ingestion- adjusted regression (of log-transformed values)

The majority of LTSU PPOIT participants had a negative (<3mm) peanut SPT wheal size (86%, 6/7). Furthermore, the majority of LTSU PPOIT participants (86-100%) also had negative (<0.35 kU/L) peanut and peanut component sIgE levels. In particular, all but one PPOIT participant with LTSU had a negative Ara h2 sIgE, which is considered to be the most specific sIgE marker for presence of peanut allergy. Interestingly, levels of peanut sIgE in PPOIT LTSU participants were uniformly low (median 0.87 kU/L, range 0.05, 4.79), however only 2 of 7 LTSU participants had a negative peanut sIgE, consistent with current understanding that peanut sIgE levels below 15 kU/L have poor specificity for clinical peanut allergy) (Table 4-12 and Table 4-13).

The only LTSU PPOIT participant who had a positive peanut SPT (idno 207, peanut SPT wheal size 5mm) had low levels of sIgE to whole peanut (0.7 kU/L) and peanut components (Ara h1 0.03 kU/L, Ara h2 0.21 kU/L, Ara h3 0.04 kU/L). Similarly, the only LTSU PPOIT participant who had a positive Ara h2 sIgE result (idno 204, Ara h2 0.68 kU/L) had negative peanut SPT result (2mm), low level whole peanut sIgE (0.87 kU/L) and negative Ara h1 and Ara h3 sIgE (0.27 kU/L and 0.14 kU/L respectively).

Table 4-12. Association between sIgE at T5 and long-term SU (LTSU)

All available, exclude Azathioprine*		Median (actual range) of sIgE (kUA/L), n	
At 4 year follow-up		SU (n=7)*	No SU (n=19)
Peanut		0.87 (0.05, 4.79), 7	24.65 (0.92, 100.01), 18
<0.35, n (%)		2/7 (29)	0/18 (0)
<0.05, n(%)		0/7 (0)	0/18 (0)
Ara h1		0.03 (0, 0.27), 7	13.15 (0, 190), 16
<0.35, n (%)		7/7 (100)	4/16 (25)
<0.05, n(%)		4/7 (57)	2/16 (13)
Ara h2		0.1 (0.01, 0.68), 7	12.075 (0.79, 433), 16
<0.35, n (%)		6/7 (86)	0/16 (0)
<0.05, n(%)		2/7 (29)	0/16 (0)
Ara h3		0.04 (0.02, 0.18), 7	2.4 (0.01, 67.5), 16
<0.35, n (%)		7/7 (100)	5/16 (31)
<0.05, n(%)		4/7 (57)	2/16 (13)

*Excluding participant who was on Azathioprine at the time of DBPCFC

Table 4-13. Association between SPT at T5 and long-term SU (LTSU)

All available, exclude Azathioprine* At 4 year follow-up	Median (actual range) of Peanut SPT (mm), n	
	SU (n=7)*	No SU (n=19)
Peanut SPT (mm)	2 (0, 5), 7	13 (2.5, 22.5), 19
< 3mm, n(%)	6/7 (86)	1/19 (5)

*Excluding participant who was on Azathioprine at the time of DBPCFC

4.5.10. Peanut sIgE and sIgG4 levels and SPT wheal size at end of treatment (T1) in PPOIT participants with initial SU (at end of treatment) who did or did not have LTSU at 4 years post-treatment (T5)

Next, the doctoral candidate investigated immune measures at end of treatment in subjects who did or did not achieve LTSU at 4 years post-treatment to explore whether end-of-treatment measures could provide an indication of LTSU status.

Focusing on the 7 subjects who attained SU immediately post-treatment and maintained LTSU (idno 103, 204, 207, 212, 214, 301, 303), results at end of treatment (T1) showed: negative peanut sIgE in 3, negative Ara h1 sIgE in 6, negative Ara h2 sIgE in 4 and negative Ara h3 sIgE in all subjects. 4 of 7 had negative peanut skin prick test (wheal size <3mm) at end of treatment. The highest peanut SPT was 11mm in an individual who had peanut, Ara h1, h2, h3 sIgE levels all <0.35kU/L.

In contrast, all three subjects who attained SU immediately post-treatment but lost SU by 4 years post-treatment (no LTSU) (idno 228, 406, 416), had high peanut and peanut component sIgEs at end of PPOIT treatment (T1) (peanut >35 kU/L; Ara h1 range 1.41 to 40.8 kU/L; Ara h2 14.5 to 27.2 kU/L; Ara h3 range 0.06 to 22.8 kU/L) despite having low peanut SPT wheal size (range 0 to 7mm), indicating suppression of peanut specific mast cell reactivity in association with OIT treatment but absence of modulation of the underlying peanut specific IgE immune response.

Full details of peanut SPT and peanut and peanut component sIgE of PPOIT-treated participants who attained 2-6 week SU early post-treatment and either maintained or did not maintain LTSU at 4 years post-treatment are presented in the Supplementary material section of this chapter.

4.6. Discussion

This is the first study to evaluate the long-term immunologic effects of a combined probiotic and peanut oral immunotherapy (PPOIT) and their relationship to persistence of SU years after treatment cessation. Peanut and peanut component sIgG4 and salivary peanut sIgA increased with PPOIT treatment but rapidly returned to study entry levels on treatment cessation. In contrast, PPOIT treatment induced reduction in peanut and peanut component sIgE levels continued beyond the active treatment phase and reduced further over time such that by 4 years post-treatment, levels were significantly lower than for participants in the placebo group, a finding that has not been reported in other peanut OIT trials to date.

These findings support the previously described short-term effects of OIT on peanut and peanut component sIgG4 and sIgA levels and peanut SPT. Studies have reported an early rise in sIgG4 within a few months of treatment, sustained elevated sIgG4 during treatment [18 20 101 102 105] and a decline in sIgG4 measured shortly after stopping treatment[18 19 106], and reduced peanut SPT within a year post-treatment[40 95]. Similarly, previous studies have shown that salivary antigen specific-IgA, a marker of mucosal response to antigen exposure[56 107], increases within weeks of commencing OIT but declines towards pre-treatment levels rapidly following treatment cessation. In a recent peanut OIT study, levels of circulating peanut and major peanut component allergen sIgE reduced in response to OIT[63]. A number of studies[40 95] previously demonstrated reduced peanut SPT wheal size in the immediate post-treatment period. These observations suggest that induction of antigen sIgA and sIgG4 and suppression of antigen-specific SPT wheal size (as a marker of cutaneous antigen specific mast cell reactivity) may mediate desensitisation but whether these factors are involved in acquisition of long-term tolerance remains unknown.

In this study, the doctoral candidate evaluated long term immunologic effects of PPOIT at 4 years post-treatment with participants who attained SU post-treatment having ad libitum peanut ingestion. It was demonstrated that peanut and peanut component sIgG4 levels are directly correlated with peanut ingestion post-

treatment, indicating that the increased levels of sIgG4 observed during allergen immunotherapy may simply reflect allergen exposure per se rather than any requisite role in the maintenance of long-term SU. This is consistent with documented increases in allergen-specific IgG4 amongst subjects with desensitisation who did not achieve SU. Adjusting for the confounding effects of ad-libitum peanut ingestion on sIgG4, it was shown that subjects with long-term SU had significantly lower peanut and peanut component sIgG4 levels at 4 years post-treatment than those who did not have long-term SU. Similarly, the ability to maintain long-term SU was associated with suppressed peanut and peanut component sIgE response and peanut specific cutaneous mast cell reactivity at 4 years post-treatment.

These novel findings suggest that long-term redirection of allergic responses towards tolerance is supported by uniform downregulation of both sIgE and sIgG4 B cell responses. Suppressed cutaneous mast cell reactivity to a food allergen, as measured by SPT wheal size, accompanies the clinical response of desensitisation during and shortly after cessation of immunotherapy treatment but its role in tolerance induction remains unclear. Syed et al found that whilst peanut OIT was associated with reduction in peanut SPT wheal size, there was no difference between those who achieved immune tolerance and those who did not[40]. An interesting observation was made here that the only long-term SU participant who had a positive peanut SPT at 4 years post-treatment reported no peanut ingestion in the post-treatment phase and yet had maintained long-term SU and had suppressed peanut component sIgE response. The doctoral candidate speculates that the suppression of antigen specific cutaneous mast cell reactivity may in part be conferred by ongoing exposure to the food allergen as a bystander effect and might not facilitate nor be critically necessary for maintenance of gastrointestinal tolerance.

Early induction of IgG4 antibodies is one of the most consistent observations of immunotherapy. Although there is some data from OIT studies to suggest that IgG4 induction may confer reduction of allergic symptoms[108] and transient

protection through either its function as a blocking antibody by preventing the cross-linking of receptor bound IgE or co-stimulation of inhibitory receptor FcγRIIb which negatively regulates FcεRI signaling[58 77], there has been no consistent correlation between sIgG4 levels and sustained clinical response to immunotherapy[76 77 109] and there is currently no evidence of sIgG4 induction being the mechanism driving treatment-induced SU or tolerance acquisition.

Similar observations can be made in other immunotherapy models. Although insect venom immunotherapy transiently induces sIgG4[110 111], it is the long-term decline in sIgE that correlates with lower disease severity[112]. Venom tolerant bee keepers have low venom sIgE, similar to healthy controls and in contrast to allergic bee keepers. The elevated levels of sIgG4 reported amongst tolerant bee keepers most likely reflect continuing allergen exposure (afforded by ongoing insect stings) given that marked elevations occur during exposure months. Indeed, bee venom sIgG4 is undetectable in non-exposed healthy controls and low although not absent in allergic bee keepers presumed to be avoiding stings[113]. The somewhat paradoxical observation that allergic individuals have low levels of sIgG4 supports the argument that sIgG4 does not facilitate tolerance[114] but merely reflects antigen exposure[115].

Data from pollen immunotherapy would further support the paradigm that increased sIgG4 reflects allergen exposure and may not play a role in tolerance induction. In pollen immunotherapy, sIgG4 rises during treatment but decreases to pre-treatment levels after discontinuation[116]. There is no association between long-term clinical response and immunoreactive sIgG4 levels[109]. In contrast, long-term SCIT resulted in reduction in sIgE concentration[117 118], a phenomenon that is proposed to support environmental allergen tolerance acquisition[76].

The observed downregulation of both sIgE and sIgG4 levels in participants with long-term SU suggests downregulation of peanut specific B cell responses. Potentiation of memory B cells has been found to be critical in maintaining allergy in a murine model[119]. Signals that drive B cell class switch recombination to IgE,

namely the production of Th2 cytokines IL-4, IL-13 along with ligation of CD40[120] and activation of downstream signals including c-Rel[121], STAT6 and NF-kappa B[122 123], are the same signals that drive class switch recombination to IgG4[124 125]. These unified IL-4 induced signalling pathways are consistent with a synergistic relationship between IgG4 and IgE[126]. Evidence of sequential class switch of naïve B cells to IgE producing B cells through IgG(1) B cells[127 128] and replenishment of IgE+ plasma cell compartment through allergen-specific long-lived memory B cells after antigen re-exposure and enhanced IL4 production by CD4 T cells[119], further support IgG and IgE relationship. On the other hand, memory B cells with IgE_{hi} phenotype may predispose to OIT non-responsiveness through high affinity antibody production and diverse repertoires of memory B and plasma cell reserves[129 130]. Therefore, successful abrogation of both sIgE and sIgG responses through suppression of the overall allergen-specific memory B cell response is expected to accompany resolution of allergy or acquisition of tolerance as demonstrated in this chapter. These cumulative findings suggest that the allergen-specific B cell response might be a critical determinant of not only long-term SU but also tolerance acquisition, and support the proposal that deletion of allergen specific memory B cells may be an effective strategy for curing allergic disease[131].

This study has a number of strengths.

Firstly, the study had access to the PPOIT randomised trial cohort which showed a high rate of SU in PPOIT-treated participants, with SU determined by stringent criteria of DBPCFC performed after a period of peanut elimination. Longitudinal bio-specimens were collected at multiple timepoints and correlated to clinical outcomes. The PPOIT RCT is one of the largest peanut OIT RCT conducted to date and is the first to report clinical outcomes to 4 years post-treatment. Participant retention rate was high with 90.3% (56/62) remaining in the trial at end-of-treatment and 77.4% (48/62 intention to treat) participating in the 4 year long-term follow-up. Availability of DBPCFC-confirmed 8-week SU clinical outcome at 4

years post-treatment uniquely allowed us to investigate the relationship between long-term tolerance and immune response.

The doctoral candidate acknowledges the limitations in association with the study design of the parent trial as discussed previously[95]. Briefly, the absence of an OIT alone arm does not allow the study to delineate the individual contributions of OIT and probiotic to the observed clinical and immune responses, hence it is difficult to definitively attribute specific immune or clinical effects to the probiotic. Nevertheless, the current findings remain entirely relevant to the acquisition of long-term SU. The absence of a probiotic only intervention is also noted, however, PPOIT failed to modify SPT wheal size for other food allergens, making it unlikely that probiotic alone produced the observed immunologic effects. A multi-centre DBPCRCT (ACTRN12616000322437) comparing allergen OIT alone, combined probiotic/allergen OIT and placebo are underway to clarify the specific contribution of probiotic to OIT in modulating the adaptive immune response to food allergens.

In this present study immunoreactive sIgG4 was measured but not its function which might correlate with clinical response. There is some evidence from an environmental allergen immunotherapy model that although post-treatment levels of sIgG4 decline, their inhibitory activities are enhanced[116]. This study has not evaluated the effect of OIT on other subclasses of IgG (namely IgG1, IgG2, IgG3), although it is widely accepted that sIgG4 is the key IgG subclass involved in immediate onset food allergy[132-134] and sIgG4 response to OIT follows a similar trajectory as sIgG[18]. Nevertheless, these other IgG subclasses and associated memory B cells[127] might be functionally and phenotypically distinct[126] and may also play a role in mediating clinical response to OIT.

The doctoral candidate has not evaluated antigen specific IgE memory B cells given that ultrasensitive methods for tracking these cells have only been developed recently. It is important to develop deeper understanding of their response to OIT generally as this may provide important insight into mechanisms of tolerance induction.

Another limitation is that the candidate did not adjust for multiple comparisons because the candidate was investigating a number of different hypotheses. It is recognised that adjusting p-values can sometimes be inappropriate and can increase the likelihood of type 2 statistical errors[97 98]. Therefore the candidate has simply described the tests of significance performed and why to enable readers to reach reasonable conclusions without the help of adjustments.

In summary, in this chapter the doctoral candidate demonstrated that long-lasting reduction in allergen-specific IgE plays an important role in acquisition of SU and persistence of long-term sustained unresponsiveness (LTSU) at 4 years post-treatment. In contrast, increased sIgG4 is a transient phenomenon observed during the active OIT treatment period, with rapid reduction back to baseline following cessation of treatment; furthermore, levels of allergen-specific IgG4 are not elevated in subjects with long-term SU. These findings question the dogma linking upregulation of sIgG4 with OIT and tolerance acquisition. Although upregulation of antigen specific IgG4 through antigen exposure may confer transient protection against clinical reactivity (that is, desensitisation), induction of sIgG4 is not required for acquisition of long-term SU. In contrast, long-term SU was associated with uniform downregulation of IgE and IgG4 response suggesting that downregulation of antigen specific IgE and IgG4 producing B cells plays an important role in the acquisition of long-term tolerance.

4.7. Supplementary material

Supplementary Table. Actual results of PPOIT early treatment responders who maintained LTSU and those who did not and their sIgE and SPT at T5

Subjects with 8-week SU at Long-term F/U

idno	8-week SU Outcome (at T9) ("T5")	Treatment group	Peanut ingestion frequency at long-term F/U	Peanut ingestion amount at long-term F/U	Peanut sIgE at T9 ("T5")	Ara h1 sIgE at T9 ("T5")	Ara h2 sIgE at T9 ("T5")	Ara h3 sIgE at T9 ("T5")	Ara h8 sIgE at T9 ("T5")	Ara h9 sIgE at T9 ("T5")	Peanut SPT at T9 ("T5")
103	Passed	Active(PP OIT)	3 or more times a week	Large amount	4.79	0.06	0.11	0.18	0.05	0.22	0
204	Passed	Active(PP OIT)	<once/week	Small amount	0.87	0.27	0.68	0.14	0.09	0.11	2
207	Passed	Active(PP OIT)	Nil	Nil/Avoiding	0.7	0.03	0.21	0.04	0.02	0.04	5
212	Passed	Active(PP OIT)	1-2 times a week	Small amount	1.36	0.01	0.06	0.03	0.01	0.05	2.5
214	Passed	Active(PP OIT)	<once/week	Large amount	0.06	0	0.01	0.02	0	0.01	2
301	Passed	Active(PP OIT)	1-2 times a week	Moderate amount	3.14	0.08	0.1	0.15	0.06	0.08	0
303	Passed	Active(PP OIT)	3 or more times a week	Large amount	0.05	0.01	0.02	0.02	0	0.01	0

Subjects with 8-week SU at Long-term F/U but on Azathioprine

idno	8-week SU Outcome (at T9) ("T5")	Treatmen t group	Peanut ingestion frequency at long- term F/U	Peanut ingestion amount at long- term F/U	Peanut slgE at T9 ("T5")	Ara h1 slgE at T9 ("T5")	Ara h2 slgE at T9 ("T5")	Ara h3 slgE at T9 ("T5")	Ara h8 slgE at T9 ("T5")	Ara h9 slgE at T9 ("T5")	Peanut SPT at T9 ("T5")
226	Passed	Placebo	Nil	Nil/Avoidi ng	5.43	0.12	0.6	0.1	0.07	0.14	13

Subjects without 8-week SU at Long-term F/U

idno	8-week SU Outcome (at T9) ("T5")	Treatmen t group	Peanut ingestion frequency at long- term F/U	Peanut ingestion amount at long- term F/U	Peanut slgE at T9 ("T5")	Ara h1 slgE at T9 ("T5")	Ara h2 slgE at T9 ("T5")	Ara h3 slgE at T9 ("T5")	Ara h8 slgE at T9 ("T5")	Ara h9 slgE at T9 ("T5")	Peanut SPT at T9 ("T5")
101	Failed	Active(PP OIT)	Nil	Nil/Avoidi ng	2.14	0.87	1.41	0.08	0.03	0.02	12.5
209	Failed	Active(PP OIT)	Nil	Nil/Avoidi ng	100.01	40.3	28	11	3.5	0.3	17
228*	Failed	Active(PP OIT)	1-2 times a week	Moderate amount	24.7	13.5	8.75	1.4	0	0	11.5
406*	Failed	Active(PP OIT)	Nil	Nil/Avoidi ng	2.63	0.15	1.44	0.02	0	0.01	19.5
416*	Failed	Active(PP OIT)	<once/we ek	Moderate amount	24.6	16.5	5.53	5.9	0.01	0.01	7.5
102	Failed	Placebo	Nil	Nil/Avoidi ng	1.62	0.03	0.79	0.07	0.05	0.05	11

105	Failed	Placebo	Nil	Nil/Avoidi ng	.	.	.	.	.	.	9
201	Failed	Placebo	Nil	Nil/Avoidi ng	100.01	1.91	17.8	6.96	0.02	0.02	7
202	Failed	Placebo	Nil	Nil/Avoidi ng	76.7	12.8	61.4	0.9	0.13	0.2	17.5
215	Failed	Placebo	Nil	Nil/Avoidi ng	0.92	0	1.08	0.01	0	0.01	2.5
216	Failed	Placebo	Nil	Nil/Avoidi ng	100.01	36.6	55.4	5.54	0.01	0.01	15
217	Failed	Placebo	Nil	Nil/Avoidi ng	100.01	20.7	88.7	6.79	0	0.01	9
219	Failed	Placebo	Nil	Nil/Avoidi ng	100.01	190	433	67.5	0.13	0.42	15
221	Failed	Placebo	Nil	Nil/Avoidi ng	7.81	.	.	.	.	.	22.5
224	Failed	Placebo	Nil	Nil/Avoidi ng	83.5	15.4	53.8	7.69	0.03	0.03	13
304	Failed	Placebo	Nil	Nil/Avoidi ng	31.6	24.9	15.4	3.09	1.09	0.01	18.5
408	Failed	Placebo	Nil	Nil/Avoidi ng	1.55	.	.	.	.	.	19
412	Failed	Placebo	Nil	Nil/Avoidi ng	6.46	4.98	3.64	1.7	0	0.01	19
432	Failed	Placebo	Nil	Nil/Avoidi ng	9.84	0.18	0.87	0.2	0.16	0.16	3

*Denote those who had 2-week SU post-treatment

4.8. Chapter summary

In this chapter, the doctoral candidate presented findings relating to the long term effects of PPOIT on antibody responses of PPOIT-001 trial participants. The doctoral candidate extends the investigation of immune effects of PPOIT and presents findings relating to the participants' cellular responses to treatment in Chapter 5.

Chapter 5. Results: Immunomodulatory effects of PPOIT on the cellular responses of participants in the PPOIT-001 randomised trial

5.1. Chapter introduction

The doctoral candidate presented long-term clinical outcomes of participants in the PPOIT-001 trial in Chapter 3. Continuing the describing of immunomodulatory effects of PPOIT, in this chapter the doctoral candidate focuses on PPOIT's effects on cellular responses of PPOIT-001 trial participants.

5.2. Abstract

5.2.1. Background

Probiotic and Peanut Oral Immunotherapy (PPOIT) is effective at inducing challenge proven sustained unresponsiveness (SU) that persists to four years post-treatment. The doctoral candidate examined the effects of PPOIT on cytokine production by in-vitro cultured peripheral blood mononuclear cells (PBMC) and peanut specific T-regulatory (Treg) cell frequency in PBMC from participants enrolled in the PPOIT-001 randomised trial. The doctoral candidate also examined the relationships between levels of peanut and peanut component specific-IgE (sIgE), cytokine production and peanut specific Treg cell frequency in children with peanut allergy.

5.2.2. Methods

Cytokine measurements

PBMC obtained from participants in the PPOIT-001 trial at study entry (T0) and at 3 months post-treatment (T3) were cultured in crude peanut extract (CPE) or media alone (resting) for 6 days. Supernatants were collected at 6 days and stored at -80C until batched processed. Levels of T-helper 2 (Th2; IL-13, IL-4, IL-5), T-helper 1 (Th1; IFN- γ) and Treg (IL-10) cytokines in PBMC culture supernatants were measured using a high-sensitivity multiplex assay (Milliplex, USA).

Peanut specific Treg

Peanut specific T-regulatory (Treg) cells were defined as proliferating (CFSElo) CD4+/CD25+/Foxp3+ lymphocytes in CPE stimulated cultures. Batch thawed PBMC obtained from participants in the PPOIT-001 trial at study entry (T0) and at 3 months post-treatment (T3) were labeled with CFSE-FITC and cultured in the presence of CPE or media for 6 days. CFSE stained PBMC were retrieved at 6 days and further incubated with CD4-Per CP, CD25-PECy 7, Foxp3-PE. CFSElo/CD4+/CD25+/Foxp3+ lymphocytes were enumerated using fluorescence activated cell sorting (FACS).

Pearson's correlation and linear regression analyses of log-transformed values were used to examine the relationship between immune indices. Age- and sex-adjusted multivariate regression analyses of log-transformed values were used for point-in-time, between-group comparisons.

5.2.3. Results

Levels of each individual cytokine measured in resting and CPE stimulated PBMC culture supernatants were similar in PPOIT- and placebo- treated participants at study entry (T0) and at 3 months post-treatment (T3). However, at T3 the change from study entry was significantly different between PPOIT and placebo groups for IL-5 (resting culture) and IL-4 (resting and CPE stimulated cultures) production [Geometric Mean (GM; 95% CI) of fold change, IL-5, resting culture: PPOIT 0.27 (0.05, 1.59) v.s. placebo 1.58 (0.70, 3.59), $p=0.050$; IL-4, resting culture PPOIT 0.54 (0.25, 1.18) v.s. placebo 1.17 (0.85, 1.61), $p = 0.046$; IL-4, CPE culture PPOIT 0.27 (0.13, 0.56) v.s. placebo 1.12 (0.88, 1.42), $p = 0.001$]. Furthermore, at T3, there was a trend towards lower frequency of peanut specific Treg cells in CPE cultured PBMC from participants in the PPOIT group compared with those in the placebo group [Mean (standard deviation, SD): 4.3 % (4.3) v.s. 11.3 % (14.7), $p = 0.07$].

At study entry, higher peanut and peanut component (Ara h1, h2, h3) sIgE levels were associated with higher ratios of IL-5:IFN- γ [$p < 0.001$ (peanut), $p < 0.001$ (Ara h1), $p < 0.001$ (Ara h2), $p < 0.001$ (Ara h3)], IL-13:IFN- γ [$p < 0.002$ (peanut), $p < 0.001$ (Ara h1), $p = 0.001$ (Ara h2), $p = 0.002$ (Ara h3)] and IL-4:IFN- γ [$p = 0.033$ (peanut), $p = 0.014$ (Ara h1), $p = 0.023$ (Ara h2), $p = 0.002$ (Ara h3)]. Surprisingly, higher peanut sIgE level was associated with higher circulating peanut specific Treg (regression coefficient 0.039, $p = 0.026$).

5.2.4. Conclusions

PPOIT was associated with reduced production of Th2 cytokines and a trend toward reduced peanut specific Treg cells frequency at the post-treatment time point. In children with peanut allergy, higher levels of peanut sIgE were associated

with higher Th2:Th1 cytokine ratios and higher frequency of circulating peanut specific Treg cells.

Together, these suggest that the clinical response induced by PPOIT treatment may be mediated by a redirection in cytokine response. A reduction in frequency of circulating Treg cells may occur as a downstream event in response to reduced allergic disease activity. Longitudinal repeated measures studies of immune indices are needed to confirm these observed effects of PPOIT on cellular immune responses.

5.3. Rationale

Combined probiotic and peanut oral immunotherapy (PPOIT) is effective at inducing clinical remission defined as 2-6 week sustained unresponsiveness (SU) immediately post-treatment[95]; and more importantly, the majority of treatment responders have persistence of SU to 4 years post-treatment[99]. Food oral immunotherapy (OIT) studies have consistently shown that gastrointestinal allergen exposure is accompanied by induction of an antigen specific-IgG4 (sIgG4) response and that sIgG4 levels continue to increase during treatment. In contrast, antigen specific-IgE (sIgE) levels increase in the first few months of treatment, followed by gradual decline towards baseline over the course of 1-2 years and levels may in some cases fall below baseline after several years of treatment. Findings reported in Chapter 4 confirmed that PPOIT is associated with increased peanut and peanut component sIgG4 levels; however, there is rapid reduction in sIgG4 levels once PPOIT treatment was discontinued. Moreover, the amount of peanut ingestion post-treatment correlated with higher sIgG4 levels at 4 years post-treatment. In addition, at 4 years post-treatment, persistence of SU after years of ad libitum ingestion was associated with reduced levels of antigen specific sIgG4 after adjusting for amount of peanut intake. Concomitantly, persistent SU was associated with reduced levels of peanut sIgE at 4 years post-treatment, suggesting that downregulation of antigen specific B cell responses may be an important mechanism supporting tolerance induction.

Redirection of B cell responses may be achieved through the induction of T-regulatory (Treg) cells and B-regulatory (Breg) cells and/or anergy or deletion of effector T cells. Surrogate measures of increased Treg cell activity and/or anergy of effector T cell activity include: (1) increased production of interleukin (IL)-10 and transforming growth factor-beta (TGF- β) which play a role in inducing IgG4[135 136] and IgA[137] production respectively, and (2) decreased production of Th2 cytokines including IL-13, IL-4 and IL-5 which are needed to support IgE production[119]. There is limited data on changes in antigen specific Treg cell

frequency and cytokine production associated with OIT and the small number of available studies report mixed findings[18 40 54 55 138].

The doctoral candidate hypothesised that (1) PPOIT treatment induces downregulation of Th2 cytokine production and upregulation of Treg cell frequency and Treg cytokine (IL-10) production, and (2) in children with peanut allergy, higher levels of serum or plasma peanut specific IgE is associated with elevated Th2:Th1 cytokine balance and reduced Treg cell frequency.

The specific aims of this chapter are to:

- Compare the levels of Th2 (IL-13, IL-4, IL-5), Th1 (interferon-gamma, IFN- γ) and Treg (IL-10) cytokines in resting and crude peanut extract (CPE) stimulated PBMC culture supernatants from PPOIT- and placebo-treated participants at study entry (T0) and at 3 months post-treatment (T3);
- Compare the frequency of peanut specific Treg cells as a proportion of total CD4+ lymphocytes in PBMC from PPOIT- and placebo- treated participants at T0 and at T3;
- Compare T0 to T3 fold change in Th2, Th1 and Treg cytokine levels in media and CPE stimulated PBMC culture supernatants from PPOIT- and placebo-treated participants;
- Compare T0 to T3 fold change in the frequency of peanut specific Treg cells in PBMC from PPOIT- and placebo- treated participants;
- Examine the association between serum or plasma levels of peanut and peanut component (Ara h1, h2 and h3) sIgE and the ratio of Th2:Th1 cytokines produced by resting and CPE stimulated PBMC culture supernatants from children with peanut allergy at T0 and at T3;
- Examine the association between serum or plasma levels of peanut and peanut component (Ara h1, h2 and h3) sIgE and the frequency of peanut specific Treg cell as a proportion of total CD4+ lymphocytes in PBMC from children with peanut allergy at T0 and at T3.

5.4. Abbreviated methods

Detailed methods including laboratory methods for the PPOIT-001 trial are presented in Chapter 2. The following is a summary of select aspects of study methodology relevant to this results chapter.

5.4.1. Participants

The Probiotic and Peanut Oral Immunotherapy (PPOIT-001) trial is a double-blind placebo controlled randomised trial that randomised 62 children aged 1-10 years with peanut allergy to receive a probiotic adjuvant [*Lactobacillus rhamnosus* CGMCC 1.3724 (NCC4007)] and peanut oral immunotherapy (PPOIT) or placebo and placebo for 18 months. Six participants withdrew during the study leaving n=56 participants at trial end. PPOIT was effective at inducing desensitisation (89.7% PPOIT versus 7.1% placebo, $p<0.001$) and 2-week SU (82.1% PPOIT versus 3.6% placebo, $p<0.001$)[95].

5.4.2. Study samples

As per registered PPOIT-001 study protocol, blood samples were collected from all eligible trial participants at study entry (T0), end of treatment (T1), 3 months post-treatment (T3) and 4 years post-treatment (T5). Saliva samples were collected from all eligible trial participants at study entry (T0), at weeks 17, 33, 49 during treatment, at 2-6 weeks post-treatment (T2) and at 12 months post-treatment (T4). Only participants who had attained desensitisation at T1 were eligible for follow-up at T2. For experiments described in this chapter, blood samples collected at T0 and T3 were used

5.4.3. Sample collection and processing

Whole blood samples were collected into sodium heparin tubes or gel tubes. Peripheral blood mononuclear cells (PBMC) from heparinized samples were separated within 2 hours of collection by density gradient centrifugation. PBMC were cryopreserved in $10\text{--}20 \times 10^6/\text{ml}$ in RPMI with 15% DMSO in fetal calf serum (FCS). At a later time they were batched thawed and either freshly stained or in-

vitro cultured before staining for Treg cell enumeration or cytokine production studies. Separated plasma and sera were cryopreserved at -80°C and subsequently batch thawed for measurement of peanut and peanut component specific-IgE (sIgE) levels.

5.4.4. Peripheral blood mononuclear cell (PBMC) processing for flow cytometry and for cytokine production assays

Batch thawed PBMCs were freshly stained with 2.5 µL CD4–Per CP, 2.5 µL CD25–PECy 7, 7 µL of Foxp3–PE per 1×10^6 cells or stained with 10 µL CFSE-FITC per 1×10^6 cells before and 2.5 µL CD4–Per CP, 2.5 µL CD25–PECy 7, 7 µL of Foxp3–PE per 1×10^6 cells after in-vitro culturing for 6 days in RPMI-1640 with 2 mmol/L L-glutamate, 25 mmol/L HEPES buffer, 10% human AB serum in the absence of antigen (that is, media alone; resting PBMC) or in the presence of either crude peanut extract (CPE; 100 µg/mL; kindly provided by Professor Wesley Burks and his research group, Duke University Medical Center), ovalbumin (OVA) or tetanus toxoid (TT) (antigen stimulated PBMC).

PBMC from participants at T0 and T3 were thawed and batch processed. Thawed PBMC were incubated with medium alone (resting PBMC culture) or crude peanut extract (CPE; 100 µg/mL; CPE stimulated PBMC culture) in RPMI-1640 medium and 10% fetal calf serum for 6 days and supernatant collected for measurement of Treg, Th1, Th2 cytokines (IL-10, IFN- γ , IL-4, IL-5, IL-13). The time course for collection of supernatants was previously validated and demonstrated to provide reliable Th1, Th2 and Treg cytokine measurements after 48 hours and 6 days of culture[139].

5.4.5. Flow cytometric enumeration of total, resting and antigen specific T-regulatory (Treg) cells

Using fluorescence activated cell sorting (FACS) analysis, frequencies of total Treg and Treg cell subpopulations were determined in freshly thawed PBMC at T0 and T3. Treg populations examined included: total Treg cell population (gating on CD4+/CD25+/Foxp3+ lymphocytes), proliferating Treg (gating on

CFSElo/CD4+/CD25+/Foxp3+ lymphocytes) in PBMC after in-vitro culture for 6 days in media alone (resting Treg) or in CPE (defined as peanut specific Treg), OVA (defined as ovalbumin specific Treg) or TT (defined as tetanus toxoid specific Treg).

5.4.6. Measurement of Th1, Th2, Treg cytokines

The doctoral candidate had sufficient funds to evaluate supernatant cytokines at 2 time points only, and selected measurement at study entry (T0) and 3 months post-treatment (T3). Cytokine levels in the T0 and T3 supernatants were measured using a high-sensitivity multiplex immunoassay (EMD Millipore, Billerica, MA) on Luminex MAP200 instrument as per manufacturers' instructions. Data was analysed using MasterPlex software (Hitachi Software Engineering America Ltd., MiraiBio Group) and expressed in pg/ml.

5.4.7. Measurement of levels of serum peanut and plasma peanut component sIgE and sIgG4

Levels of serum peanut and plasma peanut component (Ara h1, h2, h3, h8 and h9) sIgE and sIgG4 were measured using ImmunoCAP 250 (Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions.

5.4.8. Immune indices analyses

Immune indices statistically evaluated in this study include:

(1) levels of serum peanut and plasma peanut component (Ara h1, h2 and h3) sIgE (kUA/L), and

(2) frequencies, expressed as a proportion of total CD4+ lymphocytes, of total Treg (CD4+CD25+Foxp3+), resting Treg (CFSElo/CD4+/CD25+/Foxp3+, 6 days media) and peanut specific Treg (CFSElo/CD4+/CD25+/Foxp3+, 6 days CPE), OVA-specific Treg (CFSElo/CD4+/CD25+/Foxp3+, 6 days OVA), TT-specific Treg (CFSElo/CD4+/CD25+/Foxp3+, 6 days TT), and

(3) levels of in-vitro PBMC culture supernatant IL-13, IL-4, IL-5 IL-10, IFN- γ (pg/mL).

In addition, the doctoral candidate calculated and analysed ratio of Th2:Th1 cytokines as a surrogate marker of Th2:Th1 milieu[140] and the fold change in study entry (T0) to post-treatment (T3) cytokine levels in order to adjust for within individual baseline levels of cytokine production[36 55] as additional indices of interest.

5.4.9. Statistics

- **Statistical power**

Blood samples were available from 56 participants (PPOIT n=28 and placebo n=28) who completed the PPOIT-001 RCT. This sample size provides greater than 80% power to detect an effect size of 0.76 or more (that is, a difference of 0.76 standard deviations between the mean number of Treg cells in each group). Preliminary analysis of Treg cells frequency at T0 in pooled participants (n= 49) from the PPOIT-001 trial revealed a mean frequency of Treg cells (as a percentage of CD4+ cells) of 10.8% with a standard deviation of 3.7%. Based on these findings, the sample size provides sufficient power to detect a post-intervention increase in total Treg cells frequency of (0.76 times 3.7% equals) 2.8% (that is, from 10.8% to 13.6%) which is a biologically significant difference.

Given previous work on the distribution pattern of cytokine levels in healthy humans, it was anticipated that non-parametric analytical measures will be necessary. In addition, it would be difficult to anticipate the effect size that would be required. However, a number of published studies with smaller sample sizes than the PPOIT-001 RCT have demonstrated statistically significant temporal within arms and across arms differences in cytokine levels. Jones et al reported statistically significant changes in the levels of six cytokines [including IL-5 and tumour necrosis factor-alpha (TNF- α), $p < 0.05$] in the supernatants of cultured PBMC collected every six months for two years from five participants who were treated with a peanut oral immunotherapy[18]. In another study of pollen

allergy[140], statistically significant differences in the IL-10 levels and ratios of IL-5:IFN- γ , IL-13:IFN- γ , IL-4:IFN- γ in the supernatants of cultured PBMC collected from seven allergic and six non-allergic participants were detectable. Based on these studies, it was anticipated that the doctoral candidate's proposed sample sizes of n=28 per group will be sufficiently powered to detect similar differences in cytokine production.

- **Statistical analyses**

Immune indices were examined visually for normality and linearity.

As per registered PPOIT-001 trial protocol (ACTRN 12608000594325, 25/11/2008), parametric immunologic variables were analysed using standard t test with data summarised using arithmetic means and standard deviations (SD). Non-parametric immunologic variables were analysed using standard t test on logarithmic scaled values with data summarised using geometric means (GM) and 95% confidence intervals (95% CI). Pairwise correlation and age- and sex-adjusted linear regression analyses were performed to examine the association between immune indices. Between group analyses for PPOIT vs placebo were by intention to treat (ITT) where outcome data were available as per the approach adopted in previous publications relating to this study[95 99].

Given that the doctoral candidate was investigating a number of hypotheses, the candidate did not adjust p-values for multiple comparisons as per Feise and Perneger[97 98].

Data were analysed with Stata release 15 software (StataCorp, College Station, Tex).

5.4.10. Ethics

Ethics approval was obtained from The Royal Children's Hospital Human Research Ethics Committee (HREC 27086). The trial, including the a priori plan to analyse immunologic variables as secondary outcomes, was registered with the Australian New Zealand Clinical Trials Registry (ACTRN 12608000594325, 25/11/2008).

5.5. Results

To address the hypothesis that PPOIT treatment induces downregulation of Th2 cytokine production by PBMC, the doctoral candidate examined PBMC production of Th2, Th1 and Treg cytokines at study entry (T0) and at 3 months post-treatment (T3).

5.5.1. Effect of PPOIT treatment on cytokine production by resting and CPE stimulated PBMC from participants in PPOIT and placebo groups at study entry and at 3 months post-treatment

The levels of Th2, Th1 and Treg cytokines measured in resting PBMC culture supernatants were similar in PPOIT and placebo groups at T0 and at T3. Similarly, there was difference between the groups in the levels of cytokines measured in CPE stimulated PBCM culture supernatants at T0 and at T3. (Table 5-1).

Table 5-1. Between group comparisons of cytokine production at study entry (T0) and at 3 months post-treatment (T3)

IL-13 (pg/mL)		PPOIT	Placebo	
Resting PBMC (6 days media)				P-value*
T0				
Geometric mean (95% CI)		32.42 (4.52, 232.37)	15.63 (3.47, 70.43)	0.524
Median (IQR), no.		50.76 (2.48, 377.18), 13	19.63 (7.04, 53.77), 15	
T3				
Geometric mean (95% CI)		13.87 (5.15, 37.33)	32.11 (7.35, 140.33)	0.326
Median (IQR), no.		20.31 (2.49, 34.86), 14	79.48 (4.75, 206.33), 15	
CPE stimulated PBMC (6 days)				P-value*
T0				
Geometric mean (95% CI)		1415.12 (866.32, 2311.59)	753.23 (315.64, 1797.50)	0.204
Median (IQR), no.		1468.93 (721.70, 2706.98), 13	963.20 (687.20, 1828.57), 15	
T3				
Geometric mean (95% CI)		969.73 (560.15, 1678.79)	623.24 (124.55, 3118.77)	0.565
Median (IQR), no.		1160.58 (749.26, 2060.81), 14	992.97 (894.84, 2475.57), 13	

IL-4 (pg/mL)		PPOIT	Placebo	
Resting PBMC (6 days media)				P-value*
T0				
Geometric mean (95% CI)		2.18 (1.05, 4.51)	1.32 (0.84, 2.07)	0.202
Median (IQR), no.		0.92 (0.92, 3.52), 13	0.92 (0.92, 1.39), 15	
T3				
Geometric mean (95% CI)		1.16 (0.89, 1.53)	1.55 (1.05, 2.27)	0.212
Median (IQR), no.		0.92 (0.92, 0.92), 14	0.92 (0.92, 2.74), 15	

CPE stimulated PBMC (6 days)			P-value*
T0			
Geometric mean (95% CI)	4.66 (2.12, 10.23)	2.31 (1.21, 4.44)	0.148
Median (IQR), no.	4.68 (0.92, 14.93), 13	1.39 (0.92, 5.72), 15	
T3			
Geometric mean (95% CI)	1.22 (0.90, 1.66)	2.18 (1.12, 4.27)	0.091
Median (IQR), no.	0.92 (0.92, 1.53), 14	0.92 (0.92, 6.20), 13	

IL-5 (pg/mL)	PPOIT	Placebo	
Resting PBMC (6 days media)			P-value*
T0			
Geometric mean (95% CI)	9.17 (1.16, 72.61)	3.42 (1.12, 10.43)	0.354
Median (IQR), no.	3.53 (0.25, 125.31), 13	1.83 (1.20, 3.18), 15	
T3			
Geometric mean (95% CI)	2.48 (1.31, 4.67)	5.42 (1.79, 16.39)	0.207
Median (IQR), no.	2.86 (2.03, 4.74), 14	7.28 (1.39, 15.46), 15	

CPE stimulated PBMC (6 days)			P-value*
IL-5 (pg/mL), T0			
Geometric mean (95% CI)	222.73 (72.84, 681.04)	105.45 (40.48, 274.67)	0.279
Median (IQR), no.	246.56 (30.66, 1329.59), 13	92.83 (60.44, 179.07), 15	
IL-5 (pg/mL), T3			
Geometric mean (95% CI)	83.48 (44.15, 157.86)	95.41 (34.93, 260.66)	0.806
Median (IQR), no.	99.21 (50.55, 190.43), 14	200.64 (59.08, 207.81), 13	

IFN- γ (pg/mL)		PPOIT	Placebo	
Resting PBMC (6 days media)				P-value*
T0				
	Geometric mean (95% CI)	0.50 (0.26, 0.94)	0.88 (0.32, 2.40)	0.327
	Median (IQR), no.	0.30 (0.30, 0.30), 13	0.30 (0.30, 0.91), 15	
T3				
	Geometric mean (95% CI)	0.53 (0.28, 1.02)	1.81 (0.53, 6.17)	0.073
	Median (IQR), no.	0.30 (0.30, 0.30), 14	0.52 (0.30, 6.33), 15	
CPE stimulated PBMC (6 days)				P-value*
T0				
	Geometric mean (95% CI)	31.52 (11.86, 83.75)	43.54 (16.36, 115.85)	0.620
	Median (IQR), no.	31.95 (10.43, 114.68), 13	40.77 (29.08, 160.13), 15	
T3				
	Geometric mean (95% CI)	31.18 (9.98, 97.38)	54.24 (14.14, 208.07)	0.499
	Median (IQR), no.	28.26 (12.05, 85.53), 14	68.62 (34.09, 189.08), 13	

IL-10 (pg/mL)		PPOIT	Placebo	
Resting PBMC (6 days media)				P-value*
T0				
	Geometric mean (95% CI)	2.87 (1.35, 6.07)	3.52 (1.96, 6.33)	0.642
	Median (IQR), no.	3.24 (0.70, 8.41), 13	4.71 (1.22, 8.00), 15	
T3				
	Geometric mean (95% CI)	2.80 (1.54, 5.09)	4.43 (2.63, 7.46)	0.221
	Median (IQR), no.	3.66 (0.70, 7.10), 14	4.58 (2.08, 8.71), 15	
CPE stimulated PBMC (6 days)				P-value*
T0				

T3	Geometric mean (95% CI)	19.35 (9.06, 41.30)	26.68 (15.54, 45.78)	0.453
	<i>Median (IQR), no.</i>	<i>30.89 (12.76, 34.94), 13</i>	<i>25.96 (13.24, 56.80), 15</i>	
	Geometric mean (95% CI)	16.10 (10.49, 24.71)	27.75 (14.61, 52.72)	0.133
	<i>Median (IQR), no.</i>	<i>17.29 (9.89, 22.24), 14</i>	<i>32.51 (26.65, 48.33), 13</i>	

*The t test on the log scale was applied for data presented as geometric means; Resting PBMC were cultured in media for 6 days and CPE stimulated PBMC were cultured in CPE for 6 days

5.5.2. Effect of PPOIT treatment on the change in cytokine production from study entry to 3 months post-treatment

In the placebo group, Th2 cytokine production by both resting and CPE stimulated PBMC increased from T0 to T3 [Geometric Mean (GM; 95% CI) of T0 to T3 fold change, resting PBMC: IL-13 production 2.06 (0.45, 9.32), IL-4 production: 1.17 (0.85, 1.61), IL-5 production: 1.58 (0.70, 3.59); CPE stimulated PBMC: IL-4 production 1.12 (0.88, 1.42), IL-5 production 1.18 (0.42, 3.33); Table 5-2]. In contrast, in the PPOIT group there was a reduction in levels of Th2 cytokines following treatment [GM (95% CI) of T0 to T3 fold change, resting PBMC: IL-13 production 0.42 (0.05, 3.80), IL-4 production 0.54 (0.25, 1.18), IL-5 production 0.27 (0.05, 1.59); CPE stimulated PBMC: IL-13 production 0.65 (0.31, 1.37), IL-4 production 0.27 (0.13, 0.56), IL-5 production 0.37 (0.13, 1.03)]. Comparison of PPOIT versus placebo groups showed statistically significant differences in the fold change from T0 to T3 for Th2 cytokine levels (resting PBMC IL-4 production $p=0.046$, resting PBMC IL-5 production $p=0.050$, CPE stimulated PBMC IL-4 production $p=0.001$) following treatment. In contrast, there was no difference between the groups in the fold change for Th1 and Treg cytokines.

Table 5-2. Between group comparisons of the fold change from study entry (T0) in cytokine production at 3 months post-treatment (T3)

Resting PBMC	Geometric mean (95% CI) of the T0 to T3 fold change in cytokine production		Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo		
IL-13	0.416 (0.046, 3.798), 13	2.055 (0.453, 9.318), 15	0.198	0.264
IL-4	0.544 (0.252, 1.176), 13	1.171 (0.850, 1.612), 15	0.046	0.043
IL-5	0.271 (0.046, 1.589), 13	1.582 (0.696, 3.592), 15	0.050	0.071
IFN- γ	1.108 (0.519, 2.363), 13	2.055 (0.469, 8.998), 15	0.452	0.432
IL-10	1.086 (0.563, 2.095), 13	1.260 (0.894, 1.775), 15	0.655	0.707

CPE stimulated PBMC	Geometric mean (95% CI) of the T0 to T3 fold change in cytokine production		Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo		
IL-13	0.652 (0.311, 1.370), 13	0.939 (0.169, 5.206), 13	0.674	0.727
IL-4	0.268 (0.128, 0.561), 13	1.121 (0.883, 1.423), 13	0.001	0.001
IL-5	0.367 (0.130, 1.033), 13	1.182 (0.419, 3.334), 13	0.095	0.140
IFN- γ	0.915 (0.292, 2.867), 13	1.429 (0.340, 5.999), 13	0.601	0.752
IL-10	0.828 (0.348, 1.971), 13	1.199 (0.576, 2.499), 13	0.485	0.469

*Adjusted for age and sex; The t test on the log scale was applied for data presented as geometric means; Resting PBMC were cultured in media for 6 days and CPE stimulated PBMC were cultured in CPE for 6 days

5.5.3. Effect of PPOIT on resting and antigen specific T-regulatory cells at 3 months post-treatment

At study entry (T0), there was no difference between treatment groups in the frequency of proliferating (CFSE^{lo}) Treg cells in PBMC cultured in media alone or PBMC cultured in crude peanut extract (CPE), ovalbumin (OVA), or tetanus toxoid (TT). At 3 months post-treatment (T3), there was also no between group difference in resting or antigen specific Treg cells for all antigens tested (Table 5-3). In the PPOIT group as compared to placebo, there was a trend towards lower proportion of proliferating (CFSE^{lo}) Treg cells in PBMC cultured in CPE (peanut specific Treg cells) and cultured in media alone (resting Treg cells). At T3, there were no between group differences for the fold change from study entry for peanut specific and resting Treg cells (Table 5-4). These findings suggest that PPOIT has limited effect on peanut specific Treg, resting Treg or other antigen specific Treg cell frequencies when evaluated at 3 months post-treatment (T3).

Table 5-3. Between group comparison of absolute values of resting and antigen specific Treg cells at study entry (T0) and at 3 months post-treatment (T3)

6 day Media		Mean (SD) of CFSElo Treg		Unadjusted P-value	Adjusted P-value*
		PPOIT	Placebo		
	T0	8.129 (18.785), 15	1.685 (2.867), 13	0.233	0.190
	T3	0.669 (0.791), 17	2.243 (3.127), 15	0.054	0.126
6 day CPE		Mean (SD) of CFSElo Treg		Unadjusted P-value	Adjusted P-value*
		PPOIT	Placebo		
	T0	10.146 (13.910), 15	6.791 (6.517), 13	0.433	0.338
	T3	4.306 (4.251), 17	11.344 (14.743), 14	0.070	0.095
6 day OVA		Mean (SD) of CFSElo Treg		Unadjusted P-value	Adjusted P-value*
		PPOIT	Placebo		
	T0	2.161 (0.439, 5.414), 10	2.216 (1.617, 2.438), 7	0.433	0.320
	T3	1.839 (1.885), 15	3.542 (3.706), 10	0.142	0.095
6 day TT		Mean (SD) of CFSElo Treg		Unadjusted P-value	Adjusted P-value*
		PPOIT	Placebo		
	T0	6.471 (9.458), 7	5.805 (3.601), 5	0.885	0.517
	T3	7.775 (9.848), 14	8.249 (8.126), 8	0.909	0.963

*Adjusted for age and sex; The t test was applied for data presented as mean (SD).

CPE = crude peanut extract, OVA = ovalbumin, TT = tetanus toxoid.

Table 5-4. Between group comparison of the fold change from study entry (T0) in resting and antigen specific Treg cells at 3 months post-treatment (T3)

Culture condition	Mean (SD) of T0 to T3 fold change in CFSElo Treg		Unadjusted P-value	Adjusted P-value*
	PPOIT	Placebo		
6 day Media	1.482 (2.840), 12	8.866 (22.823), 10	0.278	0.181
6 day CPE	3.150 (9.104), 12	3.934 (7.263), 9	0.834	0.481
6 day OVA	1.077 (1.714), 7	2.570 (2.173), 5	0.212	0.296
6 day TT	5.041 (8.521), 4	1.117 (1.060), 3	0.474	0.946

*Adjusted for age and sex; The t test was applied for data presented as mean (SD).

CPE = crude peanut extract, OVA = ovalbumin, TT = tetanus toxoid

Next, to address the hypothesis that in children with peanut allergy, higher peanut sIgE is associated with cytokine production skewed in favour of Th2 cytokines over Th1 cytokines, the doctoral candidate examined the relationship between peanut and peanut component sIgE and the ratio of Th2:Th1 cytokine production by PBMC collected at study entry (pre-treatment, T0) and conducted pooled analyses of immune indices data from participants in the PPOIT and placebo groups prior to exposure to treatment in order to maximize sample size. For the post-treatment time point (T3), subgroup analyses stratified by exposure groups were conducted.

5.5.4. Association between levels of peanut sIgE and ratio of Th2:Th1 cytokine production by resting PBMC from participants in the PPOIT and placebo groups at study entry (pooled, pre-treatment)

As expected, levels of peanut and peanut component specific IgE correlated positively with the ratio of Th2:Th1 cytokine production by resting PBMC from children with peanut allergy at study entry (Table 5-5). Higher peanut and peanut component sIgE levels were associated with higher ratios of IL-13:IFN- γ , IL-4:IFN- γ and IL-5:IFN- γ production by resting PBMC (Table 5-6). These findings are consistent with the established understanding that pathogenic allergic antibody responses are supported by a cytokine milieu where Th2 activity dominates over Th1.

Table 5-5. Pearson's Correlation of sIgE and ratio of Th2:Th1 cytokine production by resting PBMC from children with peanut allergy (pooled PPOIT and placebo groups) at study entry (T0)

Study entry (T0), pooled PPOIT and Placebo groups (n = 56)		IL-13:IFN- γ ratio (resting PBMC)	
		Correlation, r	p-value
Peanut sIgE		0.619	0.000
Ara h1 sIgE		0.679	0.000
Ara h2 sIgE		0.597	0.001
Ara h3 sIgE		0.590	0.002
		IL-4:IFN- γ ratio (resting PBMC)	
		Correlation, r	p-value
Peanut sIgE		0.405	0.033
Ara h1 sIgE		0.475	0.014
Ara h2 sIgE		0.428	0.023
Ara h3 sIgE		0.595	0.002
		IL-5:IFN- γ ratio (resting PBMC)	
		Correlation, r	p-value
Peanut sIgE		0.725	<0.0001
Ara h1 sIgE		0.810	<0.0001
Ara h2 sIgE		0.695	<0.0001
Ara h3 sIgE		0.820	<0.0001

sIgE level as measured in kUA/L

Table 5-6. Linear Regression of sIgE and ratio of Th2:Th1 cytokine production by resting PBMC from children with peanut allergy (pooled PPOIT and placebo groups) at study entry (T0)

Study entry (T0), pooled PPOIT and Placebo groups (n = 56)

and Placebo groups (n = 56)		IL-13:IFN-γ ratio (resting PBMC)	
Resting / Media	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	0.529	<0.001	0.002
Ara h1 sIgE	0.799	<0.001	<0.001
Ara h2 sIgE	0.491	0.001	0.004
Ara h3 sIgE	0.678	0.002	0.004
IL-4:IFN-γ ratio (resting PBMC)			
Resting / Media	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	0.611	0.033	0.063
Ara h1 sIgE	1.042	0.014	0.022
Ara h2 sIgE	0.622	0.023	0.047
Ara h3 sIgE	1.255	0.002	0.010
IL-5:IFN-γ ratio (resting PBMC)			
Resting / Media	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	0.634	<0.001	<0.001
Ara h1 sIgE	0.986	<0.001	<0.001
Ara h2 sIgE	0.585	<0.001	<0.001
Ara h3 sIgE	0.915	<0.001	<0.001

*Adjusted for age and sex; sIgE level as measured in kUA/L

5.5.5. Association between levels of peanut sIgE and ratio of Th2:Th1 cytokine production by resting PBMC from participants in the PPOIT and placebo groups at 3 months post-treatment

After 18 months of treatment, placebo group participants showed weaker but still positive correlations between peanut sIgE levels and the ratio of Th2:Th1 cytokine production by their PBMC, similar to the pooled and pre-treatment findings at T0 (Table 5-7). A higher ratio of IL-5:IFN- γ production by their PBMC was associated with higher levels of peanut and peanut component (Ara h2 and Ara h3) sIgE (Table 5-8). Similar trends were observed for the ratios of IL-13:IFN- γ and IL-4:IFN- γ . In contrast, in the PPOIT group the positive association between levels of peanut sIgE and the ratio of Th2:Th1 cytokine production by PBMC was not observed at 3 months post-treatment (T3) (Table 5-9 and Table 5-10). Subgroup analyses by treatment groups involving smaller sample sizes may have contributed to the weakening of association between levels of peanut sIgE and ratio of Th2:Th1 cytokine production.

Table 5-7. Pearson's Correlation of levels of peanut sIgE and ratio of Th2:Th1 cytokine production by resting PBMC from participants in placebo group at 3 months post-treatment (T3)

Post-treatment (T3)		
Placebo group (n = 28)		
IL-13:IFN- γ ratio (resting PBMC)		
	Correlation, r	p-value
Peanut sIgE	0.475	0.074
Ara h1 sIgE	0.458	0.116
Ara h2 sIgE	0.440	0.101
Ara h3 sIgE	0.613	0.034
IL-4:IFN- γ ratio (resting PBMC)		
	Correlation, r	p-value
Peanut sIgE	0.186	0.507
Ara h1 sIgE	-0.0814	0.791
Ara h2 sIgE	0.144	0.610
Ara h3 sIgE	0.206	0.521
IL-5:IFN- γ ratio (resting PBMC)		
	Correlation, r	p-value
Peanut sIgE	0.617	0.014
Ara h1 sIgE	0.393	0.184
Ara h2 sIgE	0.580	0.023
Ara h3 sIgE	0.638	0.026

sIgE level as measured in kUA/L

Table 5-8. Linear Regression of levels of peanut sIgE and ratio of Th2:Th1 cytokine production by resting PBMC from participants in the placebo group at 3 months post-treatment (T3)

Post-treatment (T3)

Placebo group (n = 28)

Placebo group (n = 28)		IL-13:IFN-γ ratio (resting PBMC)	
	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	0.499	0.074	0.112
Ara h1 sIgE	0.725	0.116	0.170
Ara h2 sIgE	0.438	0.101	0.149
Ara h3 sIgE	0.721	0.034	0.062
IL-4:IFN-γ ratio (resting PBMC)			
	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	0.168	0.507	0.830
Ara h1 sIgE	-0.112	0.791	0.673
Ara h2 sIgE	0.123	0.610	0.963
Ara h3 sIgE	0.209	0.521	0.925
IL-5:IFN-γ ratio (resting PBMC)			
	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	0.649	0.014	0.038
Ara h1 sIgE	0.630	0.184	0.224
Ara h2 sIgE	0.578	0.023	0.056
Ara h3 sIgE	0.781	0.026	0.097

*Adjusted for age and sex; sIgE level as measured in kUA/L

Table 5-9. Pearson's Correlation of levels of peanut sIgE and ratio of Th2:Th1 cytokine production by resting PBMC from participants in the PPOIT group at 3 months post-treatment (T3)

Post-treatment (T3)			
PPOIT group (n = 28)		IL-13:IFN- γ ratio (resting PBMC)	
		Correlation, r	p-value
	Peanut sIgE	-0.040	0.891
	Ara h1 sIgE	-0.045	0.878
	Ara h2 sIgE	0.013	0.966
	Ara h3 sIgE	-0.044	0.883
		IL-4:IFN- γ ratio (resting PBMC)	
		Correlation, r	p-value
	Peanut sIgE	-0.306	0.287
	Ara h1 sIgE	-0.276	0.340
	Ara h2 sIgE	-0.185	0.526
	Ara h3 sIgE	-0.364	0.201
		IL-5:IFN- γ ratio (resting PBMC)	
		Correlation, r	p-value
	Peanut sIgE	0.227	0.436
	Ara h1 sIgE	0.312	0.278
	Ara h2 sIgE	0.340	0.234
	Ara h3 sIgE	0.177	0.545

sIgE level as measured in kUA/L

Table 5-10. Linear Regression of levels of peanut sIgE and ratio of Th2:Th1 cytokine production by resting PBMC from participants in the PPOIT group at 3 months post-treatment (T3)

Post-treatment (T3)			
PPOIT group (n = 28)		IL-13:IFN- γ ratio (resting PBMC)	
	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	-0.065	0.891	0.644
Ara h1 sIgE	-0.098	0.878	0.678
Ara h2 sIgE	0.022	0.966	0.392
Ara h3 sIgE	-0.088	0.883	0.995
IL-4:IFN- γ ratio (resting PBMC)			
	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	-0.635	0.287	0.388
Ara h1 sIgE	-0.766	0.340	0.436
Ara h2 sIgE	-0.415	0.526	0.653
Ara h3 sIgE	-0.943	0.201	0.289
IL-5:IFN- γ ratio (resting PBMC)			
	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE	0.464	0.436	0.172
Ara h1 sIgE	0.856	0.278	0.101
Ara h2 sIgE	0.752	0.234	0.046
Ara h3 sIgE	0.453	0.545	0.343

*Adjusted for age and sex; sIgE level as measured in kUA/L

Next, to address the hypothesis that in children with peanut allergy, higher peanut specific IgE is associated with reduced frequency of peanut specific T-regulatory (Treg) cell, the doctoral candidate examined the association between levels of peanut and peanut component sIgE and the frequency of peanut specific Treg cells (as a proportion of total CD4+ lymphocytes) in PBMC collected at study entry (pre-treatment, T0) and conducted pooled analyses of immune indices data from participants in the PPOIT and placebo groups prior to exposure to treatment in order to maximize analysis sample size. For the post-treatment time point (T3), subgroup analyses stratified by exposure groups were conducted.

5.5.6. Association between levels of peanut sIgE and frequency of peanut specific T-regulatory (Treg) cell in PBMC from participants in the PPOIT and placebo groups at study entry (pooled, pre-treatment)

Contrary to the doctoral candidate's hypothesis, the frequency of circulating peanut specific Treg cells was positively correlated with peanut and peanut component allergen sIgE levels in participants at study entry (Table 5-11). However, the associations weakened, and except for peanut sIgE, were no longer statistically significant after adjusting for age and sex. These findings suggest that pathogenic allergic antibody response may lead to upregulation of circulating antigen specific Treg cells rather than the alternate paradigm the doctoral candidate had hypothesized whereby reduced circulating antigen specific Treg cells leads to increased allergen-specific IgE production.

Table 5-11. Linear Regression of levels of peanut sIgE and peanut specific Treg cells from children with peanut allergy (pooled PPOIT and placebo groups) at study entry (T0)

Study entry (T0), pooled PPOIT
and Placebo groups (n = 56)

		Peanut specific Treg (CFSElo Treg, 6D CPE)		
CPE		Coefficient	Unadjusted P-value	Adjusted P-value*
	Peanut sIgE	0.039	0.026	0.050
	Ara h1 sIgE	0.048	0.049	0.084
	Ara h2 sIgE	0.035	0.046	0.090
	Ara h3 sIgE	0.043	0.057	0.075

*Adjusted for age and sex; sIgE level as measured in kUA/L

5.5.7. Association between levels of peanut sIgE and frequency of peanut specific T-regulatory (Treg) cell in PBMC from participants in the PPOIT and placebo groups, at 3 months post-treatment

At 3 months post-treatment, in both the placebo and PPOIT groups there was no significant association between levels of peanut sIgE and frequency of peanut specific Treg cells. Subgroup analyses by treatment groups involving smaller sample sizes may have contributed to the apparent loss of association between levels of peanut sIgE and peanut specific Treg cells (Table 5-12 and Table 5-13).

Table 5-12. Linear Regression of levels of peanut sIgE and peanut specific Treg cells from participants in the placebo group (n = 28) at 3 months post-treatment (T3)

Post-treatment (T3), Placebo group (n = 28)	Peanut specific Treg (CFSElo Treg, 6D CPE)		
	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE (kUA/L)	-0.014	0.492	0.136
Ara h1 sIgE (kUA/L)	0.051	0.577	0.491
Ara h2 sIgE (kUA/L)	-0.012	0.540	0.161
Ara h3 sIgE (kUA/L)	0.029	0.730	0.417

*Adjusted for age and sex; sIgE level as measured in kUA/L

Table 5-13. Linear Regression of levels of peanut sIgE and peanut specific Treg cells from participants in the PPOIT group (n = 28) at 3 months post-treatment (T3)

Post-treatment (T3), PPOIT group (n = 28)	Peanut specific Treg (CFSElo Treg, 6D CPE)		
	Coefficient	Unadjusted P-value	Adjusted P-value*
Peanut sIgE (kUA/L)	0.050	0.388	0.538
Ara h1 sIgE (kUA/L)	0.114	0.144	0.205
Ara h2 sIgE (kUA/L)	0.087	0.166	0.250
Ara h3 sIgE (kUA/L)	0.039	0.602	0.970

*Adjusted for age and sex; sIgE level as measured in kUA/L

5.6. Discussion

In this study the doctoral candidate examined the effect of PPOIT treatment on Th2, Th1 and Treg cytokine production by PBMC and the effect on Treg cell frequency in PBMC from children with peanut allergy. There were significant differences in pre-treatment to post-treatment fold change in Th-2 cytokine (IL-4) production by PBMC from participants in the PPOIT group as compared with those from the placebo group. PPOIT treatment was accompanied by a trend towards lower frequencies of peanut specific and resting Treg cells at 3 months post-treatment.

The doctoral candidate also explored the association between these immune measures and peanut sIgE levels at study entry and at 3 months post-treatment with PPOIT. At study entry, it was found that higher levels of peanut sIgE were associated with increased ratio of Th2:Th1 cytokine production by resting PBMC. At 3 months post-treatment, these associations were weaker in the placebo-treated group but no longer observed in the PPOIT treated group, which may be due to smaller sample sizes in the subgroup analyses. Contrary to expectation, the doctoral candidate found higher peanut sIgE levels being associated with a higher frequency of peanut specific Treg cells at study entry.

Th2 cytokines including IL-4, IL-5 and IL-13 support allergic antibody production and memory B cells survival[119 120]. Concurrently, excessive IL-4 signaling impairs induction of food allergen specific Treg cells and their capacity to suppress mast cell activation and reprogramming[69]. Effects of highly differentiated Th2 cells dominate over the suppressive effects of Treg cells in food allergic individuals[141]. Conversely, inactivation of Th2-like signaling pathways in Treg cells protects against food allergy[70]. Previous studies have reported mixed effects of OIT on Th2 responses, with some studies reporting downregulation of Th2 response (IL-5 and IL-4)[36 54 138] while others reported upregulation of IL-5[40]. However, given that PPOIT was associated with long-term reduction in B cell production of peanut specific antibody responses for both IgE and IgG4 isotypes, as reported in the previous chapter, the doctoral candidate hypothesised that PPOIT induces a reduction in Th2 cytokine production by PBMC or a shift

away from Th2 milieu. Findings in this chapter confirm that PPOIT treatment induced downregulation of Th2 cytokine production by PBMC.

There was no significant change in IL-10 and IFN- γ cytokine production with PPOIT treatment. The absence of any change in IL-10 production was unexpected as the doctoral candidate had hypothesised that tolerance induction would be associated with increased production of regulatory cytokines such as IL-10. A possible explanation of the unexpected negative finding with IL-10 may be that PPOIT is associated with early induction of Treg cell activity and IL-10 production while the underlying allergic response is being modulated. Yet an increased Treg cell activity may no longer be required once redirection of the allergic response is achieved. Alternatively, the findings may reflect deletion or anergy of peanut specific T cells as a key mediator of redirection of peanut specific allergic response. In addition, IL-10 production by circulating PBMC does not represent localized Treg cell activity within the gut associated lymphoid tissues (GALT) where peanut specific responses are modulated. Finally, the doctoral candidate has not measured TGF- β production. TGF- β is produced by antigen presenting cells such as CD103+ dendritic cells (DC) and by a subset of TGF- β producing T-cells (Th3). TGF- β facilitates induction of gastrointestinal tolerance through a number of mechanisms including promoting the generation of Foxp3+ Treg cells, downregulating Fc ϵ RI expression, inhibiting proliferation of B cells, upregulation of allergen specific IgA and suppressing production of allergen specific IgE[66 142]. A recent study[71] found that OIT is associated with transient effects on TGF- β regulatory pathways. A murine model of food allergen epicutaneous immunotherapy induced TGF- β production by Th3 cells and protected against anaphylaxis by suppressing mast cell activation[143]. The doctoral candidate's experiments do not exclude the presence of PPOIT induced upregulation of TGF- β production and its effect on the observed induction of salivary peanut specific-IgA production as reported in the previous chapter.

Functional Foxp3+ Treg cells have a potential role in regulating Th2 allergic responses[144]. In an animal model CD4+CD25+Treg cells inhibited the

differentiation of naïve T cells into Th2 cells and adoptive transfer of Treg protected sensitised mice from allergen challenge[145]. In addition, OIT-induced Treg cells have the functional capacity to suppress antigen specific T cell proliferation in vitro[40]. The doctoral candidate therefore hypothesised that PPOIT treatment would be accompanied by upregulation of Treg cells and that this would play a role in the downregulation of Th2 response and induction of clinical remission. Unexpectedly, the doctoral candidate did not observe this. In contrast, there was a trend towards lower Treg cell frequency post-treatment in the PPOIT group. Although this observation is counter-intuitive it is consistent with previously published findings from Varshney et al's peanut OIT study[55]. In that study, there was an initial rise in peanut specific Treg cells frequency at 6 and 12 months after commencement of the study followed by decline to baseline by 20 months after commencement. Therefore, it is possible that by only assessing Treg cell frequency at study entry and at 3 months post-treatment earlier changes in Treg cell frequency and/or their function may have been missed.

An intriguing possibility is that once the underlying allergic response is redirected towards sustained unresponsiveness and/or longer lasting tolerance, the role of Treg cells diminishes. In the present study, higher levels of peanut and peanut component sIgE, supported by a cytokine milieu favouring Th2 over Th1, were associated with higher circulating numbers of peanut specific Treg cells in children with peanut allergy. This suggests when these children are naïve to food immunotherapy, Treg cells may play a role to dampen the existing skewed cytokine and pathogenic peanut specific allergic responses; that is, Treg cells are induced in response to established allergy and play a role in limiting allergic responses to peanut. A mouse model of food allergy reported that sensitised mice had higher frequency of circulating Treg cells and increased IL-10 response to culprit antigen[146] which supports this notion that Treg cells are increased in response to allergy rather than decreased. Whilst Treg cells have been consistently demonstrated to play a role in the initiation and establishment of de novo tolerance in healthy subjects, the role of Treg cells in redirection of an established or existing allergic response remains uncertain. It is plausible that reprogramming

of a pre-existing peanut specific allergic response is instead achieved by a combination of memory T cell deletion and induction of T cell unresponsiveness, as findings from another recent study also suggest[147].

Taken together, the above findings provide supportive evidence that PPOIT treatment induced downregulation of the peanut specific IgE response may be mediated by a reduction in Th2 cytokine production without accompanying changes in Th1 or IL-10 cytokine production. Higher peanut sIgE is associated with lower frequency of peanut specific Treg cells in children with peanut allergy and who are treatment naive. The absence of upregulation of circulating Treg cells suggests that continuing upregulation of Treg cell frequency and IL-10 production may not be required for maintenance of PPOIT-treatment induced remission once this is achieved.

This study has a number of strengths.

Firstly, the high rate of SU achieved with PPOIT treatment both immediately after treatment cessation and 4 years post-treatment enabled examination of the immune effects associated with SU as against desensitisation. In contrast, peanut alone OIT studies (in the absence of co-administered probiotic) induce desensitisation while SU is only achieved in a small proportion of treated subjects[19 40 54].

Secondly, this is one of the largest studies to date investigating cytokine production by PBMC and Treg cells frequency following food oral immunotherapy. Participant retention rate was high with 90.3% (56/62) evaluated at end of treatment. The sample size and high retention rate translated to large numbers of participants in whom immune measures were available at each of the studied time points.

The doctoral candidate acknowledges the following limitations.

Firstly, due to resource constraints, the doctoral candidate was only able to examine cytokine production and Treg responses at two timepoints, being at study

entry (T0) and at 3 months post-treatment (T3). The limited time points evaluated meant that the doctoral candidate may have missed a transient rise followed by fall of Th1 and Treg cytokines as well as circulating antigen specific Treg cell frequency in response to PPOIT. In addition, given that allergen sIgE responses continued to reduce further post-treatment, by only evaluating cytokine production by PBMC collected at 3 months post-treatment, the doctoral candidate may have missed further downregulation of Th2 responses over time.

Secondly, the doctoral candidate did not use tetramer technology[148] to identify peanut specific Treg cells and instead assumed that Treg cells which had proliferated after co-culturing with CPE were indeed representative of antigen specific Treg cells. This assumption has generally been accepted in other publications[18 19 40]. The doctoral candidate has not performed suppression assays to confirm functional ability of the Treg cells identified in the present study. Nevertheless, Syed et al adopted the same FACS gating strategies when enumerating Treg cells and confirmed that Treg cells identified using these phenotypic markers were functionally suppressive in vitro, had higher Foxp3 protein and had Foxp3 transcription sites that were hypomethylated[40].

The doctoral candidate has not evaluated tissue level peanut specific cytokine production or enumerated Treg cells localized in the gut, in particular in the GALT, where the immune response to peanut is considered to be primarily regulated. The interaction between antigens, antigen presenting cells, tissue-resident Treg cells and effector cells at the tissue level may be different to what is seen in the peripheral circulation. However, obtaining tissue samples such as gastrointestinal mucosa and mesenteric or splenic lymph nodes from children participating in food immunotherapy trials is not feasible either ethically or practically. To date, researchers in this field have extrapolated findings from animal models to gain insight into tissue level immune responses to OIT.

Another limitation is that the candidate did not adjust for multiple comparisons because the candidate was investigating a number of different hypotheses. It is recognised that adjusting p-values can sometimes be inappropriate and can

increases the likelihood of type 2 statistical errors[97 98]. Therefore the candidate has simply described the tests of significance performed and why to enable readers to reach reasonable conclusions without the help of adjustments.

In summary, findings in this chapter provide supportive evidence that the PPOIT induced long-term downregulation of circulating peanut and peanut component sIgE, as reported in the previous chapter, may be mediated by reduction in Th2 cytokine production. In contrast, increased circulating Treg cell number and IL-10 production in PBMC was not observed. This raises the possibility that other cellular mechanisms may be relevant to PPOIT induced long-term SU including memory cell deletion and/or anergy and/or that changes in Treg cell activity are limited to the localised gut environment. Furthermore, this chapter's findings that peanut sIgE levels at study entry are associated with an increased frequency of peanut specific Treg cells and possible contraction of this population post-treatment, suggest that peanut specific Treg cells may play a role in controlling established peanut allergy responses in treatment naïve subjects. However, these cells may be less critical in maintaining long-lasting redirection of the immune response away from allergy towards tolerance. Larger longitudinal studies evaluating cellular responses at multiple time points both during and post food immunotherapy are required to confirm these findings.

5.7. Chapter summary

This chapter concludes the 3-chapter series reporting on the long-term clinical and immunologic effects of PPOIT on participants of the PPOIT-001 trial, a double-blind, placebo-controlled randomised trial. PPOIT treatment was associated with long-term clinical benefits including an ability to continue peanut ingestion and long-term sustained unresponsiveness (LTSU) at 4 years post-treatment. Findings from mechanistic studies suggest that the clinical response induced by PPOIT treatment may be mediated by broad reduction in peanut specific humoral immune responses through the redirection of cytokine responses.

In Part 2 of this thesis, the doctoral candidate describes the rationale, aims, methods and findings of the PPOIT-002 trial, a single-arm, open-label trial of a combined probiotic and peanut oral immunotherapy (PPOIT) using an modified (accelerated) dose escalation schedule for the buildup phase designed to improve the feasibility of PPOIT treatment.

Chapter 6. Methods: The PPOIT-002 trial, a single-arm, open-label trial of a combined probiotic and peanut oral immunotherapy (PPOIT) using an accelerated dose escalation schedule

6.1. Chapter introduction

As presented in the earlier chapters, a combined probiotic and peanut oral immunotherapy (PPOIT) using an 8-month dose-escalation schedule for the buildup phase was effective at inducing desensitisation, sustained unresponsiveness (SU), long-term sustained unresponsiveness (LTSU) and at modulating peanut specific immune responses.

In Part 2 of this thesis, the doctoral candidate explores feasibility of modifying PPOIT treatment by accelerating the dose escalation schedule of the buildup phase. Chapter 6 describes the rationale, aims and methods for conducting Part 2 of the thesis, the PPOIT-002 trial investigating PPOIT treatment which uses an accelerated dose escalation schedule.

6.2. Rationale

Food allergy affects 5-8% of children and 2% of adults, and peanut allergy affects 1.5% of children[84-86]. Reactions to peanut may be severe and peanut allergy is responsible for 30% of deaths from food induced anaphylaxis [87]. Unlike other food allergies, peanut allergy is usually lifelong[88]. Current management of food allergy requires strict avoidance of the food; however, 50% of peanut allergic patients are accidentally exposed to peanut within 2 years[89]. Therefore, children with peanut allergy are at continued risk of severe life-threatening allergic reactions throughout their lifetime.

A combined probiotic and peanut OIT (PPOIT) was shown to successfully induce desensitisation and sustained unresponsiveness (SU) in a double blind placebo controlled RCT[95]. Just over 80% of the participants who received PPOIT attained 2-6 week SU in the immediate post-treatment period compared with only 4% of those who received placebo. The trial provided a proof of principle for the effectiveness of combining probiotic and oral immunotherapy approach for the treatment of peanut allergy.

In the original PPOIT treatment schedule, doses of peanut protein were increased progressively over a period of 8 months (16 dose increases every 2 weeks). In order to improve feasibility of the PPOIT treatment in the clinical setting, a more rapid buildup schedule that is in line with other forms of immunotherapy (for example pollen immunotherapy with dose increases over 12 weeks) would be desirable. It is therefore important to evaluate whether an accelerated dose escalation schedule can be tolerated by children with peanut allergy. PPOIT-002 trial sought to evaluate a PPOIT treatment schedule where doses of peanut protein are increased over a period of 4 months rather than 8 months.

6.3. Aims

The primary aim of this trial was:

To evaluate the tolerability of PPOIT with an accelerated dose-escalation (buildup) phase, specifically:

- a) the proportion of children who can complete the buildup phase within 16 weeks, and
- b) the number of treatment emergent adverse events (AE) and serious adverse events (SAE).

The secondary aims of this trial were:

- a) To determine the rate of desensitisation, as determined by performing a 4950 mg peanut protein double blind placebo-controlled food challenge (DBPCFC) at end of treatment (T2), after completion of 18 months of PPOIT using a shortened buildup schedule;
- b) To determine the rate of sustained unresponsiveness (SU), as determined by performing a 4950 mg peanut protein DBPCFC at 8 weeks after treatment is completed (T3) with strict avoidance of peanut intake between T2 and T3, after completion of 18 months of PPOIT using a shortened buildup schedule;
- c) To determine the change in peanut skin prick test (SPT) size, from T0 to T2 and T3, after completion of 18 months of PPOIT using a shortened buildup schedule;
- d) To determine the change in blood peanut sIgE and sIgG4, from T0 to T2 and T3, after completion of 18 months of PPOIT using a shortened buildup schedule.

6.4. Methods

This section explains the methods used to conduct the PPOIT-002 trial.

6.4.1. Design

This was an open-label, single arm pilot trial of a combined probiotic and peanut OIT (PPOIT) for 18 months in 20 children with peanut allergy, using a shortened up dosing schedule for the buildup phase.

6.4.2. Participants

6.4.2.1. Inclusion Criteria

Children were eligible for the trial if they:

- o Are aged between 1 year and 12 years of age,
- o If they are >10kg (weight considered safe for the prescription and administration of an EpiPen)
- o There was a confirmed diagnosis of peanut allergy as defined by a failed double blind placebo controlled food challenge (DBPCFC) to peanut (up to a cumulative dose of 4950 mg peanut protein) and a positive SPT or sIgE to peanut at screening or in the preceding 3 months.

Provided that a participant met eligibility criteria and was not excluded on the basis of meeting an exclusion criterion, children with concomitant atopic disease including eczema were included in the trial.

6.4.2.2. Exclusion Criteria

Children were not eligible for the trial if they had a history of:

- o Severe anaphylaxis (as defined by persistent hypotension, collapse, loss of consciousness or persistent hypoxia OR ever needing three (3) or more doses of intramuscular adrenaline or needing intravenous adrenaline infusion for management of anaphylactic reaction)

- o FEV1 <85% at rest or FEV1/FVC $\leq$ 85% at rest or ongoing chronic persistent asthma (as per Australian Asthma Foundation guidelines)
- o Underlying medical conditions (eg cardiac disease) that increase the risks associated with anaphylaxis
- o Use of beta-blockers
- o Inflammatory intestinal conditions, indwelling catheters, gastrostomies, immune-compromised states, post-cardiac and/or gastrointestinal tract surgery, critically-ill and those requiring prolonged hospitalisation or other conditions that may increase the risks of probiotic associated sepsis
- o Already taking Probiotics

6.4.2.3. Withdrawal from trial

Participants were able to withdraw from the trial at any time by their own volition.

The following reasons for withdrawal are provided as examples:

1. Lack of efficacy of study treatment as determined by investigators
2. Lack of efficacy plus adverse event as determined by investigators
3. Serious adverse event to study treatment* as recorded by investigators
4. Severe adverse events to study treatment* as recorded by investigators
5. Lack of patient compliance as determined by investigators
6. Patient/Caregivers decision to withdraw
7. Patient lost to follow-up
8. Protocol Violation
9. Concurrent disease

10. Other reason

*Allocation of withdrawal reason as being related to adverse event(s)

If the participant has more than four severe (Grade 3 or greater) adverse events that are probably related to study treatment during the buildup phase, OIT treatment was discontinued.

The reason for termination was recorded on the participant's withdrawal case report form (CRF).

Every effort was made to follow-up participants who withdrew from the trial with treatment emergent adverse events (AE) in order to determine the final outcome.

6.4.3. Study visits

Study visits and assessments were scheduled as follows:

- Screening visit – Double blind placebo controlled food challenge (DBPCFC)

Eligibility for the trial was determined by screening assessments, medical history, provision of written informed consent and fulfilment of eligibility criteria. Eligibility criteria included confirmation of peanut allergy by failed DBPCFC at trial entry. Participants who had received placebo in the PPOIT-001 trial, had undergone the PPOIT-001 trial 4-year post-treatment DBPCFC[99] (cumulative dose 4000 mg peanut protein) within a 3 month period prior to Screening for PPOIT-002 and were still peanut allergic (failed PPOIT-001 trial 4-year post-treatment DBPCFC) were eligible for this trial. For these participants, data from the 4-year post-treatment DBPCFC was used as the PPOIT-002 trial screening result i.e. the DBPCFC was not repeated. For all other PPOIT-002 trial participants, a DBPCFC (cumulative dose 4950 mg peanut protein) was performed at screening visit to confirm peanut allergy.

- T0/rush day visit;

- T1 – start of maintenance phase (buildup visit dose #17 - 2 g peanut protein) visit;
- T2 – 18 months after T0/rush day (the day after the last day of treatment) visit – DBPCFC;
- T3 – 8 weeks after T2 visit – DBPCFC (with strict peanut elimination between T2 and T3);

At the conclusion of the T3 visit, participants were advised to avoid or consume peanut intake according to their allergic status as assessed at T3 DBPCFC:

- Participants who failed the T2 challenge (i.e. failed induction of desensitisation) were informed of their status and advised to continue to strictly avoid all peanut in the diet;
- Participants who passed the T2 challenge and failed the T3 challenge (i.e. desensitised but failed to achieve sustained unresponsiveness) were informed of their status and advised to continue to strictly avoid all peanut in the diet;
- Participants who passed the T3 challenge (i.e. achieved sustained unresponsiveness) were advised to incorporate peanut into their diet at libitum.

A schedule of assessments is included in the Supplementary material section of this chapter.

6.4.4. Interventions

6.4.4.1. Study treatment administered

Peanut Flour

The Peanut Flour (50% peanut protein) used in this trial was purchased from Byrd Mill, Ashland, Virginia, USA and manufactured by Golden Peanut and Tree Nut Company (GPC), Alpharetta, Georgia, USA. Peanut flour is preferred to crushed peanuts as it is easier to disguise. This was the same product as was used in the previous PPOIT RCT[95]. Peanut OIT doses were prepared by Optima Ovest (Perth,

Australia) under Good Manufacturing Practice (GMP) conditions and provided as set doses within capsules (for rush doses 1-8 and buildup doses 9-12), sachets (for buildup doses 13-16) and tubs of peanut flour with standardised measuring scoops (for maintenance dosing).

The typical analysis of the peanut powder was as follows:

Protein (N x 5.46)	50%+/- 2%
Fat	12% +/-2%
Moisture	<3.5%
Crude Fibre	10% +/-2%
Ash	4%+/- 2%
Carbohydrate	30%+/-2%
Density	21-23 lbs/cubic feet

For the rush and lower doses of buildup phase, peanut OIT was formulated in gelatin capsules (size 3, 00EL and 000) encapsulated with maltodextrin to achieve the desired strength. Strengths supplied were 0.1, 0.2, 0.4, 0.8, 1.5, 3, 6 and 12 mg (rush phase; batch numbers: P101215, P050116, P034, P035, P036, P037, P038, P039, P040, P041) and 25, 50, 100 and 200 mg (buildup phase; batch numbers: P042, P043, P044, P045, P072, P073, P074). The higher buildup phase doses, 400 mg, 800 mg, 1200 mg and 1600 mg were supplied as sachets (batch numbers P046, P047, P048, P049, P131, P132, P133). Product for use in the maintenance phase was supplied as 190 g of peanut flour packaged in a HDPE tub supplied with a standardized scoop to provide 2 g per day (batch numbers: P050, P134, P158, P214, P266, P331).

Probiotic (*Lactobacillus rhamnosus* GG; ATCC 53103)

The probiotic to be used was *Lactobacillus rhamnosus* GG ATCC 53103 (LGG®) which was manufactured under GMP as a freeze-dried powder by Health World Ltd. Supply was as dry powder in prepackaged 250ml bottles. The daily dose of 2×10^{10} cfu was measured using a standardised measuring scoop. Participants were instructed to mix the probiotic in water at a temperature NOT exceeding 38 °C (also stated on the product label). The probiotic was to be stored refrigerated (2-8°C).

6.4.4.2. Day 1 rush phase

Treatment commenced with a day of rush dosing, during which participants received increasing doses of peanut OIT every 20 minutes to reach a final dose of 12 mg of peanut protein (24 mg cumulative) as per Table 6-1. Peanut protein was sprinkled onto food (yoghurt not containing *Lactobacillus rhamnosus*). Participants also received a single dose of 2×10^{10} cfu probiotic (1 level scoop dissolved into water) prior to initiation of the peanut OIT doses during the rush phase.

Table 6-1. Dosing schedule for day 1 rush induction

Dose	Dosage Peanut Protein	Cumulative Peanut Protein
1	0.1 mg	0.1 mg
2	0.2 mg	0.3 mg
3	0.4 mg	0.7 mg
4	0.8 mg	1.5 mg
5	1.5 mg	3.0 mg

6	3.0 mg	6.0 mg
7	6.0 mg	12 mg
8	12 mg	24 mg

6.4.4.3. Buildup phase

Following the rush day, participants completed a buildup phase (target duration 16 weeks) during which the daily dose of peanut protein was increased every 2 weeks until a maintenance dose of 2 g was reached as per Table 6-2. Participants also took a fixed dose of 2×10^{10} cfu probiotic or placebo (once daily) prior to the OIT treatment.

Table 6-2. Dosing schedule for Buildup Phase (16 weeks) in the PPOIT-002 trial

Dose	Dosage Peanut Protein
9	25 mg
10	50 mg
11	100 mg
12	200 mg

13	400 mg
14	800 mg
15	1200 mg
16	1600 mg
17	2000 mg

6.4.4.4. Maintenance phase

During the maintenance phase participants took a 2 g dose of OIT (peanut protein) and 2×10^{10} cfu probiotic daily at home until completion of the OIT protocol at 18 months.

6.4.4.5. Dose adjustment rules

During the buildup phase, visits to RCH for updosing (updose visits) were scheduled every 2 weeks. The decision to proceed with a dose increase will be based on the dose adjustment rules described below. If a dose increase is deferred, the next dose increase will be at the next scheduled updose visit in 2 weeks.

The following dose-adjustment rules were applied to reactions that occurred during the rush and buildup visit days:

- If mild allergic symptoms without respiratory or cardiovascular involvement developed following a dose of OIT (urticaria, angioedema, vomiting, diarrhoea, abdominal pain), the dose was continued without adjustment. Symptoms could be treated with over the counter medicines (e.g. antihistamine, ibuprofen, paracetamol). The next scheduled dose increase proceeded uninterrupted provided the participant had been symptom free for at least 3 days prior to the scheduled updose day. The participant could be continued on antihistamine for prophylaxis against symptoms in the days leading up to the

updose day if the site investigator deemed this to be warranted. If allergic symptoms (following a dose of OIT) developed in the 3 days prior to a scheduled updose day, the dose increase didn't proceed, and the current dose was continued for a further 2 weeks (until the next scheduled updose visit).

- If moderately severe allergic symptoms developed following a dose of OIT without cardiovascular involvement (stridor, wheeze, difficulty breathing), the immediate next dose was reduced to the previous dose amount and continued for at least 10 days before proceeding with a dose increase. For example, if a moderately severe allergic reaction to OIT occurred within the first 4 days of a dose increase, the daily dose was reduced to the preceding dose amount for the remaining 10 or more days until the next scheduled updose visit whereupon the scheduled dose increase proceeded. However, if a moderately severe allergic reaction to OIT occurred 5 or more days after a dose increase, the daily dose was reduced to the preceding dose amount for the remaining 9 or fewer days until the next scheduled updose visit whereupon the reduced daily dose was repeated for a further 2 weeks (no dose increase).

- If serious allergic symptoms of reduced blood pressure (BP) or loss of consciousness developed or if a participant had more than 4 severe adverse events (AE) that were 'probably related' study treatment during the buildup phase, OIT was discontinued.

The following rules were applied for missed doses during buildup and maintenance phases:

- o If 1-4 days of OIT were missed, the participant continued with the usual dose at home. If this occurred during the buildup phase, the next scheduled updose was allowed to proceed as usual.

- o If 5-14 days of OIT were missed, OIT was recommenced at the participant's last tolerated dose and was administered at RCH under medical supervision. If this occurred during the buildup phase, the daily dose must have been taken for a minimum of 2 weeks before the next updose was allowed to proceed.

- o If 15-28 days of OIT were missed, OIT was recommenced at a reduced dose (the dose below the last tolerated dose) and was administered at RCH under medical supervision. If this occurred during either the up dosing or maintenance phase, the reduced daily dose must have been taken for a minimum of 2 weeks before the next up dose was allowed to proceed (that is, up dosing schedule resumed or the dose increased back to the 2g maintenance dose, respectively).
- o If >28 days of dosing were missed, participants had to recommence OIT at Day 1.

6.4.4.6. Oral immunotherapy treatment stopping rules

- o If severe allergic symptoms of reduced BP or loss of consciousness developed or if a participant required three or more doses of intramuscular adrenaline or required an intravenous adrenaline infusion for management of an anaphylactic reaction that was probably related to study treatment, OIT was discontinued.
- o If the participant had more than four severe adverse events that were probably related to study treatment during the buildup phase, OIT was discontinued

Participants were able to withdraw from the trial at any time by their own volition with no impact on any future care they required in the Hospital or Allergy Department.

6.4.4.7. Collection of study treatment from Pharmacy

Upon successful completion of the Day 1 rush phase, participants collected a 2 week supply of peanut protein from Pharmacy. 20 doses were provided for each 2 week period, so that they were able to continue with their OIT in the event that they missed their appointment with RCH or a dose was accidentally spilt. Unused doses had to be returned at their next visit.

The dose to be administered for each 2 week period was determined by trial staff performing the up dosing procedure and was based upon the established criteria as outlined above. When participants reached the 2 g maintenance dose, they collected peanut protein sufficient to complete three months of OIT maintenance from RCH Pharmacy.

Participants had to return unused doses and used treatment packaging to Pharmacy at each visit so that they may audit compliance.

During buildup phase, clinical review with trial staff were performed at each hospital visit (2 weekly) to monitor progress and compliance, and to address any issues that arose. At final buildup visit, a pharmacist showed parent/guardian how to measure 2 g dose of peanut dose accurately. In two weeks from this visit parent/guardian demonstrated to a pharmacist how they had been measuring doses to ensure correct dosing of participant.

During the maintenance phase, review of diary, compliance and progress occurred every 12 weeks. Clinical review; vital signs occurred every 3 months during the maintenance phase or more frequently when clinically indicated or requested by the parent/guardian or Principal Investigator.

Probiotic were provided as a freeze dried powder (Health World). Probiotic were taken daily at a dose of 2×10^{10} cfu for the 18 month period of OIT mixed in water, soy milk or cow's milk, at a temperature NOT exceeding 38 °C. Pharmacy dispensed a three month supply of probiotic each 3 months, which contained sufficient probiotic in case of spillage or delayed study visit.

6.4.4.8. Medications/treatments permitted and not permitted before and during trial

o Participants using beta blockers were excluded from the trial as this treatment can impair responsiveness to adrenaline in the event of severe allergic reactions.

- o Participants were permitted to use any other medications or treatments they were prescribed.
- o Participants had to continue to strictly avoid all foods containing peanut.
- o Participants had to continue to strictly avoid probiotic supplements and foods containing LGG probiotic, for example some yoghurt preparations

6.4.5. Outcomes

6.4.5.1. Evaluation of tolerability

Evaluation of time taken to complete the buildup phase

The number of days taken to complete the buildup phase was determined from the day the study treatment was first taken (that is, on the rush day) until and inclusive of the day immediately before the participant took the first dose of maintenance dose. The proportion of children who completed the buildup phase within a 16-week period was calculated as a proportion of all enrolled participants who took at least one dose of study treatment.

Detection of treatment emergent adverse events (AE)

Treatment emergent Adverse Events (AE) were monitored and recorded as any untoward medical occurrence in a participant which did not necessarily have a relationship with the study treatment. At each visit/assessment, all untoward AE either observed by the investigator or one of the clinical staff, or recorded in the participant's symptoms diary, or reported by the participant/caregivers spontaneously or in response to a direct question were evaluated and recorded by the investigator. Details including nature of each event, time of onset and resolution following study treatment administration, symptoms associated with the event, treatment required for the event were recorded in an electronic Adverse Event (CRF) in REDCap.

The CRF template is included in the Supplementary material section of this chapter.

Assignment and classification/reclassification of AE

In order to align practices for assignment and classification/reclassification of AE reported in PPOIT trials including PPOIT-002 and PPOIT-001[95], the following standardized procedures were followed:

1. All AEs were downloaded from REDCap.
2. Two qualified allergy specialists independently assessed each reported AE and assigned category of severity and attributed a relationship to study treatment. Severity grading for allergic reactions is summarised in Table 6-3. This was adapted from the National Health Institute (NIH) National Institute of Allergy and Infectious Diseases (NIAID) Consortium of Food Allergy Research (CoFAR) specific grading system for allergic reactions.

Table 6-3. Severity Grading for treatment emergent adverse events (AE)

	Adverse Event			Serious Adverse Event	
	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 (Life- Threatening)	Grade 5 (Death)
Impact					
Duration	Transient or mild discomforts (<48 hours).	Mild symptoms that persist > 48 hours			Death
Activity Limitation	None	Mild-Moderate	Marked	Extreme	
Symptoms					
Pruritus	x	x	x	x	
Rash	x	x	x	x	
Swelling	x	x	x	x	
Abdominal Discomfort	x	x	x	x	
Vomiting		x	x	x	
Wheezing		x	x	x	
Hoarseness			x	x	
Throat Tightness			x	x	
Dyspnea			x	x	
Hypoxia				x	

	Adverse Event			Serious Adverse Event	
	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 (Life- Threatening)	Grade 5 (Death)
Hypotension			x (transient)	x (persistent)	
Consciousness				x (decreased)	
Interventions					
Intervention	No/Minimal	No/Minimal	Parenteral required	Parenteral required	
Hospitalisation		Possible	Possible	Probable	

Note: asthma requiring change in preventer therapy or oral steroids was considered moderate severity; asthma requiring repeated cycles of Ventolin was considered moderate severity; eczema requiring antibiotics or oral steroids was considered moderate severity.

3. Non-allergic reactions were graded using the following definitions for severity:

Mild Symptom was easily tolerated by the subject, caused minimal discomfort and did not interfere with everyday activities.

Moderate: Symptom caused sufficient discomfort to interfere with normal everyday activities and may have required over the counter medications.

Severe: Symptom caused significant discomfort preventing everyday activities and may have required medical intervention.

4. Causality of allergic reactions was determined using the following definitions:

Probably Related:	Objective signs of an IgE mediated allergic reaction (hives, angioedema, vomiting, anaphylaxis) occurring within 1 hour of the study treatment in the absence of other known allergic triggers for that subject. Symptoms could not be reasonably explained by a subject's clinical state, environmental or toxic factors or other treatments taken by the subject.
Possibly Related:	Objective signs of an IgE mediated allergic reaction (hives, angioedema, vomiting, anaphylaxis) occurring more than 1

	hour but less than 4 hours after taking the study treatment. Symptoms could not be reasonably explained by a subject's clinical state, environmental or toxic factors or other treatments taken by the subject.
Unlikely Related:	Allergic symptoms occurring more than 4 hours after taking the study treatment, or Symptoms could be reasonably explained by a subject's clinical state, environmental or toxic factor or other treatments taken by the subject e.g. exacerbation of asthma or eczema, or Symptoms did not follow a known pattern of response for the study treatment.
Unrelated:	Any event where an alternate cause is identified e.g. allergic reaction following exposure to another food allergen in a subject with known allergy to that allergen, or The AE does not meet the criteria for the other categories above.

5. AEs where the independent assessors disagreed on either severity, attribution, or both were highlighted, and the assessors met in person to discuss reasons for their original decision and resolve disagreements.

6. Remaining disagreements were presented to a third independent assessor (Principal Investigator Tang) and the following decisions were made to resolve all remaining disagreements:

- If there was discrepancy between an AE report as documented by trial doctor whilst on call versus the participant's diary entry (either missing entry or missing diary), it was assumed that the trial doctor's file entry or CRF entry was accurate. The data would be included for analysis (that is, not excluded).
- If symptoms reported by participants in the context of having an intercurrent illness (e.g. abdominal pain during gastroenteritis, abdominal pain/sneezing during URTI) could be explained by the intercurrent illness, then the symptoms should first and foremost be considered to be attributed to the intercurrent illness and therefore unrelated to the study treatment.

- Abdominal pain with change in demeanour but no documented change in level of activity and without vomiting or diarrhoea would be classified as mild.

7. All AEs determined to be probably related or possibly related were included in the analysis dataset.

6.4.5.2. Evaluation of efficacy

For trial outcome measures, an oral DBPCFC with cumulative dose of 4950 mg peanut protein was performed at T2 (on the day immediately after the last day of OIT) to assess for desensitisation and at T3 (8 weeks after discontinuation of PPOIT; with a strict peanut elimination diet maintained between T2 and T3) to assess for sustained unresponsiveness (SU).

Each DBPCFC was performed in two parts, Part A and Part B, performed on 2 separate days. One part was the peanut protein (flour) and the other was a placebo (maltodextrin), each mixed in with an appropriate vehicle food. Both the trial team and participants were blinded to the contents of each part of the food challenge. The challenge was stopped if objective symptoms developed during the challenge procedure. The contents of Parts A and B of the DBPCFC were only unblinded if the participant tolerated one part and reacted to the other part of the DBPCFC; the components were not unblinded if the participants tolerated both parts of the DBPCFC or reacted to both parts of the DBPCFC. The DBPCFC was considered to be 'failed' if the participant reacted to the peanut component and not the placebo component of the challenge, 'passed' if the participant completed both parts of the DBPCFC without objective symptoms, and 'inconclusive' if the participant reacted to both parts of the DBPCFC or to the placebo component of the DBPCFC.

The food challenge dosing schedule is included in the Supplementary material section of this chapter.

Participants who failed the DBPCFC at T2 were classified as allergic; those who passed the T2 DBPCFC underwent a second DBPCFC performed 8 weeks after treatment was completed (T3), during which time they strictly avoided peanut

intake between T2 and T3, to assess for SU. Those who passed the T2 DBPCFC were classified as desensitised. Participants who passed both the T2 and T3 DBPCFCs were classified as having attained SU.

Safety assessments included measurement of vital signs and participant weight, physical examination and review of participant diary. Participants were questioned regarding any changes experienced in their health and administration of any concomitant medication throughout their participation in the trial.

6.4.5.3. Evaluation of Health-Related Quality of Life

Health related quality of life assessments were conducted at screening (T0), T2 and T3 by means of a validated Food Allergy Quality of Life Questionnaire -Parent Form (FAQLQ-PF) which was completed by the parent/caregiver of the participant. Health-Related Quality of Life data was not analysed and will not be reported as part this thesis.

The food allergy quality of life questionnaire-parent form (FAQLQ-PF) is a disease-specific health related quality of life (HRQL) questionnaire with excellent validity and reliability. This psychometric tool was developed to assess quality of life in children with food allergies (aged 0-12 years) and allows parents to report children's HRQL from the child's perspective. FAQLQ-PF is presented in a single form for all age groups (0-3; 4-6; 7-12 years) and is considered "user-friendly". It contains 30 items, and the response scale ranges from 0 (minimal impairment in HRQL) to 6 (maximal impairment in HRQL). The measure has 3 subscales assessing 3 factors found to be essential for assessing the global impact of food allergy on HRQL: general emotional impact, food anxiety and social and dietary limitations. The total score is calculated as the mean of items.

The food allergy independent measure (FAIM) is a food allergy specific measure based on 6 questions included within the FAQLQ-PF, with a response scale from 0 (extremely unlikely) to 6 (extremely likely). The questions assess the parent's perception of the chance of an adverse outcome for the child with a food allergy.

The questions relate to:

1. The chance of accidental exposure;
2. The chance of a severe reaction on food exposure;
3. The chance of dying from food exposure;
4. The chance of a child effectively treating him/herself or receiving effective treatment, following a food allergic reaction;
5. How many foods were avoided because of food allergy (categorized as single, 2, multiple >2);
6. How much food allergy limits the type of activities that the child can take part in.

6.4.5.4. Immunological assessments

The following immunological assessments, evaluating effects of combined probiotic and peanut OIT (PPOIT) on systemic immune responses, were conducted as part of PPOIT-002 study. The doctoral candidate focused on peanut and peanut component specific-IgE (sIgE) and specific-IgG4 (sIgG4) as part of this thesis.

- Serum and plasma samples were stored at -80°C for batch measurement of peanut and peanut component (Ara h1, h2, h3, h8 and h9) sIgE and sIgG4;
- Peripheral blood mononuclear cells were isolated and cryopreserved in liquid nitrogen for future immune studies. These samples were not used to generate data for this thesis.
- Stool samples were collected at T0, T1, T2 and T3. These samples were not used to generate data for this thesis.

6.4.6. Statistical methods

No formal statistical analysis plan was generated prior to commencement of the trial.

6.4.6.1. Sample size calculation

No formal calculations were made for sample size for this pilot trial. The trial aimed to recruit 20 children with peanut allergy.

6.4.6.2. Analysis populations

- Safety Analysis Set: The safety analysis set included all participants who were administered at least 1 dose of study treatment, including those who may have withdrawn prior to trial completion.

Safety data was presented for all participants in the safety analysis set and summarised by the treatment phase at onset (i.e. rush phase, buildup phase, maintenance phase).

- Efficacy Analysis Set: Efficacy data is presented for all participants who were administered study treatment, including those who may have withdrawn prior to trial completion.
- The full analysis set (FAS) for efficacy included all participants who received at least 1 dose of study treatment and who had at least one efficacy assessment for the T2 and T3 trial endpoints.

6.4.6.3. Analysis of efficacy data

Efficacy assessments were summarised with descriptive statistics.

Statistical comparisons for sIgE and sIgG4 parameters were performed between timepoints using an unmatched Mann-whitney ranksum test.

For SPT wheal size parameters, statistical comparisons between timepoints were performed using an unpaired t test.

Desensitisation to Peanut:

The number and percentage of participants who achieved desensitisation to peanut were presented for all participants. Desensitisation was defined as the ability to tolerate 4950 mg of peanut protein (cumulative dose) during the end of treatment DBPCFC following 18 months of treatment (T2). A participant was considered to have desensitisation to peanut if they passed the DBPCFC at the T2 visit.

Sustained Unresponsiveness (SU) following 18 months of treatment:

The number and percentage of participants who achieved SU at the T3 DBPCFC was presented for all participants. SU was defined as the ability to tolerate 4950 mg of peanut protein (cumulative dose) during the DBPCFC conducted 8 weeks after treatment cessation (T3). A participant was considered to have an SU to peanut if they passed the DBPCFC at the T3 visit.

6.4.7. Safety features of the trial

6.4.7.1. Prior to the dose-escalation study visit the trial nurse instructed the participant as follows:

- Participant should not do strenuous exercise for 4 hours post a dose of study treatment
- Participant should have a big meal with carbohydrates prior to the dose of study treatment.
- If the participant is unwell or having febrile illness / respiratory illness (for example uncontrolled asthma) /gastroenteritis then the dose could be withheld.
- Asthma and allergic rhinitis must be optimally controlled throughout the trial.

6.4.7.2. Monitoring of safety

Consideration was given to the safety of the participants with appropriate monitoring. All participants were monitored by a nurse (under supervision of a trial doctor) for 2 hours after a dose increase for signs of allergic reactions following OIT administered at the MCRI/RCH. All patients were contacted by phone the day following updosing of their immunotherapy. All participants were provided with an Anaphylaxis Action Plan and an EpiPen/EpiPen Jr and educated in the management of allergic reactions (standard care for peanut allergy). If a reaction occurred when the child was at home, they were to follow the Anaphylaxis Action Plan, and notify the On-Call Trial Personnel. Dose adjustment rules were developed to ensure that dose escalation only occurred if safe to proceed. Trial stopping rules were in place to ensure that participants did not continue treatment when there were safety concerns.

Frequent safety and efficacy assessments were planned during the trial period to capture relevant data.

6.4.7.3. Recording and reporting adverse events

The investigators team met regularly to monitor the conduct and progress of research. Annual reports were submitted to the RCH HREC to report on trial progress.

A data and safety monitoring committee (DSMC) was identified, comprising a biostatistician (A/Professor Lyle Gurrin), and two paediatric allergist immunologists (Professor John Ziegler, University of New South Wales and Dr Brynn Wainstein, Sydney Children's Hospital). Professor Ziegler agreed to act as the Chairperson of the DSMC and the Safety Medical Officer. The DSMC met (by teleconference) 3 monthly or as needed; any serious adverse event (SAE) was reported to both the DSMC and RCH HREC within 24hr of the principal investigator becoming aware of the event.

Serious adverse event were to be reported immediately to the Safety Medical Officer and the DSMC as well as the Human Research Ethics Committee and returned study treatment sachets would be analysed if requested by the DSMC.

Suspected Unexpected Serious Adverse Reactions (SUSARS) were to be reported to the Therapeutic Goods Association and Human Research Ethics Committee.

6.4.8. Ethics and trial registration

6.4.8.1. Independent ethics committee

The trial protocol and participant informed consent were approved by the Royal Children's Hospital (RCH) Human Research Ethics Committee (HREC) for the Murdoch Children's Research Institute (MCRI) based at the RCH (HREC 35207, 19/11/2015). The Investigators did not participate in the Committee's decisions. Approval was received in writing from the HREC prior to initiating the trial. All amendments were submitted to the HREC for review and approval prior to implementation. The trial is registered with ACTRN12615001275550, registered 23/11/2015 and was acknowledged under the Clinical Trial Notification Scheme.

6.4.8.2. Ethical Conduct of the Trial

The trial was conducted in accordance with the principles of International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline for Good Clinical Practice (GCP), (E6)(R2) and the Declaration of Helsinki (2013).

6.4.8.3. Study Participant Information and Consent

As this trial involved enrolling paediatric participants 1 year to 12 years of age, it was necessary to obtain the consent of the parent or legal guardian of the paediatric participants. The parent/guardian of all prospective participants was provided with a Parent/Guardian Information Statement and Consent form (PGISCF) approved by the RCH HREC. The Investigators or delegates provided the Information Statement to parents prior to the screening visit to read and review in a timely manner and ask any questions that he/she had in order to fully

understand the nature of the trial, and any risks and procedures involved. Full written informed consent was obtained from all parent/guardians during the pre-screening process, and before any trial-specific procedures were performed. Participants were advised that they were able to refuse to participate in, or to withdraw from the trial at any time, and that the medical care they received would not be affected by their decision regarding participation in the trial.

6.5. Risks and benefits

The key risks to participants were:

Risk of severe allergic reaction (anaphylaxis) as a result of peanut OIT or at the time of peanut challenge

Risk of discomfort during clinical procedures (cannulation, skin prick test, blood and saliva tests)

Despite the normal presence of lactobacillus species in the human gastrointestinal tract, rarely they have been reported to cause bacterial sepsis. Invasive infection due to lactobacillus species has been reported in the form of sepsis, pneumonia and liver abscesses. However such reports have exclusively been in patients with severe underlying gastrointestinal tract disease/dysfunction[149-152] and/or immunocompromised states[153 154] . A systematic review of safety of probiotics concluded that individuals at high risk of complication from use of probiotics included those with immune-compromised states, post-cardiac and/or gastrointestinal tract surgery, critically-ill and those requiring prolonged hospitalisation[155]. Such patients and patients with severe gastrointestinal tract disease or dysfunction will be excluded from this trial. There is no evidence at a population level that increased use of supplemental LGG causes an increase in population incidence of lactobacillus sepsis[156]. LGG sepsis in immunocompetent children has not been reported

6.5.1. Potential benefits

A previous HREC approved trial (HREC 27086) demonstrated the effectiveness of combined probiotic and peanut OIT (PPOIT) for the induction of sustained unresponsiveness (tolerance) and desensitisation in children with peanut allergy[25]. Just over 80% of children who received the active PPOIT treatment were able to tolerate peanut at the end of the trial compared with only 4% of children who received placebo. In that trial, the dose of peanut protein was increased over 8 months, during which time the child was required to attend

hospital every 2 weeks. A regimen of treatment with a shorter buildup phase would offer a more feasible and acceptable (to families) approach to treatment in the clinical setting.

6.6. Supplementary material

Schedule of treatment

VISIT	Screening (pre-study)	T0 RUSH day (b)	BuildUp phase (d)	T1 Maintenance phase (g)	T2 (final day of study treatment)	T3 (8 weeks after end of study treatment)
Month of Study	0	0	1-4	5-18	18	20
Week of Study	0	0	1-16	16-78	78	86
Allergy history	X	X	X	X	X	X
Vital Signs	X	X	X	X	X	X
Weight	X	X	X	X	X	X
Medical history	X					X
QOL survey	X				X	X
Skin prick test	X	X			X	X
Informed Consent	X					
Inclusion/exclusion criteria	X					
OIT		X				
Blood & faecal sample		X (e)			X (e)	X (e)
Anaphylaxis education		X	X	X	X	X
Diary dispensed		X	X	X		
Anaphylaxis action plan & EpiPen prescribed		X				
OIT dispensed		X (c)	X (c,f)	X (h)		
Diary Review			X	X		
All Medication collected for compliance			X	X		
AE & medication review			X	X	X	X
Food challenge	X(a)				X (i)	X(j)
Peanut Consumption Questionnaire						

Abbreviations: AE=adverse event; OIT=oral immunotherapy; QOL=Quality of Life

- a. DBPCFC results from PPOIT-001 T9 food challenge used if applicable and conducted within ≤ 3 months of screening date
- b. Participant admitted to Australian Paediatric Pharmacology Research Unit for rush day to receive increasing dosing of peanut protein every 20 minutes
- c. 2-week supply dispensed
- d. 1 visit every 2 weeks for 16 weeks (2-week supply dispensed)
- e. Blood samples was collected at the same time as cannulation in preparation for rush day or food challenge procedure
- f. Increased dose administered at clinic visit and continued daily at home (if no reaction at clinic visit)
- g. Diary, AE and compliance review every 12 weeks for 40 weeks (12-week supplies dispensed)
- h. Same dose of 2 g peanut protein each day for this period
- i. Food challenge on the day after the final day of treatment
- j. Food challenge 8 weeks after the final day of treatment
- k. SPT conducted at T4 visit and prior to T4 DBPCFC for those participants who opted to take the challenge
- l. Food challenge 14 months after the final day of treatment
- m. Peanut consumption questionnaire administered every 6 months from the 12-month post treatment visit (T4) through to the 3-year post-treatment visit.

Adverse Event Case Report Form (CRF) in REDCap

Confidential

[PPOIT-II] Adverse Event CRF - template
Page 1 of 3

PPOIT-II Adverse Event CRF v3

Record ID

PPOIT-II ID

Safety event category (Please select ONE)

- ☐ Study treatment (PPOIT) related
☐ Accidental food exposure related
☐ Study procedure e.g. DBPCFC related
☐ Unrelated to any of the above

Date of initial notification

Adverse Event Number (for this participant)

1. Study Dose (mg) (last dose given immediately prior to the event)

2. Date and Time dose given

3. Date and time of onset of event

4. Date and time of resolution of event

5. PPOIT treatment phase causing reaction

- ☐ RUSH
☐ Buildup
☐ Maintenance phase
☐ Other

5. Allergenic food accidentally ingested causing reaction

- ☐ Egg,
☐ Cow's milk
☐ Soy
☐ Wheat
☐ Peanut
☐ Treenuts (please specify below)
☐ Fish (please specify below)
☐ Shellfish (please specify below)
☐ Other (please specify below)

Additional description of the event

(Please provide a brief summary)

Adverse Event Symptoms

	No	Yes
Hives localised	☐	☐
Hives widespread	☐	☐
Vomiting	☐	☐
Tummy pain	☐	☐
Runny nose or sneezing	☐	☐
Occasional cough	☐	☐

Persistent cough	☐	☐
Hoarse voice	☐	☐
Wheeze	☐	☐
Shortness of breath	☐	☐
Dizziness/feeling faint	☐	☐
Collapse	☐	☐

Maximum intensity of the adverse event

☐ Grade 1 - Mild
☐ Grade 2 - Moderate
☐ Grade 3 - Severe
☐ Grade 4 - Life threatening
☐ Grade 5 - Death
 (Based upon NIH NIAD CoFAR grading system)

7a. Fulfills criteria for a Serious Adverse Event (SAE)

☐ Yes
☐ No
 (Note: a. Grade 4 and 5 reactions should be classified as SAE; b. Definition of SAE = the event was IMMEDIATELY life threatening (*this does necessary apply to an event that would have become life-threatening in a more severe form); c. All SAEs should be reported to the DSMC)

7b. Fulfills criteria for a Suspected Unexpected Serious Adverse Reaction (SUSAR) ?

☐ Yes
☐ No
 (Note: Suspected Unexpected Serious Adverse Reactions should be reported to DSMC and HREC)

ACTIONS TAKEN FOR ADVERSE EVENT

8. Treatment required (select all that applies)

☐ None
☐ Oral antihistamine
☐ I.M. adrenaline x1 dose
☐ I.M. adrenaline x2 doses
☐ I.M. adrenaline x3 doses
☐ I.M. adrenaline more than 3 doses
☐ I.V. fluids
☐ I.V. adrenaline
☐ Inhaled bronchodilator
☐ Other pharmacotherapy

9. Medical intervention (select all that applies)

☐ Observed under care of parents (i.e. none of the below)
☐ Unscheduled medical clinic visit
☐ Unscheduled Emergency Department visit
☐ Admission to Hospital
☐ Admission to ICU
☐ Other
☐ OIT Immediate next dose to be reduced
☐ OIT to be stopped

OUTCOME

10. Current status

- ☐ Recovered
- ☐ Continuing / persistent
- ☐ Worsening / deteriorating
- ☐ Death

LIKELIHOOD OF RELATIONSHIP TO STUDY TREATMENT

11. Classification of the relationship (refer to study protocol for expanded descriptions)

- ☐ Probably related (e.g. within 1 hour of treatment in absence of other triggers AND cannot be reasonably explained by other factors)
- ☐ Possibly related (e.g. more than 1 hour but less than 4 hours after treatment AND cannot be reasonably explained by other factors)
- ☐ Unlikely to be related (e.g. more than 4 hours after treatment OR can be reasonably explained by other factors)
- ☐ Unrelated (e.g. alternate cause identified OR event does not meet other categories above)
(Refer to study protocol for full definitions)

Signed off by Study Doctor

- ☐ Mimi Tang
- ☐ Dean Tey
- ☐ Marnie Robinson
- ☐ Ee Lyn Su
- ☐ Kuang Hsiao
- ☐ Other (please specify below)

Comments

Food challenge dosing schedule

Food Challenge A began with giving doses from container A as follows:

Dose	Dose (Peanut Protein)	Weight	Cumulative Dose (Peanut protein)
1	80 mg	160 mg	80 mg
2	160 mg	320 mg	240 mg
3	320 mg	640 mg	560 mg
4	640 mg	1280 mg	1200 mg
5	1250 mg	2500 mg	2450 mg
6	2500 mg	5000 mg	4950 mg

The doses were administered at 15 minute intervals, if the participant had not had a reaction to the previous dose. The participant was observed for a minimum of two hours following Food Challenge A. The patient was discharged home if no adverse reactions were noted.

Food Challenge B continued with giving doses from container B as follows:

Dose	Dose (Peanut Protein)	Weight	Cumulative Dose (Peanut protein)
1	80 mg	160 mg	80 mg
2	160 mg	320 mg	240 mg
3	320 mg	640 mg	560 mg
4	640 mg	1280 mg	1200 mg
5	1250 mg	2500 mg	2450 mg
6	2500 mg	5000 mg	4950 mg

The doses were administered at 15 minute intervals, if the participant had not had a reaction to the previous dose. The participant was observed for a minimum of two hours following Food Challenge B. The patient was discharged home if no adverse reactions were noted.

6.7. Chapter summary

PPOIT-002 was a single-arm, open-label trial of probiotic and peanut OIT (PPOIT) for 18 months in 20 children with peanut allergy, using an accelerated dose-escalation schedule for the buildup phase. All other aspects of treatment and trial designs were similar to the earlier PPOIT-001 trial.

In Chapter 7, the doctoral candidate presents findings from the PPOIT-002 trial.

Chapter 7. Results: Clinical effects of PPOIT in the PPOIT-002 single-arm, open-label pilot trial

7.1. Introduction

In Chapter 6, the doctoral candidate presented the rationale, aims and methods of the PPOIT-002 trial, a single-arm, open-label trial of a combined probiotic and peanut oral immunotherapy (PPOIT) using an accelerated dose escalation schedule. This chapter presents the findings in relation to participants' clinical response to treatment and discusses the implications of the findings.

7.2. Abstract

7.2.1. Background

Combined probiotic and peanut oral immunotherapy (PPOIT) was highly effective at inducing desensitisation (89.7% PPOIT vs 7.1% placebo, $p < 0.001$) and 2 week sustained unresponsiveness (82.1% PPOIT vs 3.6% placebo, $p < 0.001$) after 18 months of treatment as shown in a double-blind placebo-controlled randomised trial (PPOIT-001) involving 62 children with peanut allergy[95]. Accelerated immunotherapy dose escalation schedules permit more rapid achievement of a therapeutically effective dose and more rapid desensitisation protection with less inconvenience for patients than conventional schedules[157]. The PPOIT-002 trial evaluates the efficacy and tolerability of an accelerated dose escalation schedule for the buildup phase.

7.2.2. Method

20 children (aged 1-12 years) with peanut allergy, confirmed by study entry (T0) double blind placebo controlled food challenge (DBPCFC) with total cumulative dose of up to 4950mg peanut protein, were enrolled. Participants received 18 months PPOIT treatment using an accelerated dose escalation schedule for the buildup phase. All adverse events (AE) were graded using a standardised approach based on a system developed by National Institute of Health (NIH) Consortium for Food Allergy Research (CoFAR) in conjunction with Food and Drug Administration (FDA, USA). Number of participants affected by AE and rate of AE per 100 doses of study treatment taken were calculated.

Desensitisation, defined as passing a 4950 mg peanut protein DBPCFC at end-of-treatment (T1), and 8-week sustained unresponsiveness (SU), defined as passing a 4950 mg peanut protein DBPCFC 8-weeks post-treatment (T2), were assessed. A sensitivity analysis setting all non-treatment completers as non-responders was performed. Immunologic parameters including peanut skin prick test wheal size, whole peanut and peanut component (Ara h1, h2, h3, h8 and h9) specific-IgE (sIgE) and specific-IgG4 (sIgG4) were evaluated at T0, T1 and T2. Between timepoint

comparisons were done using parametric methods (t-test) for normally distributed data and non-parametric methods (Mann-Whitney) for skewed data.

7.2.3. Results

Sixteen children (75%) completed study treatment. 2 withdrew during buildup phase and 2 withdrew during maintenance phase. 30% (6/20) completed buildup phase within a 16-week period. 85% (17/20) of participants reported at least one AE. 174 probably or possibly related AE were recorded. 93.1% (162) of these AE were mild in severity. 62.6% (109) were recorded during the buildup phase. Gastrointestinal tract symptoms were the most frequently reported (67.2% of AE). 35% (7/20) of participants reported at least one AE of moderate severity or greater. Median rate of AE of any severity per 100 doses taken by treatment completers was 1.0 [interquartile range (IQR) 0.4, 2.4]. Median rate of AE of at least moderate severity per 1000 doses taken was 0.0 (range 0.0, 5.2). AE rates were comparable to PPOIT-001[95].

Desensitisation was achieved in 94% (15/16) and 8-week SU was achieved in 75% (12/16) of participants who completed study treatment. Sensitivity analysis including all enrolled participants and assigning all study withdrawals as non-responders confirmed high clinical response rates of 75% desensitisation and 60% 8-week SU. Peanut skin prick test wheal size reduced significantly by end of treatment (at T2 $p < 0.001$, at T3 $p = 0.008$). Whole peanut and Ara h2 sIgE reduced significantly by end of treatment (peanut: at T2 $p = 0.011$, at T3 $p = 0.010$; Ara h2: at T2 $p = 0.040$). Peanut, Ara h1, Ara h2 and Ara h3 sIgG4 all increased significantly by end of treatment (peanut: at T2 $p < 0.001$, at T3 $p < 0.001$; Ara h1: at T2 $p = 0.004$, at T3 $p = 0.005$; Ara h2: at T2 $p < 0.001$, at T3 $p < 0.001$; Ara h3: at T2 $p < 0.001$, T3, $p < 0.001$).

7.2.4. Conclusions

This single-arm open-label study demonstrates the tolerability of PPOIT using an accelerated dose escalation schedule. Treatment using this modified PPOIT treatment schedule induces both desensitisation and 8-week SU at rates similar to

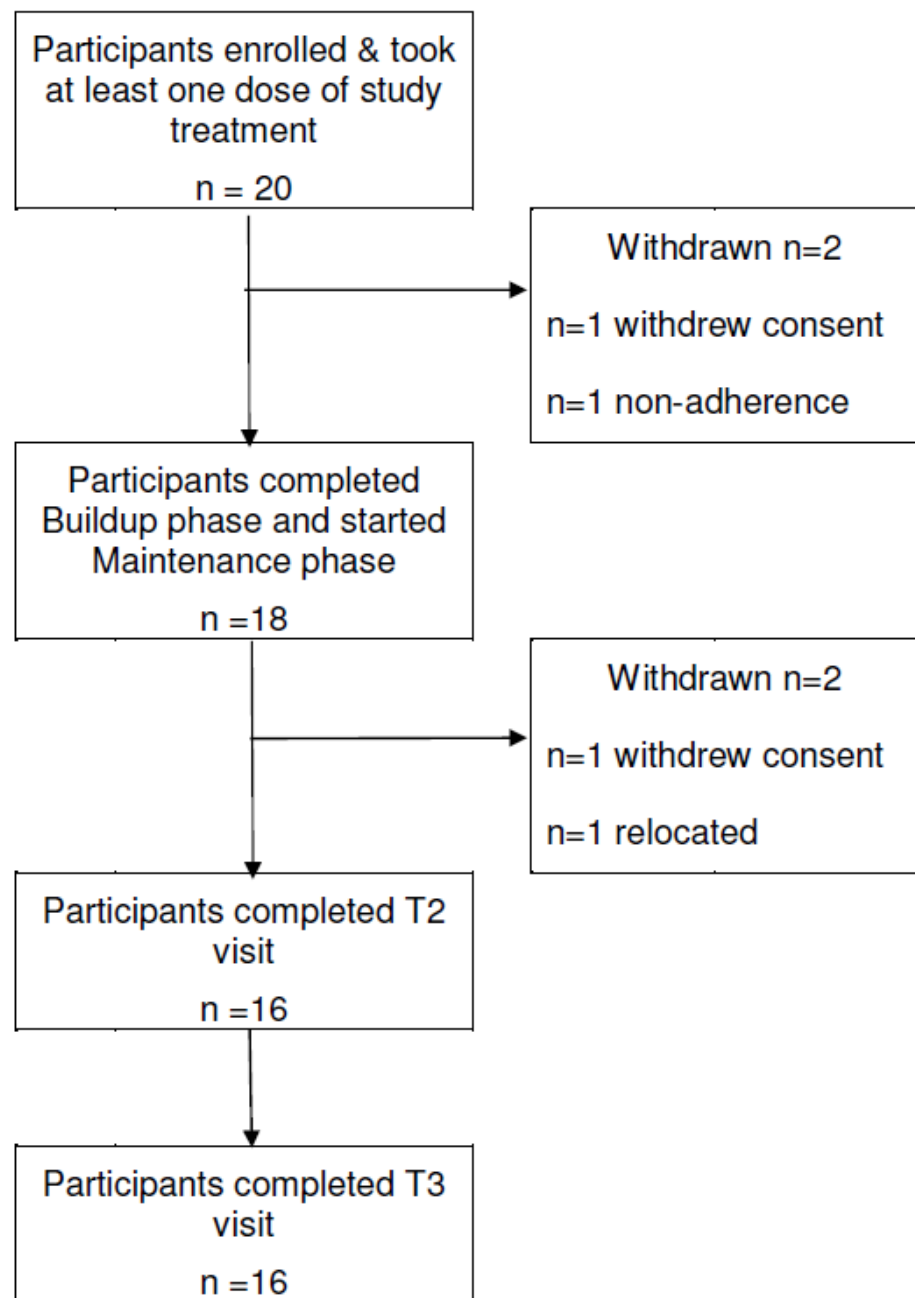
the original treatment schedule previously evaluated in the PPOIT-001 randomised trial. There is earlier achievement of 2-gram maintenance dose and the percentage of participants affected by AE is comparable to conventional schedules.

7.3. Results

7.3.1. Participants

PPOIT-002 trial participant flow is shown in Figure 7-1. Twenty children with peanut allergy [mean (SD) age 8.2 (3.0) years, 30% females] were enrolled. Study entry, T0, mean (SD) peanut skin prick test size was 12.3 (4.5) mm (Table 7-1). All enrolled participants completed rush phase. During the buildup phase, 2 participants withdrew, leaving 18 participants completing buildup phase and starting 2-gram maintenance dose. During maintenance phase, 2 participants withdrew, leaving 16 who completed treatment as per protocol. All 16 treatment completers attended the end of treatment visit (T2), and the 8 weeks post-treatment visit (T3).

Figure 7-1. Participant flow in the PPOIT-002 trial



DBPCFC=double-blind placebo-controlled food challenge; SPT=skin prick test

Table 7-1. Demographics of participants in the PPOIT-002 trial and in the PPOIT (active treatment) group of the PPOIT-001 trial

		PPOIT-002 Trial	PPOIT-001 Trial PPOIT (active treatment) group
		n=20	n=31
Age (y)	Median (IQR)	8.36 (6.34, 10.83)	6.04 (4.35, 7.50)
	Mean (SD)	8.2 (3.0)	6.2 (2.4)
Male sex	n (%)	14 (70)	17 (55)
History of doctor-diagnosed eczema (ever)	n (%)	8 (40)	24 (77)
History of doctor-diagnosed asthma (ever)	n (%)	3 (15)	16 (52)
Peanut SPT wheal size (mm) at T0	Mean (SD)	12.3 (4.5)	17.3 (6.2)

IQR = interquartile range, SD = standard deviation, SPT = skin prick test

7.3.2. Treatment withdrawals

In the PPOIT-002 trial, 4 participants withdrew during treatment, with 2 during the buildup phase and 2 during the maintenance phase. Characteristics of participants who withdrew are provided in Table 7-2. One participant withdrew 26 days after rush, whilst still on the buildup phase due to lack of compliance with treatment protocol. One participant withdrew at 176 days, or just over 25 weeks, after RUSH, during the maintenance phase due to the family's relocation to overseas. Given that adequate study oversight could not be provided and the ethics approval did not extend to continuation of treatment whilst overseas, the participant withdrew from the study. Patient/caregivers decision to withdraw accounted for the remaining two withdrawals - one of these participants (idno 20) reported frequent gastrointestinal symptoms leading to the family's initiation of withdrawal at 70 days after rush. This participant had developed sneezing, rhinorrhea and persistent cough when he was on a 25 mg buildup dose whilst at home. Dose modification rules were followed and he subsequently tolerated dose escalation to 100mg buildup dose Whilst on 100mg and despite premedication with oral antihistamine and taking treatment with food as per study protocol, he vomited within 30-60 minutes of a number of dose. There was no cutaneous, cardiovascular or respiratory manifestation. The participant discontinued treatment and subsequently withdrew consent to remain in the study. His gastrointestinal symptoms resolved shortly after treatment discontinuation. The other participant (idno 8) reported frequent Grade 1 symptoms (including cutaneous hives, sneezing, rhinorrhea and abdominal pain) whilst on 2000 mg maintenance dose and lifestyle restrictions (physical activity restriction post taking dose) leading to the family's initiation of withdrawal at 266 days or 38 weeks after rush phase, during the maintenance phase.

Table 7-2. Characteristics of participants who withdrew

Participant ID	11	20	8	15
Age (baseline)	7.6	2.8	9.7	8.7
Sex	Male	Male	Male	Male
Peanut SPT (mm) at baseline	17.5	9.5	10	12.5
Peanut sIgE (kUA/L) at baseline	100	100	100	7.8
Date of rush	11/02/2016	16/02/2016	14/01/2016	09/02/2016
Date of withdrawal	08/03/2016	26/04/2016	06/10/2016	03/08/2016
Days after rush	26	70	266	176
Withdrawal phase (Rush/BuildUp/Maintenance)	Buildup	Buildup	Maintenance	Maintenance
Reason for withdraw	Lack of patient compliance	Patient/Caregivers decision to withdraw [#]	Patient/Caregivers decision to withdraw ^{##}	Relocated while in the study

[#]Frequent gastrointestinal symptoms leading to family initiated withdrawal

^{##}Frequent symptoms and lifestyle restriction leading to family initiated withdrawal

7.3.3. Proportion of children who completed buildup phase and reached maintenance dose at 16 weeks

Eighteen (18) enrolled participants completed the buildup phase. The median time to reach maintenance phase was 18.0 weeks (IQR 2.0 weeks). Of the participants who completed the buildup phase (n=18), 6 (30%) completed buildup within a 16 week period (Table 7-3).

Table 7-3. Summary of Buildup phase completion

	All Participants (N=20)
Completion of buildup phase within a 16-week period	
Yes, completed in ≤ 16 weeks	6 (30%)
No, completed in > 16 weeks	12 (60%)
Did not complete Buildup phase	2 (10%)*

* Frequent gastrointestinal symptoms leading to family initiated withdrawal

Of the 12 participants who did not complete buildup within a 16-week period, 6 had their buildup up phase delayed due to logistic reasons, unrelated to dose modification/reduction or adverse events. Three started buildup phase at a dose lower than 25 mg (Participant 06 = 0.4mg, Participant 10 = 12 mg, Participant 17 = 3 mg), post rush day, which according to protocol extends the buildup phase due to rush doses being incorporated into the dose escalation schedule. In addition, their buildup phase was also in part delayed due to logistic reasons. One participant (idno 10)'s buildup phase was further delayed due to experiencing side effects and remained on 25mg dose for 8 weeks.

Three participants who started buildup phase at 25 mg, had delayed buildup phase due to treatment associated reactions. One participant (idno 8) had a moderate severity reaction to the 25 mg buildup dose at home and was required to reduce his daily dose within 10 days of scheduled updose, which according to protocol requires deferral of the buildup visit. This resulted in an additional buildup visit. Another participant (idno 12) had a moderate severity reaction to the 50 mg

buildup dose on the day of scheduled updose and was continued on 25 mg for 4 weeks. This participant did not have other treatment related delays. One participant (idno 16) had an allergic reaction within 3 days of a scheduled updose, necessitating extension of the same dose by 2 weeks as per treatment protocol.

7.3.4. Concordance in assignment and classification of adverse events (AE)

Classification of severity and relationship to study treatment were performed by two independent allergists. In summary, there was 62.5% (170/272) concordance between independent assessors' initial classifications, with 8.8% initial disagreement on severity, 26.5% initial disagreement on relationship and 2.2% initial disagreement on severity and relationship. 58 of the 102 disagreements were resolved after joint-review of data by the two assessors and the remaining 44 disagreements were resolved with the input of a third independent assessor. Of 272 reported events, 174 AE were assessed as probably and possibly related to study treatment and included in analyses.

7.3.5. Analysis of probably and possibly related adverse events (AE)

A summary of all probably and possibly related AE is presented in Table 7-4. A total of 174 probably or possibly related AE were reported by study participants during the treatment period. 15% (3/20) of treated participants experienced no AE throughout the treatment period. 85% (17/20) experienced at least 1 AE. The majority of AE were reported during the buildup phase (62.64%). 35.63% of adverse events were reported during the maintenance phase. Over 90% of all probably and possibly related adverse events were mild. Gastrointestinal tract symptoms were the most commonly reported (67.24%), followed by cutaneous (29.31%) and upper respiratory tract (24.14%) symptoms.

130 (75%) of all AE were reported by the 16 (80%) participants who completed the study. In both treatment completers and treatment non-completers, the majority of AE were mild (94.62% and 88.64% respectively). Moderate severity AE

were the second most common (3.08% and 9.09% respectively). In both groups, gastrointestinal tract symptoms were the most reported events (63.85% and 77.27% respectively).

PPOIT-002 treated participants experienced a median of 6 AE each (IQR 2, 13; range 0, 30). This equates to a median of 1.0 AE per 100 treatment doses taken (IQR 0.4, 2.4; range 0.0, 5.3).

Table 7-4. Characteristics of individual adverse events that were probably or possibly related to PPOIT treatment

	PPOIT-2 All enrolled	PPOIT-2 Treatment completers	PPOIT-2 Withdrawals
Number of Participants, n	20	16	4
Total number of Adverse Events	174	130	44
Study treatment phase when reaction occurred			
Rush, n (%)	3 (1.72)	3 (2.31)	0 (0.00)
Buildup, n (%)	109 (62.64)	81 (62.31)	28 (63.64)
Maintenance phase, n (%)	62 (35.63)	46 (35.38)	16 (36.36)
Allergic reactions			
Maximum intensity of the allergic reaction event			
Grade 1 - Mild, n (%)	162 (93.10)	123 (94.62)	39 (88.64)
Grade 2 - Moderate, n (%)	8 (4.60)	4 (3.08)	4 (9.09)
Grade 3 – Severe, n (%)	4 (2.30)	3 (2.31)	1 (2.27)
Grade 4 - Life threatening, n (%)	0 (0.00)	0 (0.00)	0 (0.00)
Classification of the relationship			
Probably related, n (%)	117 (67.24)	96 (73.85)	21 (47.73)
Possibly related, n (%)	57 (32.76)	34 (26.15)	23 (52.27)
Adverse event by organ involvement			
Any skin involvement, n (%)	51 (29.31)	36 (27.69)	15 (34.09)
Any gastrointestinal tract involvement, n (%)	117 (67.24)	83 (63.85)	34 (77.27)
Any upper respiratory tract involvement, n (%)	42 (24.14)	23 (17.69)	19 (43.18)
Any lower respiratory tract	12 (6.90)	8 (6.15)	4 (9.09)

involvement, n (%)			
Any cardiovascular involvement, n (%)	1 (0.57)	1 (0.77)	0 (0.00)

7.3.6. Comparison of PPOIT-002 and PPOIT-001 participants who experienced any AE of at least moderate severity

We performed post-hoc comparison of PPOIT-002 and PPOIT-001 participants who received active (PPOIT) treatment and who reported any AE of at least moderate severity (Table 7-5). The proportion of participants who reported AE in PPOIT-002 and in the active treatment arm of PPOIT-001 were similar. In PPOIT-002, 35% of participants reported at least one AE of at least moderate severity. 5% of participants experienced at least one AE during the rush phase. 20% experienced at least one adverse event during the buildup phase and 20% during the maintenance phase. These proportions are comparable to PPOIT-001 active treatment participants, 48% of whom experienced at least one AE of at least moderate severity. 35% of participants experienced at least one adverse event during PPOIT-001 buildup up phase.

Table 7-5. Number and proportion of participants affected by any adverse event of at least moderate severity

	PPOIT-002 Trial	<i>PPOIT-001 Trial</i> <i>PPOIT (active treatment)</i> <i>group#</i>
Number of Participants Enrolled	20	31
Patients who had at least one AE, N (%*)	7 (35)	15 (48)
Patients who had at least one AE during rush, N (%*)	1 (5)	1 (3)
Patients who had at least one AE during Buildup, N (%*)	4 (20)	11 (35)
Patients who had at least one AE during Maintenance, N (%*)	4 (20)	6 (19)
Number of moderate-severe-serious AE per patient		
Overall (rush, Buildup, Maintenance)		
0	13 (65)	16 (52)
1	4 (20)	7 (23)
2	2 (10)	2 (6)
3	0 (0)	3 (10)
4	1 (5)	3 (10)
During rush		
0	19 (95)	30 (97)
1	1 (5)	1 (3)
2	0 (0)	0 (0)
3	0 (0)	0 (0)
4	0 (0)	0 (0)
During Buildup		
0	16 (80)	20 (65)
1	3 (15)	6 (19)
2	1 (5)	2 (6)
3	0 (0)	2 (6)
4	0 (0)	1 (3)
During Maintenance		

0	16 (80)	25 (81)
1	2 (10)	3 (10)
2	2 (10)	2 (6)
3	0 (0)	0 (0)
4	0 (0)	1 (3)

#Data from PPOIT (active treatment) group of the PPOIT-001 trial are summarized here

7.3.7. Comparison of AE of at least moderate severity reported by PPOIT-002 and PPOIT-001 participants

Twelve AE of at least moderate severity were reported by PPOIT-002 participants (n=20). These events were evenly spread between rush/buildup phase and maintenance phase (50% maintenance). 83% of AE of at least moderate severity involved the lower respiratory tract (Table 7-6).

By comparison, 32 AE of at least moderate severity were reported by PPOIT-001 participants (n=32) in the PPOIT (active treatment) group. AE most frequently occurred during the buildup phase (63%). Gastrointestinal symptoms were the most frequently reported AE of at least moderate severity (50%).

The median overall number of AE of at least moderate severity was 0.0 (range 0.0, 3.8) per 1000 doses taken in the PPOIT-002 group as compared with 0.0 (range 0.0, 7.9) per 1000 doses taken in the PPOIT-001 group. During the maintenance phase, the median number of AE of at least moderate severity reported by PPOIT-002 and PPOIT-001 active participants were 0.0 (range 0.0, 5.2) and 0.0 (range 0.0, 17.2) per 1000 maintenance doses taken, respectively.

Table 7-6. Characteristics of individual AE of at least moderate severity

	PPOIT-002 Trial	<i>PPOIT-001 Trial</i> <i>PPOIT (active treatment) group#</i>
Total number of Adverse Events	12	32
Study treatment phase when reaction occurred		
Rush phase, n (%)	1 (8)	1 (3)
Buildup phase, n (%)	5 (42)	20 (63)
Maintenance phase, n (%)	6 (50)	11 (34)
Allergic reaction		
Maximum intensity of the allergic reaction event		
Grade 2 - Moderate, n (%)	8 (67)	29 (91)
Grade 3 – Severe, n (%)	4 (33)	2 (6)
Grade 4 - Life threatening, n (%)	0 (0)	1 (3)
Classification of the relationship		
Probably related, n (%)	8 (67)	16 (50)
Possibly related, n (%)	4 (33)	16 (50)
Adverse event by organ involvement		
Any skin involvement, n (%)	2 (17)	11 (34)
Any gastrointestinal tract involvement, n (%)	5 (42)	16 (50)
Any upper respiratory tract involvement, n (%)	5 (42)	10 (31)
Any lower respiratory tract involvement, n (%)	10 (83)	10 (31)
Any cardiovascular involvement, n (%)	1 (8)	1 (3)

#Data from PPOIT (active treatment) group of the PPOIT-001 trial are summarized here

7.3.8. Clinical efficacy of PPOIT in the PPOIT-002 trial

All participants enrolled in PPOIT-002 and who received at least one dose of PPOIT and who had at least one post-dose efficacy assessment were included for the desensitisation (T2) and 8-week SU (T3) outcome evaluation.

Desensitisation was achieved in 94% (15/16) and 8-week sustained unresponsiveness was achieved in 75% (12/16) of participants. Sensitivity analysis setting all non-treatment completers as non-responders was performed. Sensitivity analysis showed that 75% (15/20) and 60% (12/20) of participants achieved desensitisation and SU respectively.

Peanut skin prick test (SPT) wheal size decreased significantly from study entry to end of treatment ($p=0.001$). This reduction was sustained at 8 weeks post treatment completion ($p=0.008$). Desensitisation and SU rates and Skin prick test wheal size data is shown in Table 7-7. Clinical outcomes in the PPOIT-002 trial were comparable with those of the PPOIT (active treatment) group in the PPOIT-001 trial.

In addition, there was a significant positive association between cumulative dose of OIT taken and 8-week SU (coefficient 90766.66, $p=0.008$) and a trend towards a positive association between cumulative dose taken and desensitisation (coefficient 59619.41, $p=0.053$; Data included in the Supplementary material section of this chapter).

Table 7-7. Clinical outcomes of participants in the PPOIT-002 and PPOIT-001 trials

	PPOIT-002 Trial		PPOIT-001 Trial	
			<i>PPOIT (active treatment) group#</i>	
Double blind placebo controlled food challenge (DBPCFC)	n/N (%)		n/N (%)	
Sustained unresponsiveness (SU)	12/16 (75)		23/28 (82)	
Sustained unresponsiveness (SU), sensitivity analysis [^]	12/20 (60)		23/31 (74)	
Desensitisation	15/16 (94)		26/29 (90)	
Desensitisation, sensitivity analysis [^]	15/20 (75)		26/31 (84)	
	PPOIT-002 Trial		PPOIT-001 Trial	
Peanut Skin Prick Test Wheal Size (mm)			<i>PPOIT (active treatment) group#</i>	
Time point	Mean (SD), n	p-value*	Mean (SD), n	p-value*
Baseline	12.3 (4.5), 20	N/A	17.3 (6.2), 31	N/A
End of treatment	6.9 (4.1), 16	0.001	4.6 (4.1), 29	<0.001
8-weeks post-treatment	8.1 (4.4), 16	0.008	4.3 (4.5), 27	<0.001

[^]Sensitivity analysis setting all non-treatment completers as non-responders was performed.

*Unpaired t test with equal variances

#Previously published data from PPOIT (active treatment) group of the PPOIT-001 trial are summarized here

7.3.9. Immunomodulatory effects of PPOIT

PPOIT modulated the immune response to peanut allergen. There was significant reduction in peanut and major peanut component (Ara h2) sIgE levels at end of treatment (T2). The reduction was sustained for peanut sIgE at 8 weeks post treatment (T3) (Table 7-8). In contrast, there was no change in Ara h8 and Ara h9 sIgE. There was uniform induction of peanut and major peanut component (Ara h1, h2 and h3) sIgG4 levels at end of treatment (T2) (Table 7-9). This increase in sIgG4 levels was sustained at T3.

Table 7-8. Summary of sIgE response

PPOIT-002 trial participants			
	Time point	Median (IQR), n	p-value*
Peanut sIgE (kUA/L)			
	Baseline	69.9 (7.9, 100.0), 20	N/A
	End of treatment	7.4 (1.2, 33.6), 15	0.011
	8-weeks post-treatment	10.2 (1.0, 27.6), 16	0.010
Ara h1 sIgE (kUA/L)			
	Baseline	3.6 (0.0, 34.3), 16	N/A
	End of treatment	2.2 (0.0, 8.2), 15	0.735
	8-weeks post-treatment	2.0 (0.0, 6.0), 13	0.860
Ara h2 sIgE (kUA/L)			
	Baseline	34.3 (3.4, 118.7), 16	N/A
	End of treatment	7.7 (0.9, 21.8), 15	0.040
	8-weeks post-treatment	10.9 (0.5, 18.4), 14	0.071
Ara h3 sIgE (kUA/L)			
	Baseline	0.2 (0.0, 15.5), 16	N/A
	End of treatment	0.1 (0.0, 3.2), 15	0.843
	8-weeks post-treatment	0.4 (0.0, 2.3), 13	0.948
Ara h8 sIgE (kUA/L)			
	Baseline	0.0 (0.0, 0.1), 16	N/A
	End of treatment	0.0 (0.0, 0.0), 15	0.519
	8-weeks post-treatment	0.0 (0.0, 0.0), 13	0.546
Ara h9 sIgE (kUA/L)			
	Baseline	0.0 (0.0, 0.1), 16	N/A
	End of treatment	0.0 (0.0, 0.1), 15	0.764
	8-weeks post-treatment	0.0 (0.0, 0.2), 13	0.947

*Unpaired rank-sum (Mann-Whitney) test

Table 7-9. Summary of sIgG4 response

PPOIT-002 trial participants			
	Time point	Median (IQR), n	p-value*
Peanut sIgG4 (mgA/L)			
	Baseline	0.6 (0.2, 1.3), 16	N/A
	End of treatment	21.6 (6.0, 93.5), 15	<0.001
	8-weeks post-treatment	11.2 (3.3, 28.4), 14	<0.001
Ara h1 sIgG4 (mgA/L)			
	Baseline	0.1 (0.0, 0.2), 16	N/A
	End of treatment	1.3 (0.1, 4.2), 15	0.004
	8-weeks post-treatment	1.3 (0.3, 2.3), 13	0.005
Ara h2 sIgG4 (mgA/L)			
	Baseline	0.3 (0.1, 0.6), 16	N/A
	End of treatment	17.2 (4.8, 63.9), 15	<0.001
	8-weeks post-treatment	11.8 (2.6, 29.0), 13	<0.001
Ara h3 sIgG4 (mgA/L)			
	Baseline	0.1 (0.1, 0.1), 16	N/A
	End of treatment	2.4 (1.0, 7.3), 15	<0.001
	8-weeks post-treatment	1.1 (0.4, 5.6), 13	<0.001
Ara h8 sIgG4 (mgA/L)			
	Baseline	0.0 (0.0, 0.0), 16	N/A
	End of treatment	0.0 (0.0, 0.1), 15	0.115
	8-weeks post-treatment	0.0 (0.0, 0.0), 13	0.785
Ara h9 sIgG4 (mgA/L)			
	Baseline	0.0 (0.0, 0.2), 16	N/A
	End of treatment	0.0 (0.0, 0.2), 15	0.795
	8-weeks post-treatment	0.0 (0.0, 0.2), 13	0.673

*Unpaired rank-sum (Mann-Whitney) test

7.4. Discussion

It was previously demonstrated that PPOIT is effective at inducing desensitisation and sustained unresponsiveness (SU) to peanut and at modulating peanut specific immune response. This single-arm open-label trial was setup to evaluate the feasibility of delivering PPOIT using a modified (accelerated) dose escalation schedule for the buildup phase in order to reduce the number of clinic visits for patients to reach target maintenance dose. 38% (6/16) of PPOIT-002 participants were able to complete dose-escalation to the target 2000 mg maintenance dose within 16 weeks. Median rate of adverse events (AE) was 1.0% (IQR 0.4 to 2.4%) of doses taken during treatment phase. 95% of participants who completed treatment with PPOIT adopting the accelerated dose escalation schedule were successfully desensitised and 75% attained 8-week SU. Treatment induced decreases in peanut specific skin prick test (SPT) wheal size and circulating peanut and peanut component sIgE levels and increases in circulating peanut and peanut component IgG4 levels. These findings confirm previously reported changes associated with PPOIT treatment using the original dose escalation schedule previously evaluated in the PPOIT-001 randomised trial.

Peanut oral immunotherapy (OIT) has variable success at inducing desensitisation and SU[1]. Although the goal of treatment is to protect patients from experiencing peanut ingestion associated adverse events (AE), a recent meta-analysis found that treatment with peanut OIT was associated with increased risk of anaphylaxis, adrenaline use to treat reactions, serious adverse events and other non-anaphylactic reactions as compared to treatment controls adhering to allergen avoidance[43]. Of note, the rates of peanut OIT associated reactions are not dissimilar to the rates and severity of reactions associated with unmodified peanut protein subcutaneous immunotherapy (SCIT)[158]. There remains a heightened risk of reactions in association with peanut OIT despite various restrictive measures and dosing rules (including avoidance of other sources of peanut, dose omission in the presence of illness, fatigue, dose reduction in the presence of fever and during menstrual period, instructions to take dose on full stomach and/or with

food, and refraining from exercise and/or hot showers/baths following a dose) in an attempt to minimise the likelihood of reactions[43].

In the PPOIT-002 trial, over 30% of participants were able to reach the target dose within 16 weeks without compromising the tolerability of treatment. The median rate of AE of any severity was 1.0% [interquartile range (IQR) 0.4 to 2.4%, actual range 0.0 to 5.3%] which was similar to rates reported by other studies aiming for lower target maintenance doses. Whilst the frequency of AEs appears to be acceptable to most study participants given the modest participant retention rate, for 2 individuals, their parents withdrew consent to remain in the study taking into account the frequency of low grade AEs whilst on study treatment. It is possible that whilst an accelerated dose escalation schedule does not increase the risk of severe reactions that the frequency of low grade side effects still limits acceptability of OIT treatment.

Gastrointestinal symptoms whilst on OIT is commonly reported and the prevalence of biopsy-confirmed eosinophilic oesophagitis (EoE) range 2.7 to 5.3% of patients undergoing food OIT[44-46]. In the present study, no subjects had biopsy-confirmed EoE but 2 subjects withdrew their consent to remain in the study citing recurrent grade 1 symptoms including gastrointestinal symptoms suggesting reduced tolerability of the study treatment by these individuals.

A number of recent studies have adopted slower dose-escalation schedules (for example, taking more than 52 weeks to reach maintenance phase[19 36 39 40]) and/or are aiming for lower target maintenance doses (for example, 300mg or less[36 37 94]) in an attempt to further improve the tolerability of OIT treatment. It is noteworthy that all patients in a peanut OIT study which included an ultra-slow buildup phase, aiming to reach target maintenance dose of 125-250 mg of peanut protein over a period up to 14 months, experienced at least one AE[36]. 4.3% of all peanut OIT doses taken by participants were associated with AE. In a large phase-3 study evaluating a proprietary peanut OIT product (AR101)[94], also aiming for a low maintenance dose of 300mg with dose-escalation over a 26-week period, 24.5% (102/416) of participants randomised to receive active treatment

withdrew during the treatment phase. Over 95% of participants reported at least one AE. By comparison, the present study shows that PPOIT treatment using an accelerated dose escalation schedule aiming to complete buildup phase within 16 weeks and aiming for a high maintenance dose of 2000 mg was associated with a similar participant withdrawal rate (20%) and a similar proportion (95%) of participants affected by at least one AE. Taken together, these findings question whether the tolerability and safety of peanut OIT can be improved by pursuing either slower dose-escalation schedules and/or lower target maintenance doses.

The mechanisms underpinning the desensitised state whilst on OIT treatment remain elusive. IgG antibodies that share specificity with antigen specific-IgE (sIgE) may confer protection against IgE mediated clinical reactions and abrogate anaphylaxis through blocking sIgE access to FcεRI and through induction of mast cell suppression through FcγRIIb[58 159]. Induction of peanut and peanut component allergen sIgG4 has been consistently observed in peanut OIT treated participants[18 54 94 95 160] with increases observed as early as the first month of treatment[160]. Magnitude of sIgG4 induction is dose-dependent[20 34]. In a head-to-head peanut OIT trial comparing two different target maintenance doses of 300 mg per day versus 3000 mg per day of peanut protein, the rate of sIgG4 induction was more rapid and the magnitude of sIgG4 induction was greater in the higher dose group[20]. It is possible that earlier clinical protection from reactions to OIT may be conferred by treatment induced sIgG4, and therefore OIT tolerability may be improved by more rapid attainment of desensitisation to higher allergen doses.

OIT treatment success is associated with lower levels of peanut and peanut component sIgE at the post-treatment time point as reported by the doctoral candidate (in Chapter 4) and by others[19 34]. Studies adopting longer OIT treatment durations have observed significant reductions in allergen sIgE levels[18 19]; in contrast, studies adopting shorter treatment durations of 6-12 months have failed to demonstrate significant reductions [94 100].

In the PPOIT-002 trial, without altering the total treatment duration, acceleration of the buildup phase translated to an increased duration of the 2 g/day maintenance phase from 12 to 16 months. Participants in this had significant reductions in the levels of their peanut and Ara h2 sIgE at end of treatment. Clinical outcomes were similar to those reported in the PPOIT-001 trial [per-protocol analysis: PPOIT-001 desensitisation 90% (26/29), SU 82% (23/28) versus PPOIT-002 desensitisation 94% (15/16), SU 75% (12/16); intention-to-treat sensitivity-analysis: PPOIT-001 desensitisation 84% (26/31), SU 74% (23/31) versus PPOIT-002 desensitisation 75% (15/20), SU 60% (12/20)]. Taken together, whilst longer OIT treatment duration appear to be associated with more marked reduction allergen sIgE, extending the maintenance phase by 4 months does not appear to increase the likelihood of short-term treatment success as evaluated by desensitisation or SU challenges. Long-term outcome studies are necessary to evaluate if the immunomodulatory effects on sIgE response also lead to long-term beneficial effects as observed in the PPOIT-001 trial.

The major limitation of this study was the small sample size. However, this study was primarily designed to evaluate the feasibility and tolerability of a more rapid dosing schedule rather than to formally compare the different dosing schedules. The doctoral candidate recognises that between trial comparisons of efficacy and tolerability data must be done with caution given potential bias introduced by heterogeneity in study designs and conduct. In this chapter, the doctoral candidate applied the standardized CoFAR (<http://www.cofargroup.org/>) classification of AE when evaluating AE and tolerability data obtained from both the PPOIT-002 and PPOIT-001 trials as a mitigating measure against such bias.

To conclude, findings from this single-arm open-label study provides evidence that PPOIT administered using an accelerated dose escalation schedule is feasible and tolerable. The modified treatment remains effective at inducing desensitisation and SU. Further work evaluating antibody responses during the buildup phase is necessary to gain deeper understanding whether the tolerability profile of an OIT treatment adopting an accelerated dose escalation schedule is facilitated by a more

rapid induction of circulating allergen sIgG4 and sIgA in a dose-dependent manner. Findings may help inform future modifications of OIT treatment schedules aimed at improving treatment tolerability and safety without compromising efficacy.

7.5. Supplementary material

Association between cumulative dose taken during the entire treatment phase and clinical outcomes (desensitisation and sustained unresponsiveness, SU)

	Coefficient	p-value
Desensitisation (regression)	59619.41	0.053
8-week SU (regression)	90766.66	0.008
8-week SU (ranksum)	NA	0.021

7.6. Chapter summary

The PPOIT-002 trial was an open label study designed to evaluate the feasibility, tolerability and clinical effects of PPOIT using an accelerated dose escalation schedule for the buildup phase. As reported in this chapter, the study confirmed that PPOIT treatment can be improved by accelerating the dose-escalation phase, thereby reducing the number of dose escalation visits, without compromising the treatment's ability to induce desensitisation, sustained unresponsiveness (SU), or immune modulatory effects.

In the final chapter, the doctoral candidate summarises and discusses implications of the findings from the PPOIT-001 and PPOIT-002 trials.

Chapter 8. Discussion

8.1. Summary of thesis findings

There is a need for disease modifying treatment for food allergy. In this thesis, the doctoral candidate presented novel findings that improve our understanding about the clinical and immunomodulatory effects of a combined probiotic and peanut oral immunotherapy (PPOIT). As described in Chapter 3, the doctoral candidate demonstrated that at four years after discontinuation of treatment, PPOIT enabled ongoing peanut ingestion in a large proportion of participants and was associated with long-term sustained unresponsiveness (LTSU), with participants ingesting peanut ad-libitum during the four year post-treatment period. This state of persistent clinical remission from peanut reactions, maintained without the need for ongoing and regular peanut ingestion years post-treatment, may be the closest to true tolerance[42]. Findings reported in Chapter 4 and Chapter 5 showed that accompanying this clinical state, was the broad downregulation of peanut specific humoral immune responses, suggesting that abrogation of peanut specific B cell response, likely facilitated by OIT induced suppression of Th2 cytokine production, may be necessary for the attainment of tolerance following OIT treatment. Next, leveraging PPOIT's ability to induce beneficial clinical and immunomodulatory responses, the dose escalation schedule of PPOIT was accelerated in an attempt to improve treatment feasibility. Findings from the PPOIT-002 trial as reported in Chapter 7 showed that the modified treatment schedule was feasible and well-tolerated and did not compromise PPOIT's ability to induce desensitisation or SU.

8.2. Implication of thesis findings

8.2.1. Goals and clinical effects of OIT treatment

Allergen immunotherapy was designed to capitalise on the malleability of the adaptive immune response and through controlled- and graded- exposures to the culprit allergen induce allergen specific tolerance. Whilst the ultimate goal of such treatment is tolerance induction, earlier OIT studies evaluated desensitisation, that is, an ability to ingest and tolerate an allergen whilst on or shortly after withdrawal of treatment. It is increasingly recognised that desensitisation represents transient remission from allergic reactions and is a reversible state in a sizeable proportion of ‘successfully-treated’ patients. Some later studies aimed for a more persistent state or remission or ‘sustained-unresponsiveness’, that is, an ability to ingest and tolerate an allergen weeks to months after discontinuation of treatment and without ongoing ingestion of the allergen. Sustained unresponsiveness, as compared to desensitisation, is achievable in a much smaller proportion of treated patients[34 54] and has also been found to be reversible in some patients in a time-dependent manner by some groups[39 40]. More recent larger-scale OIT trials[37 94 100] now aim to achieve desensitisation. Although a state of desensitisation may be desirable by some patients, for many patients and their treating physicians, such temporary state of clinical remission may not adequately justify exposures to heightened risk of reactions and adverse events in association with OIT treatment[43].

Findings from the follow-on study of the PPOIT-001 trial demonstrated that long-term remission from allergic reactions, a state resembling tolerance, is possible. At 4 years post-treatment, a large proportion of PPOIT participants maintained their ability to ingest peanut and 58% had long-term sustained unresponsiveness (LTSU), tolerating 4000 mg of peanut protein in a peanut challenge administered after at least 8 weeks of peanut avoidance [99]. The estimated number of adverse events whilst ingesting peanut ad libitum in the four year post-treatment period was 1.1 events per 10 person-years as compared with 0.9 events per 10 person years in the placebo group who maintained allergen avoidance. The possibility of

achieving longer term and more durable remission provides stronger justification for the short-term exposure to heightened risk of reactions whilst on OIT treatment. It is important to continue pursuing the more desirable outcome of durable clinical remission.

8.2.2. Design of OIT treatment

OIT is associated with high rates of reactions and adverse events (AE). A landmark meta-analysis including 12 peanut OIT randomised controlled trials involving 1041 participants found that OIT treatment was associated with significantly increased risks of anaphylaxis, rescue treatment with adrenaline, serious adverse events and non-anaphylactic reactions[161]. There is limited head-to-head comparison of tolerability profile of high dose versus low dose OIT treatment. One trial[20] involving 40 children with peanut allergy, randomised 1:1 to receive peanut OIT at goal maintenance doses of 300 mg/day or 3000 mg/day reported an overall average AE rate of 1.1% (95% CI 0.0%, 2.3%) in the high dose arm versus 0.6% (95% CI 0.3%, 0.9%) in the low dose arm. AE rates during the maintenance phase were similar [High dose arm 0.06% (0.01%-0.1%) versus low dose arm 0.05% (0.0-0.2%)]. Contrasting the approach some recent studies have adopted aiming for lower OIT maintenance doses[36 37 94], the PPOIT Study Team has continued to aim for a higher maintenance doses of 2000 mg/day of peanut protein. Furthermore, in the PPOIT-002 trial, over 30% of participants were able to reach the target dose within 16 weeks without compromising the tolerability of treatment. The median rate of AE of any severity was 1.0% [interquartile range (IQR) 0.4 to 2.4%, actual range 0.0 to 5.3%] which was similar to rates reported by other studies aiming for lower target maintenance doses[20 100]. The doctoral candidate hypothesises that a more rapid and robust induction of blocking sIgG4 and sIgA antibodies through an accelerated dose escalation schedule to reach a high OIT maintenance dose confers protection against treatment emergent adverse events during the treatment phase. However, it remains elusive if such schedule modification successfully protects against gastrointestinal tract inflammation such as eosinophilic oesophagitis[44-46]

8.2.3. Mechanisms of action of OIT treatment

Although OIT treatment is often accompanied by the induction of circulating allergen sIgG4 and sIgA, it remains unclear whether these antibodies are necessary to support tolerance induction. Some studies have shown that peanut s IgG4 levels do not predict sustained unresponsiveness[19 40]. Findings presented in Chapter 4, suggest that suppression of peanut sIgE responses may play a role in supporting long-term sustained unresponsiveness (LTSU) or persistence of clinical remission years after discontinuation of treatment.

The doctoral candidate reported in Chapter 5, that patients' peanut allergy disease activity as measured by peanut and peanut component (Ara h1, h2, h3) sIgE levels correlated with their Th-2 cytokine levels. Treatment with PPOIT was associated with reduced production of Th-2 cytokine IL-4. Contrary to the doctoral candidate's expectations, circulating peanut specific T-regulatory (Treg) cells and IL-10 production did not increase after 18 months of PPOIT treatment. Instead, after PPOIT treatment, there was a trend towards lower frequency of circulating Treg cells and reduced production of the Treg cytokine, IL-10. Taken together, findings from Chapters 4 and 5 suggest that PPOIT induced long-term SU, a clinical state resembling tolerance, may require the broad downregulation of antibody production by allergen specific B cells through the redirection of the Th2 cytokine milieu. Reduction in disease activity is accompanied by a reduced need for circulating antigen specific Treg cells and IL-10 production. Better understanding of the mechanisms of tolerance induction helps identify potential therapeutic targets that can enhance the efficacy of OIT. Combining anti-IgE monoclonal antibody (for example Omalizumab) with OIT is already under investigation and the effectiveness of such approach was summarized in a recent review article[162]. In addition, there are case reports of monoclonal antibody therapies which target the IL-4 and IL-13 signaling pathway [163] and IL-33, an upstream Th2 cytokine[164], improving patients' ability to tolerate culprit food allergens.

8.3. Future directions

Leveraging findings from the PPOIT-001 and PPOIT-002 trials, a multi-center 3-arm DBPCRCT (PPOIT-003 Study, ACTRN12616000322437) is underway to (1) confirm the clinical and immune effects of PPOIT, (2) confirm the tolerability of the accelerated dose escalation schedule, and (3) elucidate additional effects of adding probiotics to peanut OIT. The approach of adding probiotic adjuvant to OIT is being extended to a number of other foods (PEAT Study, ACTRN12619000480189; PrEMO Study, ACTRN12619000306112).

It is important to continue the investigation of the dynamics of allergen blocking sIgG4 and sIgA induction during early phases of food immunotherapy (including OIT) treatment and to explore whether such responses can be manipulated by modifying treatment schedules. Optimising the induction of immune mechanisms that mediate early protection from clinical reactions may lead to safer and more tolerable food allergy treatments. In addition to modifying the dose escalation schedule to induce earlier desensitisation, other strategies aimed improving tolerability without compromising efficacy include (1) combining OIT with anti-IgE therapies, and (2) using modified protein based or peptide based food immunotherapy extracts warrant further investigation.

Finally, based on findings reported in this thesis, the doctoral candidate hypothesise that OIT induced long-term persistent remission from food allergic reactions is accompanied by suppression or abrogation of allergen specific humoral immune responses through the redirection of Th2 cytokine milieu. Future food immunotherapy (including OIT) research should further investigate combining immunotherapy treatment with immunomodulatory agents capable of blocking and/or redirecting the Th2 cytokine response in order to abrogate pathogenic B cell response to specific food allergens.

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Appendices

Appendix 1: Ethics approval certificates

The Royal Children's Hospital Melbourne
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Parkville Victoria 3052 Australia
TELEPHONE +61 3 9345 5522
www.rch.org.au



RCH HUMAN RESEARCH ETHICS COMMITTEE APPROVAL

HREC REF. No:	27086 Q	
PROJECT TITLE:	Long term follow-up study of the effectiveness of Probiotics and Peanut Oral Immunotherapy (OIT) in inducing desensitisation or tolerance in children with peanut allergy	
DOCUMENTS APPROVED:	PGIS & CF v2 dated Oct 2014 PIS & CF v2 dated Oct 2014 Questionnaire T9 v1 dated Jul 2014 Food Allergy QoL Questionnaire Parent Form (0-12 years) v1 dated Jul 2014	
APPROVED PROTOCOL:	Protocol v17 dated Jul 2014	
PRINCIPAL INVESTIGATOR:	Mimi Tang	
DATE OF MODIFICATION APPROVAL:	4 December 2014	
DURATION:	14 months	
DATE OF APPROVAL EXPIRY:	13 August 2015	
SIGNED:		 4 th December 2014
	COMMITTEE REPRESENTATIVE	
Comments:		
APPROVED SUBJECT TO THE FOLLOWING CONDITIONS:		
ALL PROJECTS - The study must not commence until all Research Agreements have been executed (if applicable) - Must comply with the Investigator's Responsibilities in Research Procedure and other Campus Research Policies and Procedures - Any proposed change in the protocol or approved documents or the addition of documents must be submitted to the Human Research Ethics Committee (HREC) for approval prior to implementation, including: - flyers, brochures, advertising material - Increase in recruitment target - The Principal Investigator must notify Research Development & Ethics of: - Any serious adverse effects of the study on participants and steps taken to deal with them. - Any unforeseen events (e.g. protocol violations or complaints). - Investigators withdrawing from or joining the project. - A progress report must be submitted annually and at the conclusion of the project. - RCH HREC approval must remain current for the entire duration of the project. If the project is not completed in the allocated time a renewal request must be submitted to the Research Development & Ethics. Investigators undertaking projects without current HREC approval risk their indemnity, funding and publication rights. CLINICAL TRIALS - Must comply with Good Clinical Practice (GCP) - Must report all internal (occurring in RCH participants) Serious Adverse Events (SAE) to the sponsor and the RCH HREC within 72 hours of occurrence. - Must report all Suspected Unexpected Serious Adverse Reactions (SUSARS) to the Therapeutic Goods Administration (TGA) (for sponsored studies the sponsor may take this responsibility).		

ETHICS APPROVAL & GOVERNANCE AUTHORISATION



17 November 2015

Prof Mimi Tang
Allergy & Immunology
The Royal Children's Hospital

Dear Prof Tang

Project Title: Open pilot study of Probiotic and Peanut Oral Immunotherapy (PPOIT) with shortened buildup phase: PPOIT-II

RCH HREC Reference Number: 35207A

I am pleased to advise that the above project has received ethical approval from The Royal Children's Hospital Melbourne Human Research Ethics Committee (HREC). The project has also received governance authorisation at the Melbourne Children's Campus (incorporating The Royal Children's Hospital, Murdoch Children's Research Institute and the University of Melbourne Department of Paediatrics).

The Royal Children's Hospital Melbourne HREC is organised and operates in accordance with the National Health and Medical Research Council's (NHRC) National Statement on Ethical Conduct in Research Involving Humans (2007) and all subsequent updates, and in accordance with the Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95), the Health Privacy Principles described in the Health Records Act 2001 (Vic) and Section 95A of the Privacy Act 1988 (and subsequent Guidelines).

HREC Approval Date: 17 November 2015

Please note the HREC are no longer issuing pre-determined approval periods. Ethical approval is now ongoing, subject to the submission of an annual report on the anniversary of approval.

Participating Sites:

Ethical approval for this project applies at the following sites:

Site Name
Melbourne Children's Campus (incorporating The Royal Children's Hospital, Murdoch Children's Research Institute and the University of Melbourne Department of Paediatrics)

Approved Documents:

The following documents have been reviewed and approved:

Document	Version	Date
Protocol	3	Nov 2015
PGIS & CF	2	22 Oct 2015
Telephone Script	1	Sep 2015
FAQLQ-PF	1	Sep 2015
FAQLQ-TF	1	Sep 2015
Questionnaire T0	1	Sep 2015
Questionnaire T1-3	1	Sep 2015
Questionnaire T4-5	1	Sep 2015

Conditions of Ethics Approval:

- You are required to submit to the HREC:

Page 1 of 2

- An Annual Progress Report (that covers all sites listed on approval) for the duration of the project. This report is due on the anniversary of HREC approval. Continuation of ethics approval is contingent on submission of an annual report, due within one month of the approval anniversary. Failure to comply with this requirement may result in suspension of the project by the HREC.
- A comprehensive Final Report upon completion of the project.
- Submit to the reviewing HREC for approval any proposed amendments to the project including any proposed changes to the Protocol, Participant Information and Consent Form/s and the Investigator Brochure.
- Notify the reviewing HREC of any adverse events that have a material impact on the conduct of the research in accordance with the NHMRC Position Statement: *Monitoring and reporting of safety for clinical trials involving therapeutic products May 2009*.
- Notify the reviewing HREC of your inability to continue as Coordinating Principal Investigator.
- Notify the reviewing HREC of the failure to commence the study within 12 months of the HREC approval date or if a decision is taken to end the study at any of the sites prior to the expected date of completion.
- Notify the reviewing HREC of any matters which may impact the conduct of the project.
- If your project involves radiation, you are legally obliged to conduct your research in accordance with the Australian Radiation Protection and Nuclear Safety Agency Code of Practice 'Exposure of Humans to Ionizing Radiation for Research Purposes' Radiation Protection series Publication No.8 (May 2005)(ARPANSA Code).
- The HREC, authorising institution and/or their delegate/s may conduct an audit of the project at any time.

Yours sincerely



Erica Plummer
Senior Ethics Officer
 The Royal Children's Hospital Melbourne
 Phone : (03) 9345 5044
 Email : rch.ethics@rch.org.au
 Web : www.rch.org.au

Appendix 2: Trial registration details



Trial Review

[VIEW TRIAL AT REGISTRATION](#)[VIEW HISTORY](#)[< BACK](#)

Trial registered on ANZCTR

Trial ID	ACTRN12608000594325
Ethics application status	Approved
Date submitted	31/10/2008
Date registered	25/11/2008
Type of registration	Prospectively registered

Titles & IDs

Public title	Study of the effectiveness of Probiotics and Peanut Oral Immunotherapy (P-POIT) in inducing desensitisation or tolerance in children with peanut allergy
Scientific title	Study of the effectiveness of Probiotics and Peanut Oral Immunotherapy (P-POIT) in inducing desensitisation or tolerance in children with peanut allergy
Secondary ID [1]	New secondary ID. Please modify.
Universal Trial Number (UTN)	
Trial acronym	P-POIT

Health condition

Health condition(s) or problem(s) studied:

Peanut allergy

Condition category

Inflammatory and Immune System

Condition code

Allergies

Intervention/exposure

Study type	Interventional
Description of intervention(s) / exposure	Lactobacillus rhamnosus GG (LGG) and Peanut Oral Immunotherapy LGG - 1 scoop delivering 2x10E10 Colony-forming units (cfu) of LGG taken once daily for 18 months dissolved into water, soy or cow's milk, at a temperature NOT exceeding 45 °C Peanut Oral Immunotherapy (OIT) - In the modified rush phase, subjects will receive increasing doses of peanut oral immunotherapy, starting at 0.1mg peanut protein with doubling of the dosage every 30 minutes to reach a final dose of 12mg of peanut protein (in total 8 doses delivered in 1 day, with a cumulative dose of 24mg peanut protein). In the buildup phase, the daily dose of peanut OIT will start at 24mg peanut protein and will be increased every 2 weeks until a maintenance dose of 2g is reached (expected to take 8 months). Dosage increments are as follows :- 24mg, 25mg, 50mg, 75mg, 100mg, 125mg, 150mg, 200mg, 250mg, 330mg, 425mg, 550mg, 715mg, 925mg, 1.2g, 1.55g and 2g. If mild allergic symptoms develop (urticaria, angioedema, vomiting, abdominal pain), the dose is repeated before increasing. If moderate/severe allergic symptoms occur (stridor, wheeze) the dose will be reduced by 25% before proceeding. If severe anaphylaxis develops (reduced BP, loss of consciousness) OIT will be discontinued. The dose is maintained at 2g for about 10 months, but will be shorter for subjects who take longer to reach maintenance dose. Peanut oral immunotherapy will administered orally in all phases with the peanut protein sprinkled and mixed into yoghurt. The overall duration of the intervention is 18 months.
Intervention code [1]	Treatment: Other

Comparator / control treatment	OIT placebo is maltodextrin, a glucose polymer mixed with peanut flavour and powdered brown food colouring so it looks, tastes and smells like the peanut product. OIT placebo will be given in exactly the same amount and frequency and for the same duration as the peanut protein as described above and will be administered orally, sprinkled and mixed into yoghurt. The probiotic placebo will be maltodextrin, administered as 1 scoop once daily for 18 months (the same amount, frequency and duration as the treatment group) dissolved into water, soy or cow's milk, at a temperature NOT exceeding 45 °C.
Control group	Placebo
Outcomes	
Primary outcome [1]	To examine whether combined treatment with probiotic and peanut OIT (P-POIT) in children with peanut allergy will induce tolerance to peanut in a greater proportion of children compared with placebo, where tolerance is defined as the ability to tolerate 8g of peanut protein in an oral peanut challenge performed 2 to 12 weeks after discontinuation of P-POIT (in those who pass the first challenge on the last day of P-POIT)
Timepoint [1]	2-12 weeks after discontinuation of P-POIT
Secondary outcome [1]	To examine whether combined treatment with probiotic and peanut OIT (P-POIT) in children with peanut allergy will result in desensitisation to peanut in a greater proportion of children compared with placebo, where desensitisation is defined as the ability to tolerate >2g of peanut protein in an oral peanut challenge performed on the last day of oral immunotherapy.
Timepoint [1]	On day of discontinuation of P-POIT.
Secondary outcome [2]	To examine the effects of combined treatment with probiotic and peanut OIT (P-POIT) or placebo on serum levels of peanut specific Immunoglobulin E (IgE) and peanut specific Immunoglobulin G4 (IgG4), and salivary levels of peanut specific Immunoglobulin A (IgA) in children with peanut allergy. IgE will be measured by Capsulated Hydrophobic Carrier Polymer- Radioallergosorbent Test (CAP-RAST) in our laboratory using the UNICAP system (Pharmacia Diagnostics). IgG4 and IgA will be measured by Enzyme-Linked Immunosorbent Assay (ELISA) at our collaborator, Wesley Burks' lab in Duke University Medical Centre, USA.
Timepoint [2]	4, 8, 12, 18, 22 and 23 months
Secondary outcome [3]	To examine the effects of combined treatment with probiotic and peanut OIT (P-POIT) or placebo on peripheral blood T regulatory cell (Treg) and dendritic cell (DC) populations and T helper 1 and T helper 2 cytokine production. Mononuclear cells (MNC) will be separated from heparinised blood by density gradient centrifugation within 8 hours and cryopreserved (20x10⁶ cells/ml) at each timepoint. Treg and DC populations will be assessed by flow cytometry in freshly thawed and cultured MNC stimulated with anti-CD3, Ara h 2, crude peanut extract or tetanus antigens. Treg will be identified as CD3⁺CD4⁺CD25^{hi}CD127^{lo} cells and CD3⁺CD4⁺CD25^{hi}Foxp3⁺ cells. Proliferating CD4⁺ cells will be identified by reduced Carboxyfluorescein succinimidyl ester (CFSE) staining and antigen specific Treg will be identified within this population by staining for Foxp3⁺ or CD127^{lo} cells. DC will be assessed by flow cytometry - plasmacytoid dendritic cells (pDC) and myeloid dendritic cells (mDC) will be identified as CD11c^{lo}CD123^{whi} or CD11c^{hi}CD123^{wlo} cells within the lineage⁻/HLADR⁺ cell population, respectively. T helper and Treg cytokines will be assessed in MNC cultures stimulated with PHA, anti-CD3, tetanus toxoid, crude peanut extract, Ara h 2 or media alone at each timepoint. IFNγ, IL4, IL13, TGF-β and IL10 will be assayed by multiplex array.
Timepoint [3]	4, 8, 12, 18, 22 and 23 months
Eligibility	
Key inclusion criteria	Children are eligible for the study if they: 1) Are aged between 1 year and 10 years of age 2) Are above 10kg 3) There is a confirmed diagnosis of peanut allergy as defined by: -a positive food challenge to peanut in the past 2 years AND a positive skin prick test (SPT) or Capsulated Hydrophobic Carrier Polymer- Radioallergosorbent Test (CAP-RAST) to peanut - OR -a positive food challenge or history of reaction to peanut ever AND a positive SPT greater than or equal to 8mm or a CAP-RAST greater than or equal to 15KU/I to peanut
Minimum age	1 Years
Maximum age	10 Years
Gender	Both males and females
Can healthy volunteers participate?	No
Key exclusion criteria	Children will not be eligible for the study if they have a history of: 1) severe anaphylaxis (as defined by hypotension, collapse, loss of consciousness or hypoxia) 2) Forced Expiratory Volume in 1 Second (FEV₁) of <85% at rest or ongoing chronic persistent asthma (as

per Australian Asthma Foundation guidelines)
 3) Underlying medical conditions (eg cardiac disease) that increase the risks associated with anaphylaxis
 4) Known wheat allergy (the placebo may contain traces of wheat)
 5) Use of beta-blockers
 6) Inflammatory intestinal conditions, indwelling catheters, gastrostomies or other conditions that may increase the risks of probiotic associated sepsis
 7) Cow's milk allergy, as there may be traces of cow's milk in the probiotic preparation
 8) Already taking probiotics

Study design

Purpose of the study	Treatment
Allocation to intervention	Randomised controlled trial
Procedure for enrolling a subject and allocating the treatment (allocation concealment procedures)	All screened subjects will be given a screening number and if deemed eligible, will be assigned a study number. This study number will correspond to a number on a computer-generated list to determine which treatment arm the subject is assigned to. This list will be generated at the hospital's Biostatistics Unit and delivered to the Pharmacy Department in a sealed opaque envelope. Peanut/placebo product is concealed in coloured sachets and only the Pharmacy Department has the codes to unblind the the product. Probiotic/placebo product is concealed in tins which have been randomized by the supplier (Nestle) and the randomization code was sealed in an opaque envelope and mailed directly to the Pharmacy Department.
Methods used to generate the sequence in which subjects will be randomised (sequence generation)	Randomisation will be from a computer-generated list of random numbers in short permuted blocks prepared by the Clinical Epidemiology and Biostatistics Unit (CEBU) in the Royal Children's Hospital (RCH) and will be used by the Pharmacy department to distribute oral immunotherapy and probiotic treatments. Randomisation will be stratified by: 1) age 5 years or less and 6 years or older, and 2) skin prick test (SPT) wheal size 10mm or less and greater than 10mm. The nurse delivering the oral immunotherapy will not know which intervention arm of the study the patient is in – i.e. the study will be double blind.
Masking / blinding	Blinded (masking used)
Who is / are masked / blinded?	
Intervention assignment	Parallel
Other design features	
Phase	Not Applicable
Type of endpoint(s)	Efficacy

Recruitment

Anticipated date of first participant enrolment	1/12/2008
Actual date of first participant enrolment	
Anticipated date last participant enrolled	
Actual date last participant enrolled	
Target sample size	90
Actual sample size	
Recruitment status	Recruiting

Recruitment in Australia
Recruitment state(s)

Funding & Sponsors

Funding source category [1]	Charities/Societies/Foundations
Name [1]	Food Allergy and Anaphylaxis Network (FAAN)
Address [1]	11781 Lee Jackson Hwy., Suite 160 Fairfax, VA 22033-3309
Country [1]	United States of America
Funding source category [2]	Commercial sector/Industry

Name [2]	Macquarie Bank Foundation
Address [2]	No 1 Martin Place Sydney NSW 2000
Country [2]	Australia
Funding source category [3]	Other
Name [3]	Murdoch Children's Research Institute
Address [3]	Flemington Rd, Parkville, VIC 3052
Country [3]	Australia
Primary sponsor type	Hospital
Name	Royal Children's Hospital
Address	Royal Children's Hospital Flemington Road, Parkville Vic. 3052
Country	Australia
Secondary sponsor category [1]	Other Collaborative groups
Name [1]	Murdoch Children's Research Institute
Address [1]	Royal Children's Hospital Flemington Road, Parkville Vic. 3052
Country [1]	Australia

Ethics approval

Ethics application status	Approved
Ethics committee name [1]	RCH Human Research Ethics Committee
Ethics committee address [1]	Royal Children's Hospital Flemington Road, Parkville Victoria 3052
Ethics committee country [1]	Australia
Date submitted for ethics approval [1]	
Approval date [1]	11/08/2008
Ethics approval number [1]	27086

Summary

Brief summary	A two arm randomised-controlled trial to test a novel therapy (Peanut Oral Immunotherapy with a Probiotic adjunct) against placebo. The findings of this study will provide much needed evidence on whether OIT combined with a probiotic adjunct can induce tolerance in children with peanut allergy.
Trial website	
Trial related presentations / publications	
Public notes	

Contacts

Principal investigator

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Country
Phone
Fax
Email

Contact person for public queries

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Trial Review

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Trial registered on ANZCTR

Trial ID	ACTRN12615001275550
Ethics application status	Approved
Date submitted	28/10/2015
Date registered	23/11/2015
Type of registration	Prospectively registered

Titles & IDs

Public title	Open study of Probiotic and Peanut Oral Immunotherapy (PPOIT) with shortened buildup phase for the treatment of peanut allergy in children.
Scientific title	Safety and efficacy of Probiotic and Peanut Oral Immunotherapy (PPOIT) with shortened buildup phase for the induction of sustained unresponsiveness in children with peanut allergy.
Secondary ID (s)	NIL
Universal Trial Number (UTN)	
Trial acronym	PPOIT-II

Health condition

Health condition(s) or problem(s) studied:

Peanut allergy
Food Allergy

Condition category
Inflammatory and Immune System

Condition code
Allergies

Intervention/exposure

Study type	Interventional
Description of intervention(s) / exposure	PEANUT FLOUR Peanut Flour (50% peanut protein) is purchased from a US company (Golden Peanut Company, LTD). PROBIOTIC (Lactobacillus rhamnosus GG; ATCC 53103) The probiotic to be used is Lactobacillus rhamnosus GG. Probiotic will be prepared under strict Food Manufacturing Regulations. Supply will be as dry powder. The daily dose of 2×10^{10} cfu will be measured using a 4g scoop. Participants will be instructed to mix the probiotic in water, soy milk or cow's milk, at a temperature NOT exceeding 38 degrees Celsius. TREATMENTS AND DOSAGE DAY 1 RUSH INDUCTION * In the rush phase, subjects will receive increasing doses of peanut oral immunotherapy every 20 minutes to reach a final dose of 12mg of peanut protein (cumulative dose 24mg peanut protein). * There will be a maximum of 8 doses administered at a starting dose of 0.1mg. * Peanut protein will be sprinkled onto food (yoghurt not containing LGG). * Subjects will receive a single dose of 2×10^{10} cfu LGG (one level scoop mixed into 100 ml of water, at a temperature NOT exceeding 38 degrees Celsius) prior to initiation of the rush phase. * Probiotic will be administered just prior to the first dose of peanut protein on the rush day and once daily just prior to peanut protein doses throughout the build up and maintenance phases. * Subjects will be monitored (including at least half hourly vital signs and general nursing assessment of the skin and chest) for 2 hours after the last dose during modified rush. * The study doctor and study nurse will be present at all times during the rush phase

- * The rush phase will be performed in hospital.
- * Spirometry will be performed on all participants aged 8 or older, and younger participants who are capable of doing spirometry reproducibly, before the participant receives their first dose of peanut oral immunotherapy and 2 hours after receiving the final dose of Day 1 rush induction.
- * Subjects who complete the rush protocol without reaction will commence the Buildup Phase at a daily dose of 25mg peanut protein (Dose g) on the day after the rush induction day.
- * If a subject reacts to one of the doses during rush induction, the rush schedule will be ceased and they will commence the Buildup Phase at the dose immediately below the reaction-eliciting dose starting on the day after the rush induction day. The remaining RUSH doses that were not completed on day 1 will be incorporated into the buildup phase (modified buildup schedule for that subject) and subsequent incremental dose increases will proceed through all remaining doses of the RUSH schedule followed by the doses in the buildup schedule. For example, if a reaction occurs following dose 6, the subject will commence the buildup phase at the dose 5 amount and will be instructed to start this reduced dose on the following day).

BUILD UP PHASE (16 WEEKS DURATION)

In the buildup phase, the daily dose of peanut OIT will be increased every 2 weeks until a maintenance dose of 2000mg is reached (expected to take 16 weeks).

- * Each dose increase will be administered in hospital under medical supervision.
- * Hospital visits for dose increases (updose visits) will be scheduled every 2 weeks. Where indicated, dose adjustments will result in deferment of a dose increase to the next scheduled visit.
- * Spirometry will be performed on all participants aged 8 or older, and younger participants who are capable of doing spirometry reproducibly, before the participant receives their first increased dose of peanut oral immunotherapy and 2 hours after receiving the dose.
- * Peanut protein will be sprinkled onto food (e.g. yoghurt not containing LGG).
- * Subjects will also take a fixed dose of 2×10^{10} cfu LGG (one level scoop mixed into 100 ml of water, at a temperature NOT exceeding 38 degrees Celsius) once daily prior to the OIT treatment.
- * Subjects will be monitored for 2 hours after the treatment has been administered.

MAINTENANCE PHASE

A maintenance phase, during which participants take a daily dose of 2g peanut protein and a daily dose of 2×10^{10} cfu LGG at home, is continued until a total of 18 months PPOIT treatment is completed. If the subject has not completed a minimum of 6 months on maintenance dosing at 18 months, the total duration of treatment will be extended to ensure a minimum of 6 months maintenance dosing.

STRATEGIES TO MONITOR ADHERENCE

We will monitor adherence by daily dosing diary and weighing / counting contents of returned treatment packages.

Intervention code [1]	Treatment: Other
Comparator / control treatment	No control group
Control group	Uncontrolled

Outcomes

Primary outcome [1]	a. The proportion of children who can complete the buildup phase within 17 weeks - this is assessed by review of study records and allows an additional 1 week to suit family schedules.
Timepoint [1]	17 weeks from the first day of OIT.
Primary outcome [2]	b. The number of adverse events (AE) and serious adverse events (SAE) per participant during the buildup phase.
	Definitions and examples of known/possible adverse reactions and how they will be assessed is detailed below.
	Adverse Event (AE): Any untoward medical occurrence in a patient enrolled into this study regardless of its causal relationship to study treatment. Adverse events are classified as serious or non-serious (see below for definition of a serious adverse event).
	Serious Adverse Event (SAE): An SAE is defined as any AE that: * Results in death; or * Is immediately life threatening; or * Requires inpatient hospitalisation; or * Requires prolongation of existing hospitalisation; or * Results in persistent or significant disability/incapacity; or * Is a congenital anomaly/birth defect.
	AE and SAE will be assessed either observed by the investigator or one of the clinical staff at the study visit/assessment, or reported by the patient spontaneously or in response to a direct question will be evaluated by the investigator, or from the daily dosing diary that captures doses taken and any reactions experienced. The parent will be asked a non-leading question such as: "In general, how has your child been feeling since starting the new treatment/ the last assessment?"
	Examples of AE are: *Mild: Transient or mild discomforts (<48 hours), no or minimal medical intervention/therapy required. These symptoms may include pruritus, swelling or rash, abdominal discomfort or other transient. * Moderate: Symptoms that produce mild to moderate limitation in activity some assistance may be needed; no or minimal medical intervention/therapy is required. Hospitalisation is possible. These symptoms may include persistent hives, wheezing without dyspnea, abdominal discomfort/ increased vomiting or other symptoms. * Severe: Marked limitation in activity, some assistance usually required; medical intervention/therapy

required, hospitalization is possible. Symptoms may include Bronchospasm with dyspnea, severe abdominal pain, throat tightness with hoarseness, transient hypotension among others. Parenteral medication(s) are usually indicated.

Examples of SAE are:

Extreme limitation in activity, significant assistance required; significant medical therapy / intervention is required; hospitalisation is probable. Symptoms may include persistent hypotension and/or hypoxia with resultant decreased level of consciousness associated with collapse and/or incontinence or other life threatening symptoms.

Timepoint [2]	The number of AE and SAE reported during buildup phase.
Secondary outcome [1]	a. The proportion of children who are desensitized – this will be determined by performing double blind placebo controlled food challenge (DBPCFC) at end of treatment (T2)
Timepoint [1]	T2: 18 months after the first day of OIT (the last day of treatment)
Secondary outcome [2]	b. The proportion of children who attain sustained unresponsiveness – this will be determined by performing DBPCFC 8 weeks after treatment is completed (T3) with strict avoidance of peanut intake between T2 and T3
Timepoint [2]	T3: 8 weeks after treatment has ceased
Secondary outcome [3]	c. The proportion of children who attain sustained unresponsiveness at T3 and maintain long-term sustained unresponsiveness – this will be determined by performing DBPCFC three years after treatment is ceased (T5) after a period of peanut elimination for at least 8 weeks
Timepoint [3]	T5: 3 years after treatment has ceased
Secondary outcome [4]	d. Change in peanut skin prick test wheal size from T0, T1, T2, T3, T4 & T5
Timepoint [4]	T0: First day of OIT T1: 16 weeks after the first day of OIT T2: 18 months after the first day of OIT (the last day of treatment) T3: 8 weeks after treatment has ceased T4: 12 months after treatment has ceased T5: 3 years after treatment has ceased
Secondary outcome [5]	e. Change in immunological measures (sIgE and sIgG4) from T0, T1, T2, T3, T4 & T5 - this will be determined by measurement of these parameters in blood samples.
Timepoint [5]	T0: First day of OIT T1: 16 weeks after the first day of OIT T2: 18 months after the first day of OIT (the last day of treatment) T3: 8 weeks after treatment has ceased T4: 12 months after treatment has ceased T5: 3 years after treatment has ceased
Secondary outcome [6]	f. Quality of life at T0, T2, T3, T4 & T5 – this will be measured using validated Food Allergy Quality of Life Questionnaires. The Parent Form (FAQLQ-PF) will be used for children 0-12 years and the Child Form (FAQLQ-TF) for children > 12 years during the follow up period (ref: Dunn-Galvin et al CEA 2008; 38:977).
Timepoint [6]	T0: First day of OIT T2: 18 months after the first day of OIT (the last day of treatment) T3: 8 weeks after treatment has ceased T4: 12 months after treatment has ceased T5: 3 years after treatment has ceased

Eligibility

Key inclusion criteria	Children are eligible for the study if they: * Are aged between 1 year and 12 years of age. * If they are >10kg (weight considered safe for the prescription and administration of an EpiPen) * There is a confirmed diagnosis of peanut allergy as defined by a failed DBPCFC to peanut and a positive SPT or sIgE to peanut at screening.
Minimum age	1 Years
Maximum age	12 Years
Gender	Both males and females
Can healthy volunteers participate?	No
Key exclusion criteria	Children will not be eligible for the study if they have a history of: * Severe anaphylaxis (as defined by hypotension, collapse, loss of consciousness or hypoxia OR ever needing three (3) or more doses of intramuscular adrenaline or needing intravenous adrenaline infusion for management of anaphylactic reaction) * FEV1 <85% at rest or FEV1/FVC < 85% at rest or ongoing chronic persistent asthma (as per Australian Asthma Foundation guidelines) * Underlying medical conditions (eg cardiac disease) that increase the risks associated with anaphylaxis * Use of beta-blockers * Inflammatory intestinal conditions, indwelling catheters, gastrostomies, immune-compromised states, post-cardiac and/or gastrointestinal tract surgery, critically-ill and those requiring prolonged hospitalisation or other conditions that may increase the risks of probiotic associated sepsis * Already taking Probiotics

Study design

Purpose of the study	Treatment
Allocation to intervention	Non-randomised trial
Procedure for enrolling a subject and allocating the treatment (allocation concealment procedures)	Allocation is not concealed
Methods used to generate the sequence in which subjects will be randomised (sequence generation)	Not applicable
Masking / blinding	Open (masking not used)
Who is / are masked / blinded?	
Intervention assignment	Single group
Other design features	Open study
Phase	Not Applicable
Type of endpoint(s)	Safety/efficacy

Recruitment

Anticipated date of first participant enrolment	15/12/2015
Actual date of first participant enrolment	15/12/2015
Anticipated date last participant enrolled	10/02/2016
Actual date last participant enrolled	
Target sample size	20
Actual sample size	
Recruitment status	Recruiting
Recruitment in Australia	
Recruitment state(s)	VIC
Recruitment hospital [1]	The Royal Childrens Hospital - Parkville
Recruitment postcode(s) [1]	3052 - Parkville

Funding & Sponsors

Funding source category [1]	Charities/Societies/Foundations
Name [1]	Murdoch Childrens Research Institute
Address [1]	The Royal Children's Hospital Flemington Road, Parkville VIC 3052
Country [1]	Australia
Primary sponsor type	Charities/Societies/Foundations
Name	Murdoch Childrens Research Institute
Address	The Royal Children's Hospital Flemington Road, Parkville VIC 3052
Country	Australia
Secondary sponsor category [1]	None
Name [1]	
Address [1]	
Country [1]	

Ethics approval

Ethics application status	Approved
Ethics committee name [1]	The Royal Children's Hospital Human Research Ethics Committee
Ethics committee address [1]	Flemington Road Parkville VIC 3052
Ethics committee country [1]	Australia
Date submitted for ethics	09/09/2015

approval [1]
 Approval date [1] 19/11/2015
 Ethics approval number [1] HREC 35207A

Summary

Brief summary

At present there is no cure for food allergy. People with a food allergy need to avoid the food they are allergic to in order to stay safe. However we know that accidental exposure is common. Research shows that 50% of children with a peanut allergy are accidentally exposed to peanut within 2 years.

Researchers have begun to look at the effectiveness of 'oral immunotherapy' as a treatment for food allergy. In oral immunotherapy, patients with food allergy are given small amounts of the food they are allergic to and tested for food allergy after a set amount of time. Results have been mixed. Studies suggest that oral immunotherapy can induce desensitization (short term ability to tolerate the food allergen while the patient continues on therapy) but has a limited ability to induce sustained unresponsiveness (longer term ability to tolerate the food allergen after treatment is stopped for at least 2-4 weeks or longer). We conducted a RCT to evaluate a novel combination treatment approach involving administration of probiotic together with oral immunotherapy - Probiotic and Peanut Oral Immunotherapy (PPOIT). In our study we found that just over 80% of children who received PPOIT tolerated peanut after stopping treatment for more than 2 weeks compared with only 4% in the placebo group. PPOIT was highly effective at inducing sustained unresponsiveness - if 9 children were treated with PPOIT, 7 would benefit.

PPOIT participants received a daily dose of probiotic together with peanut protein (peanut flour) for 18 months. The probiotic was taken as a fixed daily dose. The dose of peanut protein was commenced at very low levels then increased every 2 weeks over a period of 8 months to reach a maintenance dose of 2g peanut protein.

This study (PPOIT-II) will build on our previous PPOIT study. PPOIT-II is an open study to investigate whether we can increase the dose of peanut protein over 4 months (instead of 8 months), similar to other forms of immunotherapy (e.g. for bee venom or pollen immunotherapy). Specifically we want to assess whether this approach is safe and tolerated by participants. 20 children with peanut allergy will take part in the study. Children will take increasing doses of peanut protein and a set amount of probiotic for 16 weeks. Children will then continue on a set maintenance dose until a total of 18 months treatment is completed. Children will be tested for peanut allergy at the start of the study, at the end of PPOIT treatment (18 months) and 2 months and 3 years after treatment.

The discovery of a safe and tolerable treatment for food allergy will have great public health benefit. If this immunotherapy protocol incorporating a shorter buildup phase is well tolerated, this will provide a more acceptable and feasible immunotherapy schedule for patients and families.

Trial website

Trial related presentations / publications

Public notes

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Appendix 3: Follow-on study of PPOIT-001 Trial Parent/Guardian Information Statement and Consent Form

The Royal Children's Hospital Melbourne
50 Flemington Road
Parkville Victoria 3052 Australia
TELEPHONE +61 3 9345 5522
www.rch.org.au



HREC Project Number: 27086

Research Project Title: Long term follow-up study of the effectiveness of Probiotics and Peanut Oral Immunotherapy (OIT) in inducing desensitisation or tolerance in children with peanut allergy

Principal Researcher: Professor Mimi Tang

Version Number: 2 **Version Date:** October 2014

Thank you for taking the time to read this **Parent/Guardian Information Statement and Consent Form**. We would like to invite your child to participate in a research project that is explained below. This document is 5 pages long. Please make sure you have all the pages.

If you speak a language other than English and would like this Information Statement and Consent Form in your language, please ask the person explaining this research project to you.

What is an Information Statement?

These pages tell you about the research project. It explains to you clearly and openly all the steps and procedures of the project. The information is to help you to decide whether or not you would like your child to take part in the research. Please read this Information Statement carefully.

Before you decide to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like your child to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

- understand what you have read
- had a chance to ask questions and received satisfactory answers
- consent to your child taking part in the project.

We will give you a copy of this information and consent form to keep.

1. What is the research project about?

Peanut allergy is common and affects up to 1.5% of children. Reactions may occur with exposure to very small amounts of peanut. Reactions to peanut may be severe and life threatening. Unlike other food allergies, peanut allergy is usually lifelong.

Current treatment of peanut allergy is to strictly avoid peanut in the diet. However this can be difficult and we know that around 50% of peanut allergic people are accidentally exposed to peanut within 1 year of being diagnosed with peanut allergy. Therefore, children with peanut allergy may be placed at continued risk of severe or life threatening reactions.

This research study is trying to find out whether peanut oral immunotherapy (giving small but increasing amounts of peanut protein at regular intervals) plus a probiotic will result in:

- **tolerance** to peanut - the longstanding ability to tolerate peanut after immunotherapy is ceased or after not eating peanut for at least 4 weeks ie, a cure for peanut allergy; or
- **desensitisation** to peanut - the ability to tolerate peanut whilst on peanut immunotherapy or whilst still

- eating peanut regularly, or
- **no change** to peanut allergy – the treatment may prove ineffective.

Studies have shown that peanut oral immunotherapy may bring on desensitisation to peanut in peanut allergic children. In this research study we are trying to find out whether the use of a probiotic may help in building tolerance and desensitisation to peanut.

Probiotics are live microorganisms which are beneficial to your health when administered in adequate amounts. The probiotic supplement has not been registered/licensed/approved for marketing in Australia and will be used in this study for experimental purposes only.

2. Who is funding this research project?

This study is not sponsored by a company and has been initiated by Professor Mimi Tang from The Royal Children's Hospital, Melbourne. The researchers have no financial gain to make from this project.

3. Why am I being asked to be in this research project?

We are asking your child to take part in this project because your child took part in the Study of the effectiveness of Probiotic and Peanut Oral Immunotherapy (OIT) in inducing desensitisation or tolerance in children with peanut allergy (PPOIT study). If your child was found to be 'tolerant' to peanut at the end of the PPOIT study (passed both peanut challenges), we now want to see if they have remained peanut tolerant. If your child was found to be peanut allergic at the end of the PPOIT study (failed one of the peanut challenges), we now want to see if they have remained allergic to peanut or if they have grown out of their peanut allergy.

4. What does participation in this research involve?

We would like you to sign a new consent form to allow us to conduct a follow-up visit with your child at the RCH Hospital which would involve the following:

- A Quality of Life questionnaire and General food questionnaire
- A skin prick test
- A double blind placebo controlled peanut food challenge (over 1 or 2 days)
- Providing a stool sample/rectal swab and a saliva sample
- Blood Test (optional)
- Assessment by one of the allergy nurses/doctors - at this interview we will also discuss the findings of the study and what this means for you and your child.

It will take 7 hours to complete the food challenge together with a clinical assessment by an allergy nurse and/or doctor.

Participation in this study will be to complete the above assessments at the RCH hospital so we can assess the impact the study has had on your child and their family's life. We would like to know how the children in the study are going - both those who now eat peanut and those who do not. If your child was told they were tolerant at the end of the PPOIT study, we want to see if tolerance has been maintained. If your child was told that they were allergic to peanut at the end of the PPOIT study, we want to find out if they have grown out of their Peanut Allergy.

You will not know which treatment group your child was in. The research team will not know which group your child was in.

Blood, saliva swab and stool samples will be stored and batch analysed once samples from all consenting participants have been collected. You have the option of consenting to or not consenting to the storage of any leftover blood, saliva, swab and stool samples for use in **future ethically approved** research related to the study of probiotic effects on the immune response.

5. What are my alternatives to taking part?

Participation in a research project is voluntary. It is your choice whether your child will take part in this research. You do not have to agree if you do not want to.

If you give your consent and change your mind, you can withdraw your child from the project. You do not need to tell us the reason why you want your child to stop being in the project. If your child leaves

the project we will use any information already collected unless you tell us not to.

Your decision will not affect any treatment or care your child will get, or your child's relationship with The Royal Children's Hospital.

6. What are the possible benefits for me and other people in the future?

You and your child will have contact with the Research team and the RCH Allergy Immunology Department and have an accurate assessment of your child's peanut allergic status. For other people in the future, the findings from this study will help to show if peanut oral Immunotherapy may be a useful treatment for peanut allergy.

7. What are the possible risks, side-effects, discomforts and/or inconveniences?

Blood test:

We will use some cream to numb your child's skin before taking a blood sample. However, your child may still feel some discomfort. A bruise may appear at the site of the blood test and we will use cotton wool and a band-aid to reduce the bruising.

Saliva sample

Saliva sample is collected by chewing on a piece of gauze to soak up the saliva, there should be no discomfort from this.

Skin prick test:

This may cause your child some discomfort. The skin may become itchy. We can use some cream to reduce the itching. There is a small risk (about 1 in 10,000) of a serious allergic reaction. If this happens we will treat your child with the appropriate medicines.

Stopping eating peanut for 4-8 weeks before the Food Challenge:

Children who are desensitised (that is, those that are not truly tolerant, but have maintained their ability to eat peanuts by eating them very regularly) may be at risk of losing their desensitised state when they stop eating peanut for 4-8 weeks prior to the Food Challenge.

However, from a long term safety point of view, it is important that children in this study find out whether or not they have become 'truly tolerant', that is being able to tolerate peanut after stopping peanut in the diet for at least 4 weeks.

If your child has been eating peanut regularly, but fails the peanut Food Challenge in this follow-up study, this will mean that they were only desensitised (not tolerant). If they are found to be desensitised but not tolerant, they can choose to either stop eating peanut in their diet completely or recommence peanut at the last dose they tolerated without reaction during the Food Challenge. The study team will discuss these options with you and your child at the end of their challenge.

Food Challenge:

Your child may have a reaction when challenged to peanut. *It is difficult to quantify the actual likelihood or risk of having a reaction. **The likelihood of having a reaction during the peanut challenge is very high if your child did not gain tolerance to peanut in the first part of this study.*** If your child is tolerant they will not react to the peanut challenge.

If your child reacts during the challenge, the reaction may be mild (rash, facial swelling, vomiting, diarrhoea, tummy pain) or severe (involving breathing problems or low blood pressure or collapse). ***Very rarely patients may die as a result of an allergic reaction.***

All challenges will be performed at The Royal Children's Hospital. A doctor will be present at all challenges and be able to give medications to treat the reaction immediately.

Mild allergic reactions will be treated with antihistamine medicines.

Severe reactions may be life threatening. Severe reactions will be treated with adrenaline (EpiPen) and you may need to be admitted to hospital overnight.

There may be unknown or unforeseen risks associated with this study.

Injury during the trial

In the unlikely event that your child suffers an injury as a result of participating in this trial, hospital care and treatment will be provided by the public health service at no extra cost.

8. What will be done to make sure my child's information is confidential?

Any information we collect about your child will remain confidential. Only the researchers involved with this study and The Royal Children's Hospital Ethics Committee can have access to this information. We can disclose the information only with your permission, except as required by law.

You have the right to look at, and ask correction of, your child's information in accordance with the Freedom of Information Act 1982 (Vic).

The information will be de-identified. This means that we will remove your child's name and give the information a special code number. Only the research team will be able to break the code to match your child's name to his/her code number.

All information will be stored securely in a locked filing cabinet in the Department of Allergy and Immunology and also within the hospital pharmacy at The Royal Children's Hospital. Your child's information will also be stored on a password-protected computer database.

We will keep the information until your child turns 25 years old. We may use your child's information for other research studies during this time, without personal or identifying details. After this time, we will destroy the information by shredding documents and/or deleting computer files.

Blood and saliva samples will be destroyed at the end of the study.

When we write or talk about the results of this project, we will report information about the whole group of participants. This means that no one will be able to identify your child.

9. Will I be informed of the results when the research project is finished?

We will send you a short report of the overall results when the study is finished. The report will only contain information of the whole group of participants.

If you would like more information about the project or if you need to speak to a member of the research team in an emergency please contact:

Christine Axelrad, PPOIT study coordinator
Tel: 0414 577 213 or (03) 9345 6974
Email: Christine.axelrad@rch.org.au

If you have any concerns and/or complaints about the project, the way it is being conducted or your rights as a research participant, and would like to speak to someone independent of the project, please contact:

Director, Research Ethics & Governance, The Royal Children's Hospital Melbourne on telephone: (03) 9345 5044.

CONSENT FORM

HREC Project Number: 27086

Research Project Title: Long term follow-up study of the effectiveness of Probiotics and Peanut immunotherapy (OIT) in inducing desensitisation or tolerance in children with peanut allergy

Version Number: 2 **Version Date:** October 2014

- I voluntarily consent for my child to take part in this research project.
- I believe I understand the purpose, extent and possible risks of my child's involvement in this project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I understand I will receive a copy of this Parent/Guardian Information Statement and Consent Form.

OPTIONAL CONSENT

☐ I do	☐ I do not	consent to doing a Double Blind Placebo Controlled Food Challenge(DBPCFC)
☐ I do	☐ I do not	consent for skin prick testing, saliva collection, stool or rectal swab and blood collection.
☐ I do	☐ I do not	consent for the storage of my information as well as the collected blood, saliva, swab and stool samples for use in <u>future ethically approved</u> research related to the study of probiotic effects on the immune response

Child's Name

Parent/Guardian Name

Parent/Guardian Signature

Date

Name of Witness to Parent/Guardian's Signature

Witness Signature

Date

I have explained the project to the parent/guardian who has signed above, and believe that they understand the purpose, extent and possible risks of their child's involvement in this project.

Research Team Member Name

Research Team Member Signature

Date

Note: All parties signing the Consent Form must date their own signature.

Appendix 4: PPOIT-002 Trial Parent/Guardian Information Statement and Consent Form

The Royal Children's Hospital Melbourne
50 Flemington Road
Parkville Victoria 3052 Australia
TELEPHONE +61 3 9345 5522
www.rch.org.au



HREC Project Number: 35207

Research Project Title: Open pilot study of Probiotic and Peanut Oral Immunotherapy (PPOIT) with shortened buildup phase: PPOIT-II

Principal Researcher: Professor Mimi Tang, Group Leader, Allergy and Immune Disorders Group, Murdoch Childrens Research Institute

Version Number: 3 **Version Date:** 28/11/2015

Thank you for taking the time to read this **Parent/Guardian Information Statement and Consent Form**. We would like to invite your child to participate in a research project that is explained below.

This document is 10 pages long. Please make sure you have all the pages.

What is an Information Statement?

These pages tell you about the research project. It explains to you clearly and openly all the steps and procedures of the project. The information is to help you decide whether or not you would like your child to take part in the research. Please read this Information Statement carefully.

Before you decide if you want your child to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

Important things you need to know

- It is your choice whether or not your child can take part in the research. You do not have to agree if you do not want to
- If you decide you do not want your child to take part, it will not affect the treatment and care your child gets at The Royal Children's Hospital

If you would like your child to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

- understand what you have read
- had a chance to ask questions and received satisfactory answers
- consent to your child taking part in the project

We will give you a copy of this information and consent form to keep.

1. What is the research project about?

Peanut allergy affects around 1.5% of children. At present there is no cure for food allergy. People with a food allergy need to avoid the food they are allergic to in order to stay safe.

Researchers have begun to look at the effectiveness of '**oral immunotherapy**' as a treatment for food allergy. Oral immunotherapy involves feeding patients with food allergy the food they are allergic to, starting at very low doses and then increasing to a higher maintenance dose that is continued for months to years. We have recently completed a randomized trial involving the combined administration of a probiotic together with oral immunotherapy - **Probiotic and Peanut Oral Immunotherapy (PPOIT)**. We found that PPOIT was highly effective at inducing 'sustained unresponsiveness' - just over 80% of children who received PPOIT tolerated peanut after stopping treatment as compared to only 4% of children who received placebo (dummy treatment).

This study (PPOIT-II) will build on our completed PPOIT study. In the previous PPOIT study, the dose of peanut immunotherapy was increased over a period of 8 months, and children had to return to hospital every 2 weeks to have their dose increased during this 8 month period – a total of 16 visits. We now want to see if the dose of peanut immunotherapy can be increased over a shorter period of time - 4 months; this is similar to other forms of immunotherapy (such as bee venom or pollen immunotherapy) where the doses are increased over 3 to 4 months. This will also reduce the number of visits to hospital for families and make the treatment more feasible to administer when it is used in the clinic.

20 children with peanut allergy will take part in the study. In order to be eligible for this study, your child must complete a double blind placebo controlled peanut challenge to confirm that they have peanut allergy. Once they are enrolled in the study, children will take a dose of probiotic AND a dose of peanut protein, in the form of food grade defatted peanut flour, once daily every day for 18 months. The dose of probiotic will be the same every day for the whole 18 months; however the dose of peanut protein will be started at very small amounts, increased every 2 weeks for at least 16 weeks (the buildup phase) to a top maintenance dose of 2g peanut protein. This maintenance dose will then be continued until a total of at least 18 months is reached.

The **main study aim** will be to see how many children can complete the PPOIT buildup schedule within the 16 weeks. This will tell us whether a shorter buildup phase of PPOIT treatment can be tolerated by children with peanut allergy. **The effect of the PPOIT treatment** will also be assessed by performing up to three further double blind placebo controlled peanut challenges at the end of the 18 month treatment period. All children will undergo a food challenge on the day after stopping treatment (end of treatment challenge); however, only those children who pass this first end of treatment challenge will go on to have a further challenge 8 weeks later.

- If your child passes both the end of treatment challenge and the subsequent challenge 8 weeks after stopping treatment, they have developed 'sustained unresponsiveness'. They are considered to be 'tolerant' to peanut and can introduce peanut into their diet.
- If your child passes the first end of treatment challenge but fails the subsequent challenge 8 weeks after stopping treatment, they are desensitized – this means that they are still allergic to peanut and are only able to tolerate peanut while they are receiving the PPOIT treatment. They will therefore need to avoid peanut in their diet, as is recommended for all children with peanut allergy.
- If your child fails the first end of treatment challenge, they are allergic to peanut and will need to avoid peanut in their diet.

Children who develop 'sustained unresponsiveness' and go on to include peanut in their diet will undergo a final double blind placebo controlled peanut challenge 3 years after PPOIT is stopped to see if the sustained unresponsiveness that was induced by PPOIT is long-lasting. This is very important for safety reasons – it is vital to know if your child has maintained their sustained unresponsiveness or if their peanut allergy has returned.

2. Who is funding this research project?

This study is not sponsored by a company and has been initiated by Professor Mimi Tang at the Murdoch Childrens Research Institute and The Royal Children's Hospital. The study will be supported by funds from Professor Tang's research group at the Murdoch Childrens Research Institute.

3. Why is my child being asked to take part?

We are asking your child to take part in this project because he/she has a peanut allergy and is aged between 1-12 years old.

4. What does my child need to do in this research project?

Study length

This study will continue for 5 years from the time your child is accepted to the study.

Screening

We would like you and your child to come to an interview at The Royal Children's Hospital / Murdoch Childrens Research Institute. This interview is to find out if your child is suitable to take part in the study. At this interview we will:

- Take a history of any previous reaction to peanut and take a detailed medical history.
- Perform a skin prick test on your child.

This interview will take around 40 minutes.

If your child is found to be suitable for the study, we will arrange for them to have a double blind placebo controlled peanut challenge to confirm the presence of peanut allergy and eligibility for the study. This challenge is not required if your child has already completed and failed a double blind placebo challenge in the past 3 months. If a challenge is required, it will be completed in 2 parts over 2 separate days - one day for the placebo part of the challenge and one day for the peanut protein part of the challenge. The challenge will involve:

- 2 visits to the hospital for half a day on each visit.
- 6 increasing doses of peanut protein will be given on one half day and 6 increasing doses of placebo will be given on a different half day. The study team and your child / you will not know whether your child is being given the peanut protein or the placebo on each day.
- On each half day, your child will have an intravenous cannula (a small flexible straw put into a vein using a needle) placed to help with administering any treatments if they have an allergic reaction to the challenge. A blood sample will be taken when the cannula is placed. A topical anaesthetic cream will be used to numb the area before inserting the cannula.

Study treatment

Your child will need to take two study preparations (the probiotic and the peanut protein) everyday for the entire 18 month treatment period. The dose of the peanut protein will be increased during the first 4 months of the study (as described below) however the dose of the probiotic will be the same throughout the entire study.

Visits and Procedures

Day 1 of the treatment:

Your child will be admitted as a day-stay patient to The Royal Children's Hospital.

During the day, we will give your child a dose of probiotic and 8 separate doses of peanut protein. These doses will be given every 20 minutes. The amount of peanut protein given will be increased with each dose.

At the end of the day your child can go home. He/she will be asked to take a dose of peanut protein (based on the amount of peanut protein that your child has tolerated on this day) together with a dose of probiotic once a day for the next 2 weeks.

Buildup phase of the treatment:

During the buildup phase, your child will need to return to the hospital every two weeks to have the dose of peanut protein increased (updose visit). At each updose visit, we will increase the amount of peanut protein to the next dose level. Your child will be asked to take this increased dose of peanut protein together with a dose of probiotic once daily for the next 2 weeks, before returning again for a further dose increase (updose visit). This will continue until your child reaches the maintenance dose of 2g peanut protein. We expect this buildup phase to take about 4 months. During this buildup phase, your child will have 9 visits to the hospital. Some children will have reactions to the peanut protein doses and may take longer than 4 months to reach the maintenance dose – this will mean there are more than 9 visits during the buildup phase.

All increases in the peanut protein dose will take place in the hospital. We need to watch your child in hospital 2 hours after the dose is given.

After each updosing visit, we will ask you to collect a 2-week supply of peanut protein from The Royal Children's Hospital Pharmacy. You will collect a monthly supply of probiotic every second visit.

Dose diary:

A dose diary will be provided at each visit where information on daily doses, accidental ingestion and reactions are recorded. You must fill in these details and return it to the study nurse at the following visit. This information is very important for your child's safety.

Maintenance phase of the treatment:

Following the buildup phase, your child will take a daily 2g maintenance dose of peanut protein together with the probiotic for up to 14 months to complete a total of 18 months of PPOIT treatment. The maintenance phase will be least 6 months. If the buildup phase for your child takes longer than 12 months, the PPOIT treatment will be continued beyond 18 months in order to allow a minimum of 6 months on the top maintenance dose.

We will ask you to collect your child's study treatments from The Royal Children's Hospital Pharmacy every 12 weeks during the maintenance phase (expected to be 14 months).

End of treatments

When the study treatments are stopped:

- We will ask you to complete a quality of life questionnaire
- We would like your child to undergo peanut challenges to assess the effect of the treatment

Peanut challenges:

We would like your child to complete four peanut challenges during the course of the study. All challenges will be performed at The Royal Children's Hospital. Each challenge procedure will take place over two half days, up to one week apart.

- The first challenge will be to confirm your child's eligibility to participate in the study. This is because we need to be sure that your child has peanut allergy to be eligible to participate in the study.
- The second challenge will take place at 18 months, on the day after your child's last study treatment dose (end of treatment challenge).
- The third challenge will take place 8 weeks after treatment is stopped and will only proceed if your child has passed the second food challenge. Your child will not be allowed to eat any peanut or peanut containing food for 8 weeks prior to this food challenge.

- The fourth challenge will take place at 3 years after your child's last dose of study treatment. This will only proceed if your child passed the third food challenge. Your child will not be allowed to eat any peanut or peanut containing food for 8 weeks prior to this food challenge.

Quality of Life Questionnaires

We would like you and your child to complete 5 quality of life questionnaires during the course of the study. You and your child will be asked to complete 4 of these questionnaires on the days when you come in for your child's peanut challenges, before you know the outcome of the peanut challenge. You will be asked to complete 1 of these questionnaires when you come in for your child's non-challenge review appointment, 12 months after completion of treatment. Each questionnaire will take approximately 30 minutes to complete.

Test samples

During the course of the treatment and after the treatment is finished, we will need to collect a total of 6 blood samples and 6 stool samples from your child. The samples will be tested to assess how your child's immune system is responding to the therapy.

We will collect a 20 mL (the equivalent of approximately 4 teaspoons) blood sample from your child:

- on the day of the first peanut challenge, before starting treatment
- approximately 4 months after starting treatment (at the final up dosing visit during the buildup phase)
- on the day of the end of treatment peanut challenge, approximately 18 months after starting treatment
- on the day of the third peanut challenge, approximately 8 weeks after stopping treatment
- on the day of the fourth peanut challenge, approximately 3 years after stopping treatment

Before the blood sample is taken, we will put anaesthetic cream on your child's hand or elbow to numb the skin. About 20 minutes later we will take the blood.

We will provide you with stool collection containers and/or swabs and ask you to collect samples from your child at home and to bring the sample in at the next scheduled visit.

Blood and stool samples will be stored until the immune studies are completed.

Your responsibilities

Participation in this study will be for a total of 5 years, including 18 months of receiving study treatment. Compliance with study procedures, including but not limited to attendance at scheduled visits, adherence to treatment and completion of dose diaries, is critical. In the interest of the safety of your child, non-compliance may result in the study treatment being terminated prematurely for your child.

Informing your GP

We will notify your child's GP at the beginning of the study if your child is eligible to participate. We will also inform your GP of the outcomes of the food challenges at the end of study treatment.

After the study ends

We will notify your child's GP and if you wish, your child's allergy specialist, of your peanut allergy status. We will ask your child's GP and allergy specialist to continue monitoring your child's health.

5. What are the alternatives to taking part?

The alternative to taking part is for your child to get the standard medical care and treatment for their condition. This includes:

- *Continuing strict avoidance of all peanut intake, and*
- *Maintaining an allergy / anaphylaxis management plan, and*
- *Regular monitoring as recommended by your child's allergy specialist*

6. Can my child withdraw from the project?

If you give your consent and change your mind, your child can withdraw from the project. You do not need to tell us the reason why you or your child wishes to stop being in the project. However, please tell us if your child plans to leave the research study so we can let you know if there are any health risks

or special requirements linked to withdrawing. If your child leaves the study, we will use any information already collected unless you tell us not to.

7. What are the possible benefits for my child and other people in the future?

Your child may benefit from this study by developing 'sustained unresponsiveness' (becoming tolerant) to peanut. This means that they will be able to eat peanut after the treatment has finished and include peanut into their diet.

It is possible that the PPOIT treatment will not work for your child. This means your child will not get any benefits from the study and will continue to have peanut allergy.

The greater community may benefit from this study by the discovery of a safe and tolerable treatment for food allergy.

8. What are the possible risks, side-effects, discomforts and/or inconveniences?

Oral peanut immunotherapy treatment:

Allergic reactions may happen during any phase (RUSH, buildup and maintenance phases) of the study.

The treatment may cause mild allergic reactions like a raised, itchy rash, facial swelling, vomiting, diarrhoea or tummy pain. If these symptoms happen, we may need to give your child antihistamine medicine. We may need to repeat the treatment dose before the next buildup dose is given.

More severe allergic reactions (***anaphylactic reactions***) could include difficulty breathing, wheezing, coughing, tongue swelling, hoarse voice or difficulty talking, paleness, low blood pressure or collapse. If your child has airway symptoms, we will reduce the dose of peanut protein before continuing on the dosing schedule. If your child has a very severe reaction (drop in blood pressure or collapse) we will stop the treatment. ***Anaphylactic reactions may in extremely rare instances result in death.***

Anaphylactic reactions require treatment with adrenaline. Adrenaline is administered into the thigh muscles. Administration of adrenaline may be associated with pain at the site of injection, palpitations, tremor, restlessness, anxiety, dizziness, headache and cold hands and feet. The use of adrenaline may very rarely cause irregularities of heart beat – this is most commonly seen in the elderly and people with existing heart conditions.

The first dose of each increased dose level during the buildup phase will be given at the hospital to allow us to closely monitor your child for allergic reactions. In our initial PPOIT Study, we found that allergic reactions can occur following a dose taken at home. Therefore, it will be important for you to know how to recognise and treat an allergic reaction should this occur in your child. To help you with this, you will be provided with a written anaphylaxis management plan at study entry. Study staff will review this information with you at each visit. You will be asked to monitor for reactions and manage these reactions according to the action plan. Should your child experience an allergic reaction, it is very important that you notify our on-call study staff that this has occurred by calling the on-call study phone. You will also have access to members of the study team to discuss any concerns or queries during business hours.

If other peanut allergic people (such as siblings) come into contact with the immunotherapy preparation it is likely that they will have a reaction, in the same way that they would if they accidentally ate some food containing peanut. It is important that the immunotherapy preparation that you keep at home is in a labeled container, securely away from young children.

Probiotic Treatment

The probiotic treatment is called LGG (*Lactobacillus rhamnosus* GG). Probiotics have been shown to both increase immune responses in the intestine and decrease inflammation in the intestine. Taking a probiotic may occasionally cause tummy cramps and tummy bloating. There is also a theoretical risk of causing an infection which may require treatment with antibiotics.

Your child will not be allowed to take any other probiotic supplements and foods (for example some yogurt preparations) containing LGG probiotic for the 18 months period whilst receiving study treatment.

Blood test:

We will use some cream to numb your child's skin before taking a blood sample. However, your child may still feel some discomfort. A bruise may appear at the site of the blood test and we will use cotton wool and a band-aid to reduce the bruising.

Skin prick test:

This may cause your child some discomfort. The skin may become itchy. We can use some cream to reduce the itching. There is a small risk (about 1 in 10,000) of a systemic allergic reaction. If this happens we will treat your child with the appropriate medicines.

Food Challenges:

Your child may have a reaction when challenged to peanut. The first challenge performed to confirm your child is allergic to peanut will cause a reaction. Subsequent challenges may or may not cause a reaction. *It is difficult to quantify the actual risk involved if your child has a reaction.*

The reaction may be mild (rash, facial swelling, vomiting, diarrhoea, tummy pain) or severe (involving breathing problems or low blood pressure or collapse). ***There is a theoretical risk of death as a result of an allergic reaction during a challenge, however there are no reported deaths from a food challenge.***

All challenges will be performed at The Royal Children's Hospital. A doctor will be present at all challenges and be able to give medications to treat the reaction immediately.

Mild allergic reactions will be treated with antihistamine medicines.

Severe reactions may be life threatening. Severe reactions will be treated with adrenaline (Epipen) and your child may need to be admitted to hospital overnight.

There may be unknown or unforeseen risks associated with this study.

9. What will be done to make sure my child's information is confidential?

In this study we will collect and use personal and health information about your child for research purposes. Any information we collect that can identify your child will be treated as confidential and used only in this project unless otherwise specified. We can disclose the information only with your permission, except as required by law.

All information will be stored securely in the Allergy Immune Disorders Research at Murdoch Childrens Research Institute.

The information will be re-identifiable. This means that we will remove your child's name and give the information a special code number. Only the research team can match your child's name to their code number, if it is necessary to do so.

We are required to keep information collected as part of a research project for a certain length of time. Because the participants in this project are under 18 years old, we must keep information until a participant turns 25 years old or 15 years following the study closure - whichever date is latest. The research information may be destroyed or kept indefinitely in secure storage after this time.

In accordance with relevant Australian and/or Victorian privacy and other relevant laws, you have the right to access and correct the information we collect and store about your child. Please contact us if you would like to access this information.

The following people may access information collected as part of this research project:

- the research team involved with this project
- The Royal Children's Hospital Human Research Ethics Committee

These groups may need to inspect and/or copy your child's research records for data analysis or to check that study procedures are followed correctly. Your child's name and personal details will not be released unless required by law.

Some of the information collected as part of this research may be important for your child's medical treatment and health. The following information will be placed in your child's hospital medical record to help the people who care for him/her:

- Blood test results

Any blood and stool sample collected as part of this study will be stored in Murdoch Childrens Research Institute. Blood and stool samples will be destroyed at the end of the study.

Your child's sample may be sent to another laboratory for testing. Any samples sent from The Royal Children's Hospital will be labelled with your child's code number only. It will not be possible for the testing laboratory to identify your child's sample. Any samples sent overseas are not protected by Australian laws and regulations.

At the end of the study, results will be presented at conferences or published in medical journals. This will be done in such a way that your child cannot be identified.

10. Will we be informed of the results when the research project is finished?

We will send you a short report of the overall results when the study is finished. Please note that this will not be until after 2020. The report will only contain information of the whole group of participants.

11. What happens if my child is injured during the project?

If your child suffers any injury or complication as a result of this research project you must contact the study team as soon as possible. The study team will help to arrange appropriate medical treatment for your child. If your child is eligible for Medicare, they can get medical treatment to treat the injury or complication as a public patient in any Australian public hospital at no cost to you.

12. Will my child be able to claim compensation if injured?

By signing the consent form, you are not giving up any legal rights to seek to obtain compensation for injury.

13. Who should I contact for more information?

If you would like more information about the project please contact:

Name: Christine Axelrad (Study Nurse Coordinator)

Contact telephone: (03) 93456974

Email: Christine.axelrad@mcri.edu.au

Alternatively, if you wish to contact Professor Mimi Tang for additional information, please email mimi.tang@rch.org.au.

If you have any concerns and/or complaints about the project, the way it is being conducted or your child's rights as a research participant, and would like to speak to someone independent of the project, please

contact: Director, Research Ethics & Governance, The Royal Children's Hospital Melbourne on telephone: (03) 9345 5044.

CONSENT FORM

HREC Project Number: 35207

Research Project Title: Open pilot study of Probiotic and Peanut Oral Immunotherapy (PPOIT) with shortened buildup phase: PPOIT-II

Version Number: 3 **Version Date:** 28/11/2015

- I have read, or someone has read to me in a language that I understand, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my child's involvement in this project.
- I voluntarily consent for my child to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital Melbourne Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007) – including all updates.
- I understand I will receive a copy of this Information Statement and Consent Form.

Child's Name

Parent/Guardian Name

Parent/Guardian Signature

Date

Name of Witness to
Parent/Guardian's Signature

Witness Signature

Date

Declaration by researcher: I have explained the project to the parent/guardian who has signed above, and believe that they understand the purpose, extent and possible risks of their child's involvement in this project.

Research Team Member Name

Research Team Member Signature

Date

Note: All parties signing the Consent Form must date their own signature.

Appendix 5: Laboratory Standing Operating Procedures (SOPs)

PBMC Isolation and Cryopreservation

Make up fresh:

Transport media	100ul heparin : 10ml RPMI	(stock of heparin 5000U in 5ml)
RPMI/2% HI FCS	49ml RPMI : 1ml HI FCS	(need 50ml per 10ml blood sample)

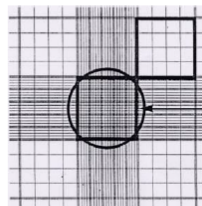
Freezing mix 42.5ml HI FCS : 7.5ml DMSO (add DMSO into HI FCS dropwise)

Separation of Plasma:

- Spin at 700g for 10 mins (brake off) (Frances centrifuge 79 9)
- Collect plasma into 10ml tube
- Centrifuge at 700g for 10 min (brake on)
- Aliquot 1ml into cryovials and store at -80°C

Separation of PBMCs:

- Fill blood tube to 9ml (if 20ml blood) or 10ml of (if 10ml blood) with Transport media
NOTE: can be left in Transport for 18hours at RT before separation
- Layer blood onto 10ml tubes with 2.5ml (or 3ml for P POIT) Ficoll-Plaque
NOTE: add blood along side of tube – DO NOT MIX
- Spin at 400g for 30 mins **BRAKE OFF** (Frances centrifuge 79 9)
- Carefully aspirate PBMCs at the interface with a transfer pipette into new 10ml tube
- Top up to 10ml with RPMI/HI FCS
- Spin at 500g for 10 mins (brake on)
- Discard supernatant, top up to 10ml with RPMI/HI FCS
- Spin at 500g for 7 mins (brake on)
- Repeat above two steps
- Resuspend in 1ml RPMI/HI FCS
- Cell count:



Count 5 boxes in a row

No. of cells x 10^5 cells/total

Cryopreservation of cells:

- Put cell suspension on ice
- Add Freezing Mix, dropwise into cell suspension at 1:1 ratio
- Store in 'Mr Frosty' overnight at -80°C
- Transfer the cryovials to liquid nitrogen within 24 hours

eg. 25×10^6 cells/total in 1ml

Divide into 3 vials 8.3×10^6 – need to make up to 3ml

1.5ml cells : 1.5ml Freezing media

Add 0.5ml extra RPMI/HI FCS, then slowly add 1.5ml Freezing mix

Thawing out Cells for Culture

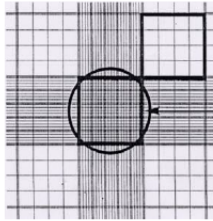
Make up fresh:

RPMI/2% HI FCS

49ml RPMI : 1ml HI FCS

(need 50ml per 10ml blood sample)

- Collect cryovials from tank into liquid nitrogen canister
- Thaw out vials in 37°C waterbath – until small block of ice left inside vials
- Transfer to 10ml tube on ice
- Add 1ml of RPMI/ HI FCS to tube, dropwise ~over 1 min
- Add 2ml of RPMI/ HI FCS to tube, dropwise ~over 1 min
- Rinse out cryovials
- Top up to 10ml, dropwise – DO NOT MIX
- Spin at 600g for 10mins (brake on)
- Resuspend in ~800ul (to make up to 1ml)
- Cell count:



Count 5 boxes in a row

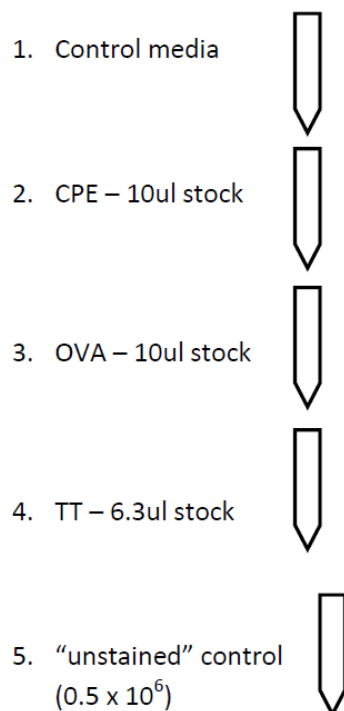
No. of cells x 10⁵ cells/total

- Check for viability and recovery from the initial cell counts

Peripheral Blood Mononuclear Cell (PBMC) culture

6-day cultures

- Take out 4.5×10^6
- Aliquot out 0.5×10^6 cells into a FACS tube for “unstained”
- For remaining 4ml of cell suspension add 40ul of CFSE
- Vortex
- Incubate at 37°C in incubator for 5 mins
- Top up to 10ml with RPMI + 1% autologous serum
- Spin at 600g for 10 mins
- Resuspend and top up to 10ml with RPMI + 0.1% autologous serum
- Spin at 600g for 10 mins
- Resuspend cells in AIMV + 5% autologous serum (4ml for 4×10^6)
- Divide 1ml cell suspension into 4 FACS tubes for culture conditions



- Culture cells at 37°C in incubator for 6 days

Tetanus Toxoid Preparation

Stock = 635 LF/ml = 1.6mg/ml (1:10 dilution, 100ul in 900ul sterile PBS; $0.16\text{mg/ml} = 0.16\mu\text{g}/\mu\text{l}$
1μg in culture = 6.3μl per 1ml culture

CFSE stock preparation

Stock A + Stock B (18μl) = 5mM (5000μM) (1:50 dilution, 18μl in 882μl sterile PBS)

For a stock concentration of 100μM. 5μl in 1ml of culture for a final working concentration of 0.5μM.

Fresh Treg FACS

Make up:

RPMI + 2% HIFCS	20ml RPMI: 400ul HIFCS
FACS Buffer	48.5ml PBS: 1ml HIFCS: 0.5ml sodium azide
FOXP3 Buffer A (diluted)	(1:10) 10X Buffer A: dH ₂ O
FoxP3 Buffer C	(1:50) Buffer A (diluted): Buffer B

Make up Compensation Beads before starting FACS staining (PerCP, CD25-PECy7, APC, V450, PE)

1. Take out cells.
2. Add approximately 1 mL RPMI + 2 % FCS (to wash).
3. Spin at 600 g (0.6 r.c.f.) for 10 minutes (brake ON).
4. Transfer pipette out media and discard, leaving cell pellet
5. Resuspend in 100ul of antibodies and FACS buffer

2.5ul	CD4 – PerCP	}	per 1 x 10 ⁶
2.5ul	CD25 – PE-Cy7		
2ul	CD45RA – APC		
1ul	CD27 – V450		
91.5ul	FACS Buffer		

6. Vortex.
7. Incubate at RT for 20 minutes (dark) – put foil over tubes and put in cupboard.
8. After incubation, add 2 mL FACS buffer
9. Incorporate Compensation Beads here → Spin at 600 (0.6 r.c.f.) for 10 minutes (brake ON).
10. Tip excess out, leaving cell pellet.
11. To fix the cells, resuspend in 2 ml FoxP3 Buffer A per tube.
12. Vortex.
13. Incubate at RT for 10 minutes (dark) – put foil over tubes and put in cupboard.
14. Top up with FACS buffer, approximately 2 mL.
15. Spin at 500g (0.5 r.c.f.) for 10 minutes (brake OFF) – cell pellet is BUOYANT so transfer pipette out solution **(DON'T POUR!)**
16. Top up with FACS buffer, approximately 2 mL.
17. Spin at 500 g (0.5 r.c.f) for 10 minutes (brake ON).
18. Tip out excess, leaving cell pellet.
19. To permeabilise the cells, resuspend in 0.5 mL FoxP3 Buffer C per tube
20. Vortex.
21. Incubate at RT for 30 minutes (dark) – put foil over tubes and put in cupboard.

22. Add 2 mL FACS buffer
23. Spin at 500g (0.5 r.c.f.) for 7 minutes (brake ON).
24. Tip out excess.
25. Repeat steps 21-23.
26. Resuspend in 7 μ L of FoxP3 – PE Ab (Mouse anti-IgG PE)
27. Vortex.
28. Incubate at RT for 30 minutes (dark) – put foil over and into cupboard.
29. Add 2 mL FACS buffer
30. Spin at 500g (0.5 r.c.f.) for 7 minutes (brake ON).
31. Tip out excess.
32. Resuspend in 200 μ L FACS buffer and mix with transfer pipette

6-Day Treg FACS

Make up:

FACS Buffer	48.5ml PBS: 1ml HIFCS: 0.5ml sodium azide
FOXP3 Buffer A (diluted)	(1:10) 10X Buffer A: dH ₂ O
FoxP3 Buffer C	(1:50) Buffer A (diluted): Buffer B

Make up Compensation Beads before starting FACS staining (PerCP, CD25-PECy7, APC, V450, PE)

1. Take out cells.
2. Add approximately 1 mL RPMI + 2 % FCS (to wash).
3. Spin at 600 g (0.6 r.c.f.) for 10 minutes (brake ON).
4. Transfer pipette out media and discard, leaving cell pellet
5. Resuspend in 100ul of antibodies and FACS buffer

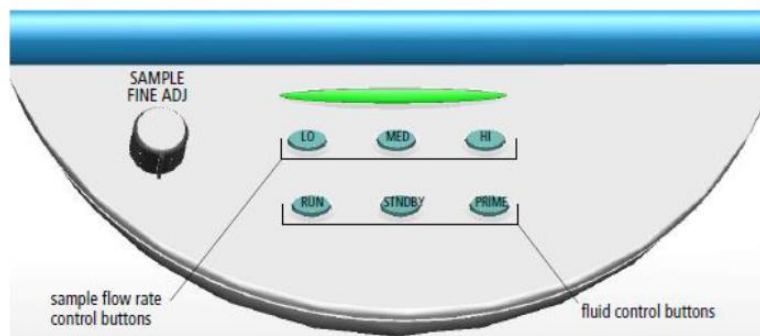
2.5ul	CD4 – PerCP	}	per 1 x 10 ⁶
2.5ul	CD25 – PE-Cy7		
2ul	CD45RA – APC		
1ul	CD27 – V450		
91.5ul	FACS Buffer		

6. Vortex.
7. Incubate at RT for 20 minutes (dark) – put foil over tubes and put in cupboard.
8. After incubation, add 2 mL FACS buffer
9. Incorporate Compensation Beads here → Spin at 600 (0.6 r.c.f.) for 10 minutes (brake ON).
10. Tip excess out, leaving cell pellet.
11. To fix the cells, resuspend in 2 ml FoxP3 Buffer A per tube.
12. Vortex.
13. Incubate at RT for 10 minutes (dark) – put foil over tubes and put in cupboard.
14. Top up with FACS buffer, approximately 2 mL.
15. Spin at 500g (0.5 r.c.f.) for 10 minutes (brake OFF) – cell pellet is BUOYANT so transfer pipette out solution (**DON'T POUR!**)
16. Top up with FACS buffer, approximately 2 mL.
17. Spin at 500 g (0.5 r.c.f.) for 10 minutes (brake ON).
18. Tip out excess, leaving cell pellet.
19. To permeabilise the cells, resuspend in 0.5 mL FoxP3 Buffer C per tube
20. Vortex.
21. Incubate at RT for 30 minutes (dark) – put foil over tubes and put in cupboard.
22. Add 2 mL FACS buffer

23. Spin at 500g (0.5 r.c.f.) for 7 minutes (brake ON).
24. Tip out excess.
25. Repeat steps 21-23.
26. Resuspend in 7 μ L of FoxP3 – PE Ab (Mouse anti-IgG PE)
27. Vortex.
28. Incubate at RT for 30 minutes (dark) – put foil over and into cupboard.
29. Add 2 mL FACS buffer
30. Spin at 500g (0.5 r.c.f.) for 7 minutes (brake ON).
31. Tip out excess.
32. Resuspend in 200 μ L FACS buffer and mix with transfer pipette

BD LSR II Flow Cytometer

Control panel



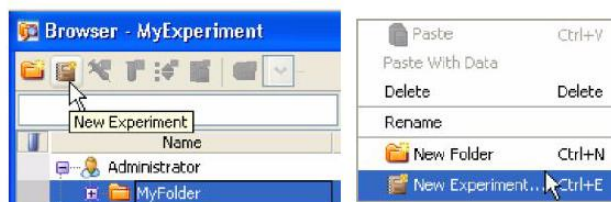
When not running sample, leaving settings on LO/STNDBY

When running sample, put settings on LO/RUN → change to HI/RUN when recording data

Running Samples

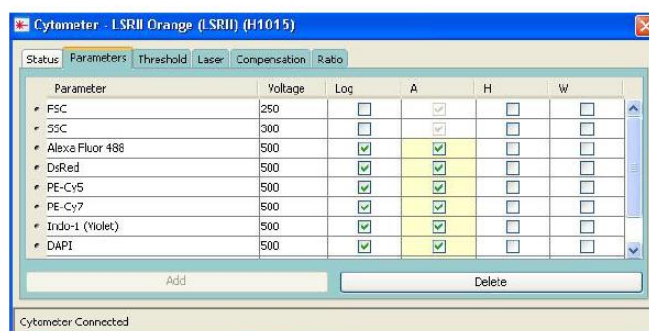
If setting up a New Experiment

1. Click the New Folder button () on the Browser toolbar to add a new folder.
2. Click the folder and rename it *MyFolder*.
3. Click MyFolder, then click the New Experiment button on the Browser toolbar, or right-click the new folder and select New Experiment from the menu.



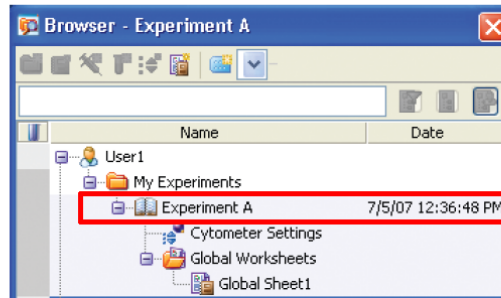
4. Click the new experiment in Browser and rename it

If you create a New Experiment, you will need to adjust the voltages under Cytometer Settings to ensure the population of interest is within detection

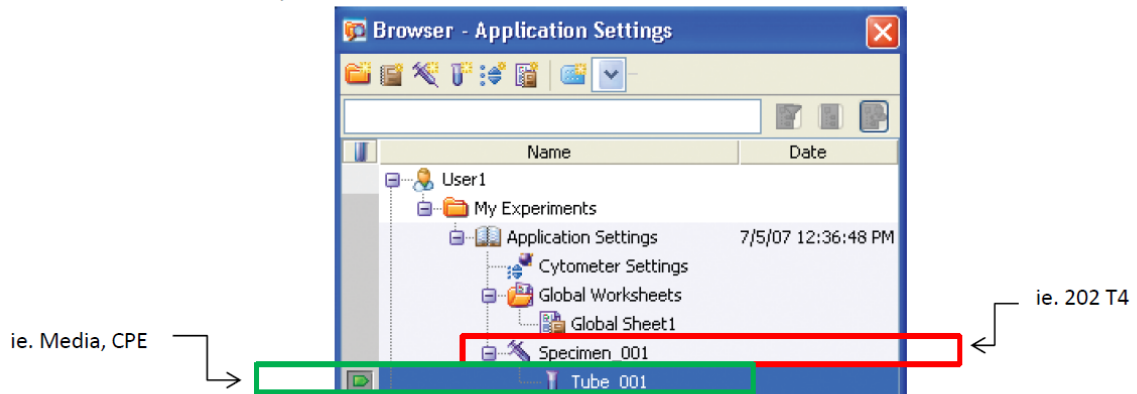


Verify that the FSC, SSC and threshold settings are appropriate for your experiment

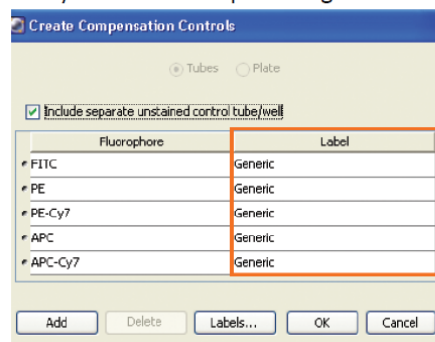
If duplicating an Old Experiment



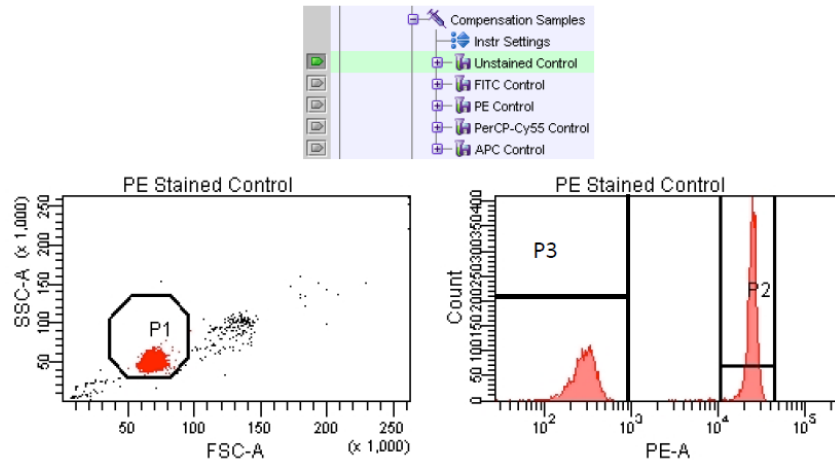
1. Open experiment you want to duplicate
2. Right click 'Experiment A'
3. Select 'Duplicate without data'
4. Rename Specimen and add tubes for the different conditions



5. Select Experiment > Compensation Setup > Create Compensation Controls
6. Delete the fluorochromes you are not compensating



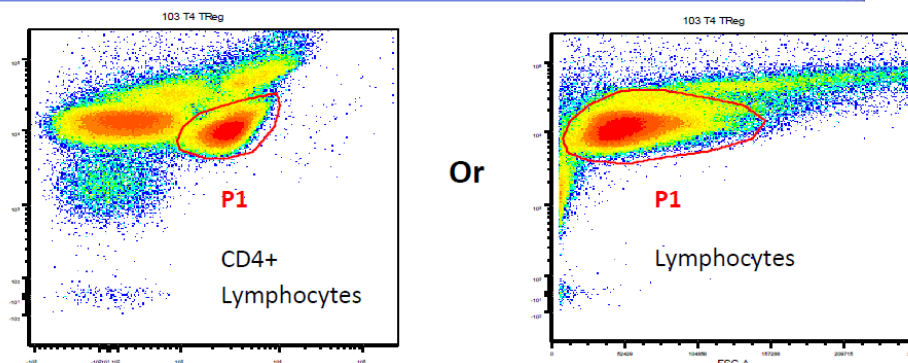
7. Run the Compensation Bead Controls – collect 5000 evts



8. Assign both the positive and negative bead populations gates. Make sure the negative gate is dragged off the graph past 0
9. Select Experiment > Compensation Setup > Calculate Compensation > Link & Save
10. Make sure compensation has been calculated by looking under Cytometer Settings
(If compensation done after test samples go to Cytometer Settings > Copy Highlight all the Test samples you want to compensate. Right click > Paste Spectral Overlap with Zeros)

11. Now run all Test samples

MAKE SURE
Parameters are set



Multiplex Assay for detection of IL-10, IL-4, IL-5, IL-13, IFN- γ

Milliplex MAP Human High Sensitivity Magnetic Bead Panel kit (EMD Millipore, St Charles, MO, USA)

Background

Cytokines are immunomodulatory polypeptides that play key roles in both adaptive and innate immune responses. A generic term, “cytokines” includes myokines (produced by muscle cells), lymphokines (produced by activated Th cells), interleukins (acting as mediators between T cells) and chemokines (responsible for T-cell migration). One of the regulatory mechanisms of the immune system, cytokines act at the recognition, activation, or effector phases of an immune response, modulating the development and functional activities of the subtypes of T cells, B cells and myeloid cells. Consequently, research involving cytokines plays a significant role in achieving a deeper understanding of the immune system and its multi-faceted response to most antigens, especially those responses that make up the inflammatory process.

Low levels of inflammation are involved in many clinical and sub-clinical disease states, such as autoimmune disease, cardiovascular disease, diabetes, neurological disorders and cancer. Measuring picogram levels of cytokines, therefore, is critical for understanding the pathogenesis of these diseases.

EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:

The Luminex analyzers Luminex 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.

The Luminex analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.

Working protocol

Preparation:

Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- Avoid multiple (>2) freeze/thaw cycles.
- Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay. Tissue/cell extracts should be done in neutral buffers containing reagents and conditions that do not interfere with assay performance. Excess concentrations of detergent, salt, denaturants, high or low pH, etc. will negatively affect the assay. Organic solvents should be avoided. The tissue/cell extract samples should be free of particles such as cells or tissue debris.

NOTE:

- A maximum of 25 μL per well of neat or one to two diluted serum or plasma can be used. Tissue culture or other media may also be used.
- All samples must be stored in polypropylene tubes. DO NOT STORE SAMPLES IN GLASS.

- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anti-coagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

If premixed beads are used, sonicate the premixed bead bottle 30 seconds and then vortex for 1 minute before use.

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 70 µL from each antibody-bead vial to the Mixing Bottle and bring final volume to 3.5 mL with LBD. Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to one month.

(Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

Example : When using 10 antibody-immobilized beads, add 70 µL from each of the 10 bead vials to the Mixing Bottle. Then add 2.8 mL LBD

B. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 1.0 mL deionized water to the bottle containing lyophilized serum matrix (Cat# MXHSM-7). Mix well. Allow at least 10 minutes for complete reconstitution. Add 3 mL Assay Buffer (Cat# L-ABIR) to the bottle for a final volume of 4 mL. Unused reconstituted matrix should be stored at ≤ -20°C for up to one month.

C. Preparation of Quality Controls

For serum and plasma samples, reconstitute Quality Control 1 (QC1) and Quality Control 2 (QC2) vials with 250 µL MXHSM-7. These are the Stock QC Vials. Invert the Stock Vials several times to mix and vortex. Allow the vials to sit for 5-10 minutes. Label two tubes QC1 and QC2 and add 150 µL MXHSM-7 to each tube. Remove 50 µL from QC1 or QC2 Stock Vials and add to the 150 µL MXHSM-7 in the respective QC1 and QC2 tubes and vortex. Use these one to four diluted QCs in the assay. Unused portions may be stored at ≤ -20°C for up to one month.

For culture samples, substitute the appropriate sample media for the MXHSM-7 used for serum and plasma samples above.

D. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer (two bottles) with 540 mL deionized water. Store the unused portion at 2-8°C for up to one month.

E. Preparation of Human High Sensitivity T Cell Standard

1.) For serum and plasma samples, reconstitute the Human High Sensitivity T Cell Standard with 250 µL MXHSM-7. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This is the Stock Standard Vial NOT Standard 7. Unused Standard may be stored at ≤ -20°C for up to one month.

2). Preparation of Working Standards

For serum and plasma samples, label seven polypropylene microfuge tubes as Standard 7, Standard 6, Standard 5, Standard 4, Standard 3, Standard 2 and Standard 1. Add 150 µL of MXHSM-7 to each of the seven tubes. Prepare serial dilutions by adding 50 µL of the Stock Standard to the Standard 7 tube, mix well and transfer 50 µL of the Standard 7 to the Standard 6 tube, mix well and transfer 50 µL of the Standard 6 tube to the Standard 5 tube, mix well and transfer 50 µL of the Standard 5 tube

to the Standard 4 tube, mix well and transfer 50 μ L of the Standard 4 tube to the Standard 3 tube, mix well and transfer 50 μ L of the Standard 3 tube to the Standard 2 tube, mix well and transfer 50 μ L of the Standard 2 tube to the Standard 1 tube and mix well. The 0 pg/mL standard (Background) will be MXHSM-7 or appropriate sample media.

3). For other samples (tissue culture, cell culture etc.) substitute the appropriate media for the MXHSM-7 used for serum and plasma samples above.

PLATE WASHING PROCEDURE

If using a solid plate, use either a handheld magnet or magnetic plate washer.

1.) Solid Plate

If using a solid plate, use either a handheld magnet or magnetic plate washer.

A.) Handheld magnet (EMD Millipore Catalog #40-285) - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 μ L of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

B.) Magnetic plate washer (EMD Millipore Catalog #40-094, #40-095, #40-096 and #40-097) - Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 μ L of residual wash buffer in each well. This is expected when using the BioTek plate washer and this volume does not need to be aspirated from the plate.

If using an automatic plate washer other than BioTek® 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.

2.) Filter Plate (EMD Millipore Catalog #MX-PLATE)

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 μ L/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Repeat wash steps as recommended in the Assay Procedure.

IMMUNOASSAY PROCEDURE

Day 1

- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
 - Diagram the placement of Standards [0 (Background), standards 1 through 7], Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
 - If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.
1. Add 200 μ L of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
 2. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
 3. Add 50 μ L of each diluted Standard or Quality Control into the appropriate wells (NOT from Stock Vials). The Serum Matrix should be used for 0 pg/mL standard (background). When assaying tissue culture or other supernatant, use appropriate control culture medium as the background.
 4. Add 25 μ L of Assay Buffer to the sample wells.

5. Add 25 μ L of sample into the sample wells.
6. Vortex Mixing Bottle and add 25 μ L of the Mixed or Premixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
7. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hrs) at 4°C Collect

Day 2

8. Gently remove well contents and wash plate 3 times following instructions listed in the PLATE WASHING section.
9. Add 50 μ L of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
10. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). DO NOT ASPIRATE AFTER INCUBATION.
11. Add 50 μ L Streptavidin-Phycoerythrin to each well containing the 50 μ L of Detection Antibodies.
12. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
13. Gently remove well contents and wash plate 3 times following instructions listed in the PLATE WASHING section.
14. Add 150 μ L of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
15. Run plate on Luminex 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.
16. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples and Controls. (Note: Because of the built-in two-fold sample dilution, for all neat samples, multiply the calculated concentrations by two. For two-fold diluted samples, multiply the calculated concentrations by four. Calculated Quality Control concentrations do not require multiplication by a dilution factor.)

Materials

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalog #40-50000) or Luminex Drive Fluid (Luminex Catalog #MPXDF-4PK)

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 25 μ L to 1000 μ L
2. Multichannel Pipettes capable of delivering 5 μ L to 50 μ L or 25 μ L to 200 μ L
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Rubber Bands
6. Aluminum Foil
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
10. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
11. Luminex 200™, HTS, FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex Corporation

12. Automatic Plate Washer for magnetic beads (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

Note: If a plate washer or handheld magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (EMD Millipore Catalog #MX-PLATE) to run the assay using a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog #WP6111560 or equivalent).

Fluorezyme immunoassay (FEIA) for measurement of specific IgE and IgG4

Summary of ImmunoCAP Test Principle

The ImmunoCAP 250 is a fully automated system. The specific allergen of interest, covalently coupled to ImmunoCap cellulose carrier (sponge), reacts with the specific IgE in the patient serum sample. After washing away non-specific IgE, enzyme-labeled antibodies against IgE are added to form a complex. After incubation, unbound enzyme anti-IgE is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the sponge is compressed and the fluorescence of the resulting eluate is measured. The higher the response value, the more specific IgE is present in the sample. To evaluate the test results, the response for the patient samples is compared directly to the response for the calibrators.

Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

- 1) Stop solution and development solution, 2 bottles each
- 2) Loading tray for 38 EliA or ImmunoCAP carriers in addition to the positions in the storage chamber
- 3) Bar code controlled storage chamber for 180 EliA and ImmunoCAP carriers
- 4) Sample diluent (6 bottles) and dilution plates (2x96 wells) for onboard dilution of samples
- 5) Bar code reader for reagents and samples
- 6) Conjugate compartment (6 bottles)
- 7) Strip tray (8 positions) for calibrator strips and/or curve control strips
- 8) Instrument management via touch screen
- 9) Containers for wash and rinse solutions and solid and liquid waste
- 10) Sample racks (5 positions) each holding up to 10 patient and quality control samples
- 11) PC for Phadia IDM software

Consumables and materials required

F422 Ara h 1
F423 Ara h 2
F424 Ara h 3
F352 Ara h 8
F427 Ara h 9
Development solution, 11 ml
Development solution, 11 ml
Specific IgE conjugate 100, 5.3 ml
Specific IgE conjugate 100, 5.3 ml
Stop solution, 119 ml
Stop solution, 119 ml
Washing Solution Additive, 86 ml
Washing Solution Concentrate, 400 ml
Specific IgG4 Calibrator strip
Specific IgG4 Curve Control Strip
Specific IgG/IgG4 Control L
Specific IgG/IgG4 Control H
Specific IgG4 Conjugate 50

Calibration and Calibration verification Procedures

A. Calibration of Instrument

Calibrators are provided in a strip with 6 positions, one for each calibrator point. A sealing foil covers the strip. The calibrators are pipetted with the sample pipette.

The 6-point calibration is performed every 28 days or when a new lot of conjugate is to be used or as indicated by curve control results. The calibration curve is set up for a specific lot number of conjugate by using six samples of known concentrations (calibrators), each sample run in duplicate. The curve is verified with each subsequent run using 2 point curve controls (CC-1 & CC-2). The patient results are calculated from this curve.

Fluorometer is calibrated by using Fluoro C (a reagent). This is performed when the glass cuvette has been replaced as instructed by a Phadia instrument engineer.

B. Instructions for Calibration of Instrument

1. Remove calibrator strips from refrigerator and allow it to come to room temperature. Gently invert to mix.
2. In Load and Start field change status from Curve Control to Calibration.
3. Load calibrator strip in reagent tray.
4. Select OK.

Procedure Operating Instructions; Calculations; Interpretation of Results (Setting up instrument, steps)

A. Operating Procedure

1. Retrieve the samples to be tested from the refrigerator to bring to room temperature.
2. Switch ImmunoCAP Data Manager (IDM) computer on.
3. Switch ImmunoCAP Instrument system power on.
4. Perform daily maintenance.
5. Import test requests using the IDM.
6. Make a list of samples using consumable page in the IDM, make a tasklist, or use LIS pending log.
7. In the IDM, Request window, review the reagents needed for the assay run by highlighting all requests to be included in the assay run, then select "Menu" and "Consumables".
8. In the ISW, select "Utilities" and "Load Reagents"
9. Load necessary reagents by touching the appropriate area on the screen.
 - a. Pipette tips
 - b. Calibrator/Curve Control Strip
 - c. Conjugate
 - d. Development
 - e. Stop Solution
 - f. Immunocap carrier
 - g. There must be full Washing Solution and Rinse Solution bottles loaded
10. Select "Load and Start"
11. Verify methods selected and status of Curve control or Calibration.
12. Select OK
13. The instrument will perform Assay initializing, assay priming, Blank measurement.
14. Load the QC rack
15. Vortex samples. Rim sample with a wooden stick if clot is present.
16. Load the Sample Racks.

17. Instrument will process Curve Controls/Calibration, QC and CDC Samples.
18. Confirm Calibration Curve or Curve Controls are OK and Quality Controls are within range.
19. Select "Samples Tab" to approve sample results.
20. After all sample pipetting is completed in Assay Processing press "End Assay"
21. Select Finish with Rinse and Shutdown. Instrument will complete testing, calculation of results, rinse and shut down.

B. Procedure following completion of testing.

1. Print laboratory report including Calibration information (if performed), Quality control information, Sample results, Lot # information (if selected), errors, and comments.
2. Return samples to their designated place in the box.

C. Calculations

The IDM software uses Rodbard 5 parameter calculations to graph the calibrator concentrations as a loglog graph. The software then plots the sample response on the calibration curve to calculate the concentration into kUA/L (for sIgE) or mgA/L (for sIgG4).

Reportable Range of Results

Specific IgE

Measuring range for specific IgE for an undiluted sample is 0.35 – 100 kU/L.

Reportable range undiluted sample is <0.35 kU/L - 100 kU/L. Dilution of sample is required for determination of values higher than 100 kU/L IgE. Values >100 kU/L are verified by re-assay after the sample has been diluted 1:5 with sample diluent. If the repeat value is still >100 kU/L the sample may be diluted further to obtain the end point value up to 1,000 kU/L

Quality Control (QC) Procedures

The Phadia 250 is a fully automated system(<http://www.phadia.com/en/Products/Phadia-Laboratory-Systems/Phadia-250/>). This permits the positive-negative threshold to be narrower and a more rapid and robust chemistry. However, since all the results are generated by a computer, there is a need for quality control with established positive control sera, with defined binding ranges for the type of antibody to be analysed.

The following quality control material are used:

- Optitrol Multiallergy Food (total IgE) (external QC material)
- Previous samples (specific IgE) (internal QC material)

Run the external QC material at the beginning of each run. This usually takes about an hour. Input QC results into Unity Realtime QC data database. Proceed to assaying samples if the QC results are acceptable.

When the results are documented into Unity Realtime, they are compared to a mean. If the result deviates > +/-2SD, it is not acceptable.

Acceptable ranges: Mean +/- 15 % of mean (i.e. one SD entered as 7.5 %).

If the QC fails, the scientist performing the assay must fill out the form IM-F-191Failed QC Investigations, and have it signed by a senior scientist.

The laboratory performs quality controls for total, soy and peanut IgE.

For IgG4, quality controls are not performed on a regular basis, since IgG4 is evaluated exclusively for research purposes, and not diagnostic.

The following PPOIT internal QC material for IgE are used with every run.

Sample name	Expected value	Sample name	Expected value
101 T4	11.4	101 T9	16.0
406 T4	35.6	406 T9	30.3

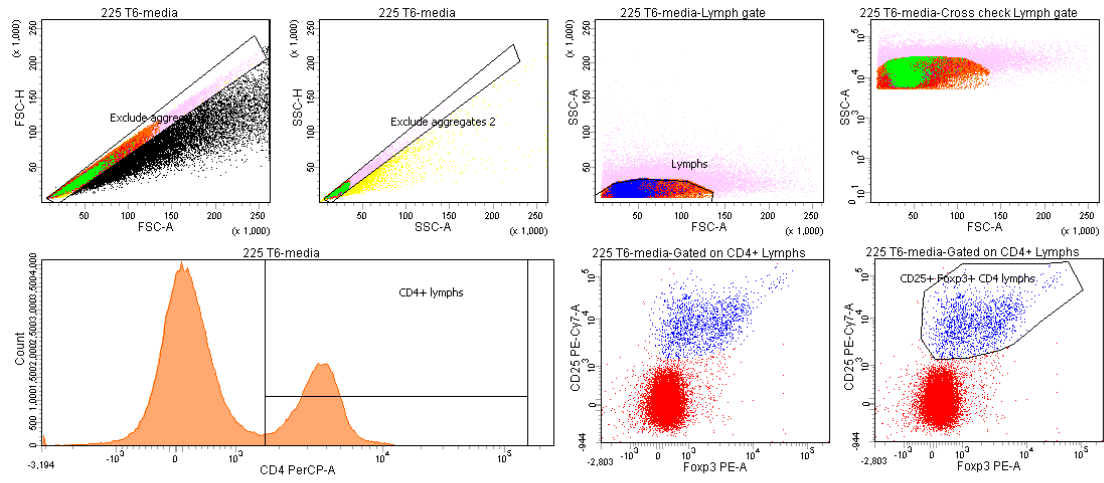
The laboratory participates in **external quality assurance programs including** RCPA (Royal College of Pathologists of Australasia) and UKNEQAS (United Kingdom National External Quality Assessment Service) quality assurance programs (QAP)

References

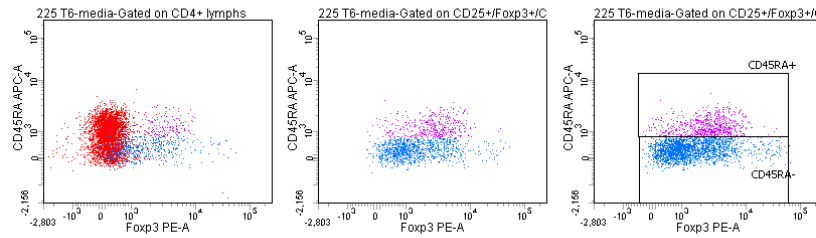
Phadia 250 User Manual, version 1.4. Revised November 2012.

The Royal Children's Hospital, Immunology Laboratory. ImmunoCAP Method.

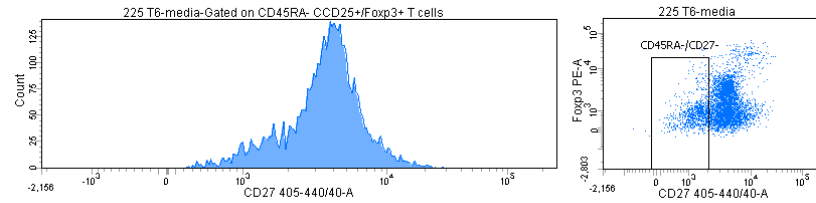
Appendix 6: Fluorescence Activated Cell Sorting (FACS) gating strategies



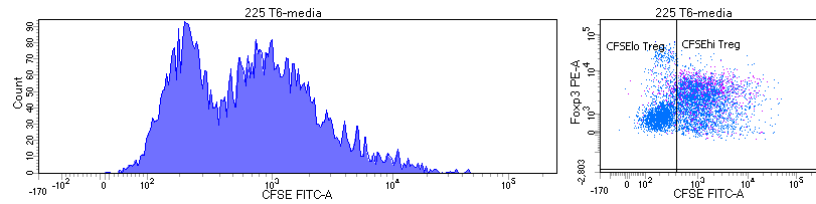
3. & 4. Tregs: Naive (CD45RA+) v.s. Activated (CD45RA-)



5. & 6. Activated Tregs: CD27-ve population



7. CFSElo Tregs



6D cultured Treg parameters to report:

1. #Events CD4+ Lymphs
2. #Events CD25+ Foxp3+ CD4 lymphs (Total Tregs)
3. #Events CD45RA+ CD25+Foxp3+/CD4 lymphs (nTreg)
4. #Events CD45RA- CD25+Foxp3+/CD4 lymphs (total aTreg)
5. #Events CD45RA- CD27- (CD27- aTreg 1)
6. #Events CD45RA-CD27- (CD27- aTreg 2)
7. #Events CFSElo CD25+ Foxp3+ CD4 lymphs
8. #Events CD45RA-CD25+ CD4 lymphs (total activated T cells)]

Experiment Name:	Kuang
Specimen Name:	225 T6
Tube Name:	media
Record Date:	18/02/2013 5:13:21 PM

Population	#Events	%Parent	%Total	Mean
All Events	269,253	###	100.0	616
Exclude aggregates 1	238,763	88.7	88.7	365
Exclude aggregates 2	178,532	74.8	66.3	426
Lymphs	165,801	92.9	61.6	387
CD4+ lymphs	47,663	28.7	17.7	574
CD25+ Foxp3+ CD4 lymphs	6,008	12.6	2.2	2,791
CD45RA+	1,522	25.3	0.6	3,225
CD45RA+/CD27-	39	2.6	0.0	1,443
CD45RA-	4,570	76.1	1.7	2,615
CD45RA-/CD27-	888	19.4	0.3	1,232
CFSElo Treg	2,329	38.8	0.9	2,964
CFSEhi Treg	3,679	61.2	1.4	2,682
Q3	0	0.0	0.0	###
Q4	0	0.0	0.0	###
CD45RA-CD25+ CD4 Lymphs	4,509	9.5	1.7	2,645
CD25+/CD45RA-/CD27-	884	19.6	0.3	1,456

Tube:	media

Population	#Events	%Parent	%Total
All Events	269,253	###	100.0
Exclude aggregates 1	238,763	88.7	88.7
Exclude aggregates 2	178,532	74.8	66.3
Lymphs	165,801	92.9	61.6
CD4+ lymphs	47,663	28.7	17.7
CD25+ Foxp3+ CD4 lymphs	6,008	12.6	2.2
CD45RA+	1,522	25.3	0.6
CD45RA+/CD27-	39	2.6	0.0
CD45RA-	4,570	76.1	1.7
CD45RA-/CD27-	888	19.4	0.3
CFSElo Treg	2,329	38.8	0.9
CFSEhi Treg	3,679	61.2	1.4
Q3	0	0.0	0.0
Q4	0	0.0	0.0
CD45RA-CD25+ CD4 Lymphs	4,509	9.5	1.7
CD25+/CD45RA-/CD27-	884	19.6	0.3

Appendix 7: Publications presented in this thesis



Novel Treatments for Established Food Allergies

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Abstract

Purpose of review Current management of food-allergic children centres on allergen avoidance and emergency management of allergic reactions due to accidental ingestion. These measures minimise harm to patients, but the need for constant vigilance and dietary restriction may have a major long-term negative impact on quality of life.

Recent findings Research has focused on searching for a curative treatment that can modify the natural history of disease. Both non-antigen-specific and antigen-specific immune modulation approaches have been explored. Oral immunotherapy (OIT) has drawn the greatest interest as a potential treatment for food allergy. OIT is effective at inducing desensitization; however, its ability to induce long-lasting tolerance or sustained unresponsiveness remains to be confirmed. There is growing evidence that the addition of immune-modifying adjuvants may enhance efficacy of OIT in inducing long-lasting sustained unresponsiveness. This article reviews recently published literature on this rapidly evolving field.

Keywords Food allergy · Desensitization · Tolerance · Sustained unresponsiveness · Oral immunotherapy · Adjuvants · Quality of life

This article is part of the Topical Collection on *Allergy*

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Introduction: Food Allergy Prevalence and Current Management

Globally, there has been a significant increase in the prevalence of food allergy over the past two decades. Epidemiologic studies suggest that factors contributing to this epidemic may include timing and route of allergen exposure, sunlight exposure, vitamin D levels and microbial diversity and composition. While allergies to egg, milk, wheat and soy generally resolve during childhood, the age at resolution appears to be increasing with many cases resolving in adolescence. Furthermore, peanut, tree nut, shellfish and fish allergies tend to persist throughout life. The current standard of care for children with food allergy includes allergen avoidance and education of patient and parent or carer in the recognition and emergency management of allergic reactions. Although these measures are designed to minimise harm to a food-allergic child, they have a major negative impact on the child's and their family's quality of life due to the constant vigilance required to avoid foods, impact on participation in social events and increased fear and anxiety of having a potentially life-threatening allergic reaction. There is a need for disease-modifying therapy. This article reviews the literature on novel approaches to treatment of IgE-mediated food allergy in children published in recent years. Mechanisms of oral tolerance and immune changes associated with oral immunotherapy, covered in a recently published article [1], will not be reviewed here.

The Search for an Effective Treatment

There are two outcomes of interest when evaluating the clinical effectiveness of potential food allergy treatments—'desensitization' and 'tolerance'. Desensitization describes the ability to ingest a food without reaction while maintaining

regular exposure to that food (for example continuing on oral immunotherapy). Successful desensitization in the absence of tolerance is mediated by changes in effector cells such as mast cells and basophils, without modulation of underlying pathogenic immune mechanisms. 'Tolerance' on the other hand is the ability to ingest a food without reaction even after discontinuation of exposure to the food for a period of time. Tolerance is believed to reflect sustained reprogramming of the immune response to allergen, involving T regulatory (Treg) cells and/or allergen-specific anergy and clonal deletion. Desensitization can be evaluated clinically by administering a food challenge while a subject is still receiving oral immunotherapy or eating regular doses of the food. In contrast, tolerance is confirmed by performing a food challenge after oral immunotherapy or intake of the food has been stopped for an extended period of weeks to months. Currently, there is no consensus on the exact duration of secondary elimination that is required to demonstrate true long-lasting tolerance. For this reason, some experts have introduced the term 'sustained unresponsiveness' (SU) in place of tolerance to acknowledge that the ability to tolerate a food after discontinuation of oral immunotherapy (OIT) may not be long-lasting and may not represent a permanent state of immune tolerance.

Non-Antigen-Specific Immune Modulation

One approach to treatment of food allergy is to induce desensitization or tolerance in a non-antigen-specific way.

Monoclonal antibodies (mAb) targeting IgE induce suppression of basophil FcεRI expression which may confer clinical protection against a reaction following allergen exposure. Early studies of anti-IgE mAb (either HU-901 or omalizumab) for treatment of peanut allergy described enhanced tolerability of peanut in peanut-allergic individuals. A recent pilot study [2] treated 14 peanut-allergic subjects (aged 18 to 50 years) with omalizumab for 6 months and demonstrated a greater than 50-fold increase in the median reaction-eliciting dose of peanut double-blind placebo-controlled food challenge (DBPCFC) (80 vs 5080 mg, $p = 0.005$). Mechanistic studies showed suppression of basophil histamine release within 8 weeks of treatment and later onset suppression of mast cell reactivity by 6 months. These findings suggest that anti-IgE treatment can improve tolerability of allergen and is associated with suppression of effector cell function. However, it remains unclear whether such treatment modulates adaptive immune responses or can induce long-lasting tolerance.

FAHF-2 is a nine-herb Chinese herbal formula, which in animal model studies and phase 1 trials was found to be safe and well tolerated. In a multicentre double-blind placebo-controlled (DBPC) phase 2 randomised clinical trial [3], 68 subjects (aged 12–45 years) with challenge-confirmed allergies to

one or more foods including peanut, tree nut, sesame, fish and/or shellfish were randomised to receive FAHF-2 ($n = 46$) or placebo ($n = 22$) for 6 months without concurrent exposure to the offending allergen. FAHF-2 was not superior compared to placebo at improving tolerance to food allergens. Surprisingly, the placebo-treated subjects had a high rate (45.5 %) of treatment success (the study's primary outcome), defined as being able to consume 2 g of allergen protein on DBPCFC or a greater than four-fold increase in allergen dose required to induce a reaction when compared to the baseline reaction-inducing dose. Even using more stringent criteria of clinical response, i.e. a 10–20-fold increase in the amount of allergen protein tolerated, the placebo-treated group attained a high rate of success (13.6 %) within a short period of time. The authors could not explain the high rate of treatment success amongst placebo participants given the generally accepted rates of spontaneous resolution of allergy to peanut (0–15 %), tree nut (up to 10 %), fish (1 %) and shellfish (2 %). This highlights the importance of including a placebo group in clinical trials evaluating food allergy treatments. Interestingly, despite a lack of clinical effect in vivo, in vitro culture of participants' peripheral blood mononuclear cells (PBMCs; obtained at study entry) with food allergen showed significant differences in interleukin (IL)-5 (lower), IL-10 (higher) and CD4+CD25+Foxp3+ Treg cell numbers (higher) in the presence of FAHF-2 compared with media control. This suggests that co-exposure to antigen and FAHF-2 may be required to induce antigen-specific switch from allergic to tolerogenic responses and/or that the concentrations of FAHF-2 achieved in vivo were insufficient to reprogramme the immune response to allergen.

Antigen-Specific Immune Modulation

Noon and Freeman first described allergen-specific immunotherapy over 100 years ago. Since then, immunomodulation of antigen-specific responses through the gradual exposure to offending allergen by subcutaneous injection has become a standard of care for patients with severe allergic rhinitis or asthma and for insect venom anaphylaxis. Attempts at food allergen-specific subcutaneous immunotherapy were associated with safety concerns. Subsequent studies of sublingually delivered food immunotherapy did not confer adequate levels of protection. Therefore, OIT has remained the major focus of ongoing study in the search for an effective food allergy treatment.

OIT involves the oral administration of increasing doses of food allergen starting at very low doses and building up to a higher maintenance dose. Modern OIT regimens generally comprise a rapid induction phase, a gradual build-up phase and a maintenance phase. During the rapid induction phase, the dose is rapidly increased from fractions of milligrams to

tens of milligrams over several hours. In the build-up phase, the patient continues to take the last tolerated dose on a daily basis, with dose increases performed periodically (usually every 2 weeks) over a number of months until the target maintenance dose is reached. After reaching the maintenance dose, the same dose is maintained on a daily basis for months to years.

OIT Using Whole Food Protein

Inducing Desensitization

Early studies of OIT focused on evaluating the effectiveness of OIT at inducing desensitization. Results of these earlier studies have been summarised in recent meta-analyses and systematic reviews [4–6].

A meta-analysis of five cow's milk randomised controlled trials (RCT) published up to October 2012 involving a total of 196 patients (106 active OIT treatment and 90 controls) reported successful desensitization, defined as being able to tolerate a 200-ml serve of cow's milk, in 62 % of children in the OIT group compared to 8 % of the control group [4]. More recent studies report similar desensitization rates of 61–88 % [7, 8].

A Cochrane review of DBPC RCT of egg OIT published up to December 2013 included four studies involving 167 egg-allergic children (100 active OIT treatment and 67 controls) [5]. Thirty-nine percent of children in the OIT group were successfully desensitized, defined as being able to tolerate a full serving of egg (10–13.6 g of egg protein or 10 mL raw egg white) compared to 11.9 % of the control group, with a pooled relative risk ratio (RR) of 3.39 (95 % confidence interval (CI) 1.74–6.62). Partial desensitization (being able to tolerate 1 to 7.5 g of egg protein) was achieved in 79 % of the OIT group, resulting in a pooled RR of 5.73 (95 % CI 3.13 to 10.50). However, the range of treatment duration in the included studies was wide (6 to 22 months) and the meta-analysis did not adjust for the potential effect of treatment duration on outcome. Reporting of adverse events (AE) was highly heterogeneous. Sixty-nine percent of children reported mild to severe AE during OIT treatment with pooled RR of 6.06 (95 % CI 3.11 to 11.83). Overall, 5 out of 100 (5 %) OIT-treated participants required adrenaline.

A meta-analysis of all peanut OIT and sublingual immunotherapy (SLIT) DBPC RCT published up to March 2013 included one OIT study (19 active treatment and 9 placebo treatment, children 2–11 years) and two SLIT studies (31 active treatment and 27 placebo treatment, children and adults) [6]. Random effect model analysis of pooled results found that OIT or SLIT had a positive effect on peanut allergy, as defined by the ability to tolerate an increased amount of peanut protein from the start of therapy, with 86 % (43/50) in the active treatment group achieving the primary outcome compared to

8.3 % (3/36) in the control group (odds ratio [OR], 38.44; 95 % CI, 6.01–245.81). There was no difference in the number of patients needing adrenaline between groups (OR, 0.51; 95 % CI, 0.03–10.20).

A more recent RCT evaluating the effect of low-dose peanut OIT at a target maintenance dose of 0.8 g/day of peanut protein for 6 months included 99 peanut-allergic children aged 7 to 16 years [9•]. Sixty-two percent (24/39) of the active treatment group compared to none (0/46) in the placebo-treated group achieved the primary outcome of passing a desensitization DBPCFC to a cumulative dose of 1.4 g of peanut protein. The median increase in peanut threshold after OIT was 1345 mg (range 45–1400; $p < 0.001$) equating to a 25.5-fold increase (range 1.82–280; $p < 0.001$). Limited mechanistic studies performed after 6 months of treatment demonstrated reduced peanut skin prick test wheal size and increased peanut sIgE but no change in basophil reactivity.

Overall, reported rates of desensitization from cow's milk, egg and peanut OIT studies range from 40 to 90 % in actively treated subjects, compared to 0–12 % of control or placebo-treated subjects, indicating that OIT can induce desensitization. However, it is important to highlight that there is significant heterogeneity in treatment protocols including duration of treatment, study design and outcome measures, which may limit comparability of reported outcomes.

Long-Term Outcomes in Subjects Achieving Desensitization

It is important to understand long-term outcomes following OIT when considering the risk vs benefit of this treatment approach. Keet et al. presented long-term follow-up data on 32 children from two early cow's milk OIT studies [10•]. In both studies, desensitization was the primary outcome and participants were advised to continue regular cow's milk intake following completion of the studies. After a median of 4.5 years in the first study and 3.2 years in the second study, 7 of 32 (22 %) participants were limiting their cow's milk intake due to symptoms and only 25 % of children had no symptoms with long-term cow's milk intake. Moreover, 22 % (6/27) of participants who continued regular cow's milk intake reported at least one episode of anaphylaxis and one subject who maintained regular cow's milk intake reported using intramuscular adrenaline at least twice a month for reactions to cow's milk. Similarly, two other long-term follow-up studies reported that 58–81 % of cow's milk-desensitized children were able to maintain regular cow's milk intake up to 7 years after completion of treatment; however, cow's milk-induced symptoms were reported by 18–50 % of the participants [11, 12].

These findings suggest that desensitization may not be an optimal outcome for patients with food allergy in the longer term as allergic reactions are common and can occur unexpectedly. Further studies evaluating quality of life measures as well

as the frequency and severity of allergic reactions in desensitized individuals compared with those maintaining allergen avoidance are required to clarify the benefits and safety of desensitization as an outcome in food-allergic patients.

Sustained Unresponsiveness

The ability to tolerate a food allergen after OIT has been discontinued for a period of time, referred to as sustained unresponsiveness (SU), and has been assessed in a small number of studies.

An early egg OIT RCT [13••] reported that 11 of 40 (27.5 %) egg OIT-treated children (receiving 2 g/day of egg protein) achieved SU (secondary elimination period of 4 to 6 weeks) after 22 months of treatment compared with 0 % (0/15) of placebo-treated participants. It is important to note that placebo participants were unblinded after 10 months and assessment for SU was only performed if their egg sIgE fell below 2 kU/L; hence, only 1 of 15 placebo participants underwent the second oral food challenge to assess for SU at 22 months, whereas SU food challenges were completed for all active (OIT-treated) participants who had not withdrawn from the study by that time point. This study design introduces bias, and findings should be interpreted with caution. In a follow-up study, OIT was continued in OIT-treated participants who failed to achieve SU at 22 months until such time as their egg sIgE fell below 2 kU/L. SU was achieved in 20 of 40 subjects (50 %) after up to 4 years of treatment; however, once again, this finding should be interpreted with caution since there was no parallel placebo-treated comparison group to control for natural resolution of egg allergy [14•]. Two other studies in which participants received active egg OIT for shorter periods (one egg or approximately 6 g of egg protein every 48 h for 3 months [15]; 4 g/day for 4 months followed by 6 months ad libitum egg containing diet [16]) reported lower rates (31–37 %) of SU. These findings suggest that duration of treatment and/or cumulative OIT dose (as a product of maintenance dose and the duration on maintenance dose) may affect the likelihood of achieving SU.

An open uncontrolled study of peanut OIT [17•] involving 39 peanut-allergic children (age 1–16 years) (maximum maintenance dose of 4 g peanut protein/day) reported that 12 of 24 children evaluated at trial end achieved SU and passed a 5 g peanut protein DBPCFC following 4 weeks of secondary peanut elimination [18]. However, it should be noted that the criterion for stopping a patient's treatment and administering the SU challenge was a reduction in peanut sIgE to below a certain threshold which was changed (from <2 to <15 kU/L) half way through the study, resulting in significant variability in treatment duration (mean 4.0 years, standard deviation 1.8 years). Furthermore, the study lacked a placebo group to control for the natural rate of resolution, which is relevant given the extended follow-up period. A recent peanut OIT study involving younger

children (age 9–36 months) employed a similar study design, whereby treatment was continued until and SU assessed only upon achievement of pre-specified benchmarks—at least 12 months in the maintenance phase, peanut sIgE ≤ 15 kU/L, skin prick test (SPT) ≤ 8 mm and no severe peanut-related symptoms in the previous 6 months. After a median duration of treatment of 2.4 years (IQR 2.1–3.9 years) at maintenance doses of 300 mg ($n = 20$) or 3000 mg ($n = 17$), 78 % (29/37) of participants passed a 5-g peanut protein DBPCFC following 4 weeks of secondary peanut elimination [19]. Several caveats should be noted when interpreting these findings. Firstly, the study did not include a 'no-treatment' or placebo control group, but included a retrospective cohort of age-matched peanut-allergic patients receiving standard care ($n = 154$) followed for an average of 3.6 years (95 % CI 3.3–3.8 years). The rate of natural resolution of peanut allergy amongst controls was estimated to be 4 %; however, the accuracy of this estimate is uncertain given the likely bias in both the selection of the control group and the decision to perform a food challenge to assess for tolerance. Indeed, the rate of resolution of peanut allergy over a similar time frame was reported to be 22 % (over ~3 years) in the HealthNuts study, which uniformly evaluated all participants for resolution of challenge-proven peanut allergy at age 4 years, and 23 % (over 4.6 years) in a study by Fleischer et al. which challenged patients to assess for tolerance if their peanut sIgE had dropped to <5 kU/L [20, 21]. Secondly, baseline peanut sIgE levels amongst OIT-treated participants tended to be lower than values in the control group, which may influence outcomes as lower peanut sIgE is associated with greater likelihood of natural resolution of peanut allergy [20]. Moreover, the investigators observed that OIT treatment failures had significantly higher baseline peanut sIgE than treatment successes. To address these caveats, Vickery et al. performed a worst-case analysis, where they reclassified OIT-treated participants who had baseline peanut sIgE <5 kU/L as treatment failures and recalculated the rate of resolution of peanut allergy in the controls based on sIgE levels, and found a much smaller difference in estimated rate of resolution of peanut allergy between groups (57 % in OIT participants vs 31 % in controls; (RR 1.8, 95 % CI 1.3–2.6, $p = 0.007$)). Hence, for both the peanut OIT studies described above, significant variability in treatment duration and the lack of appropriate controls introduce bias and make reported findings difficult to interpret.

An open pilot peanut OIT study involving 23 patients (aged 5–45 years) who received peanut OIT at a maintenance dose of 4 g/day and 20 age-matched control patients (aged 6–20 years) who maintained avoidance of peanut for 24 months reported SU (13 weeks after cessation of OIT) in 35 % (7 of 20, excluding 3 who were not able to complete 24 months of treatment) of participants in the active treatment group compared with 0 % in the placebo group. After a further 3 months of peanut avoidance (6 months after cessation of peanut OIT), only 15 % (3 of 20) OIT-treated participants maintained SU

[22]. Immune studies revealed upregulation of antigen-specific Treg cells, enhanced in vitro suppressor function and epigenetic changes in T cells of subjects achieving SU. However, no difference in peanut-specific antibody levels or basophil response was found.

Taken together, these studies suggest that OIT may have a limited ability to induce SU in around 30–50 % of patients with egg or peanut allergy and may be more effective if commenced in children under the age of 3 years.

Long-Term Outcomes in Subjects Achieving SU

Only one study has evaluated long-term clinical outcomes in participants who achieved SU at least 1 year after discontinuation of treatment.

Jones et al. administered two follow-up questionnaires to assess long-term effects of egg OIT. The first questionnaire was administered after the last subject completed OIT and study challenges, and the second questionnaire was administered around 1 year later. The range of follow-up period following discontinuation of study treatment at the time of administration of the questionnaires, although not described in the study, is expected to be wide (estimated to be 1–5 years after stopping OIT for the second questionnaire) and introduces potential reporting bias. Acknowledging this limitation, at the time of the first questionnaire, a significantly higher proportion of actively treated subjects was ingesting any egg as compared to placebo-treated subjects (82 vs 36 %, $p = 0.07$). One year later, there was no difference in the rate of ingestion of any egg between groups (85 vs 67 %, $p = 0.22$). It is notable that 4 of 18 (22.2 %) OIT subjects who achieved SU reported symptoms on egg ingestion in the 12-month period between the first and second follow-ups, despite maintaining regular baked and unbaked egg ingestion, indicating resensitisation to egg [14•].

These recent studies confirm that OIT can induce desensitization in a majority of patients; however, the ability to induce tolerance (SU) remains uncertain and appears to be limited in most reports. Furthermore, Syed et al.'s study demonstrates that SU may be lost following longer duration of secondary elimination. This suggests that ongoing allergen intake may be required for maintenance of a tolerance state, consistent with recent findings in murine models [23]; nevertheless, continued allergen intake may not necessarily provide absolute protection against resensitisation [14•].

Combining OIT with Immune-Modifying Adjuvants or Anti-Allergy Therapies

Combined Administration of Probiotic and OIT

Probiotics are live microorganisms that confer a health benefit to the host and may act by directly interacting with mucosal

immune cells and/or indirectly through modulation of the intestinal microbiota. Tang et al. recently completed a randomised placebo-controlled trial [24••] evaluating a combined probiotic (*Lactobacillus rhamnosus*) and peanut oral immunotherapy (PPOIT) in 62 peanut-allergic children who were randomised to receive either PPOIT or placebo for 18 months. SU (assessed by DBPCFC performed 2–5 weeks after treatment cessation) was achieved in 82.1 % (23/28) of PPOIT and 3.6 % (1/28) of placebo-treated children. Immunomodulatory changes were observed in the active treatment group with reduction of peanut SPT and sIgE and increase in peanut sIgG4 suggesting modulation of the peanut-specific allergic response.

This rate of SU is notably higher than previously reported with OIT and suggests that combined administration of the probiotic immune-modifying adjuvant may enhance effectiveness of OIT in inducing SU. A further multicentre RCT comparing PPOIT vs peanut OIT vs placebo is underway to clarify whether addition of a probiotic confers greater benefit than OIT alone. It should also be noted that the duration of secondary peanut elimination before assessment of SU in the study by Tang et al. was relatively short (2–5 weeks) so it is possible that the rate of SU would be lower if assessed a longer time after stopping PPOIT. The multicentre RCT that is underway will assess for SU after 12-week peanut elimination to evaluate the effectiveness of PPOIT to induce longer-term SU compared with placebo or OIT alone.

Omalizumab and OIT

Omalizumab is a monoclonal antibody against IgE. It is expected that administration of omalizumab with OIT should improve the tolerability of OIT. A pilot study involving 13 peanut-allergic children reported that 12 of 13 children pretreated with omalizumab for 12 weeks reached the maximum peanut OIT maintenance dose of 4 g/day after 8 weeks. Clinical effectiveness of OIT was not compromised with 12 subjects tolerating 8 g of peanut protein on either DBPCFC or open challenge after a further 12 weeks of peanut OIT without omalizumab [25]. Similarly, in a multicentre DBPC RCT [26••] of omalizumab combined with cow's milk OIT (28 subjects) vs placebo with cow's milk OIT (29 subjects) in milk-allergic patients aged 7–35 years, tolerability of OIT was improved by the use of omalizumab. Subjects who received omalizumab reported that 91.5 % doses taken during the escalation phase were symptom-free compared to 73.9 % in the placebo group ($p < 0.0001$). Furthermore, frequency of needing adrenaline treatment was higher in the placebo-treated group vs omalizumab-treated group (18 doses in 9 subjects vs 2 doses in 2 subjects) ($p = 0.052$). Rates of desensitization (omalizumab with milk OIT 85.71 % (24/27) vs placebo with milk OIT 71.43 % (20/28)) and SU after a secondary elimination period of 8 weeks (omalizumab with milk OIT 48.1 %

(13/27) and placebo with milk OIT 35.7 % (10/28)) were comparable. These findings suggest that omalizumab may be used to enhance the safety and tolerability of OIT, without compromising the ability to induce desensitization or SU.

Ketotifen and OIT

Ketotifen is a benzocycloheptathiophene with histamine receptor antagonist and mast cell stabilising properties. Coadministration of ketotifen with OIT would be expected to reduce allergic reactions to OIT. In a small study involving six peanut-allergic children (median age 10 years), ketotifen premedication from 2 weeks prior to and continued until 4 weeks after commencement of peanut OIT improved the tolerability and reduced the rate of gastrointestinal symptoms in subjects receiving OIT (reaction rate of 40 % of all doses taken in the ketotifen group vs 76 % of all doses in the placebo) [27].

OIT Using Heat-Treated Food Proteins

Heating induces conformational changes to allergenic epitopes responsible for IgE binding and therefore may improve the tolerability of an allergenic protein in subjects who would otherwise react to natural and unheated forms of the protein. Linear epitopes, unaltered by the heating process, retain their immunomodulatory effects, most probably through induction of allergen-specific IgG and T cell responses. Therefore, OIT using extensively heated forms of a protein may confer clinical effects with improved tolerability profile. Observational studies describe resolution of egg allergy in children who can tolerate and incorporate baked egg in their diets, although the absence of a control group prevents assessment of effectiveness above and beyond natural resolution of disease. In a recent pilot study of cow's milk-allergic children who had failed to complete the parent unheated cow's milk OIT study, 6 (out of 14) subjects who were placed on baked milk OIT for 12 months achieved a significant increase in the median reaction-eliciting dose during an unheated milk challenge (6 vs 360 mg, $p = 0.005$) [28]. However, the majority of subjects could not tolerate the target maintenance dose of 1.3 g/day of baked milk protein nor could they complete the study per protocol, suggesting limited tolerability of extensively heated proteins in allergic subjects.

Boiling of nuts may similarly result in loss of allergenic proteins—particularly Ara h 2, 6 and 7—from peanut seeds, possibly through leaching of allergens into cooking water or conformational change. A small observational study involving four peanut-allergic children showed varying ability to tolerate boiled peanut (which contained 2S albumins) suggesting that boiled peanut OIT may be better tolerated than native peanut OIT [29]. Larger controlled studies are underway

(BOPI-1: NCT02149719) to confirm these findings and to evaluate effectiveness of these approaches for induction of SU.

OIT Using Multiple Foods

Food-allergic patients often have more than one food allergy. Hence, the ability to perform OIT with several foods concurrently would be more desirable than sequential single-food immunotherapy treatments, which may take many years to complete. A single centre open pilot study compared tolerability of single-food and modified multi-food OIT schedules in 15 monoallergic (defined as sensitisation and positive reaction to a cumulative dose of 182 mg peanut protein on DBPCFC) and 25 polyallergic (defined as having peanut allergy and at least one additional allergy to a tree nut, sesame, milk or egg, with sensitisation and positive reaction to a cumulative dose of 182 mg on DBPCFC) participants. Participants receiving single-food OIT underwent rapid desensitization on day 1 followed by fortnightly dose escalations until the maintenance dose of 4 g/day was reached. Participants receiving multi-food OIT received the same total amount of allergenic protein for each allergen as per the standard schedule. Dose-escalation phase continued until a daily maintenance dose of 4 g/day of each allergen (up to 20-g cumulative dose for five allergens) was reached [30]. There was no difference in reaction rates between multi-allergen vs single-allergen desensitization groups (median of 3.3 and 3.7 % in multi and single OIT group, respectively; $p = 0.31$). Adrenaline was used twice in each group (8 % of multi-allergen-treated group and 13 % of single-allergen-treated group). Clinical efficacy (induction of desensitization or SU) was not assessed.

Sublingual Immunotherapy

Early food allergen SLIT trials reported limited clinical efficacy. A more recent multicentre study randomised 40 peanut-allergic subjects, aged 12 to 37 years (median, 15 years), 1:1 to daily peanut or placebo SLIT. After 44 weeks of SLIT, 14 (70 %) of 20 subjects receiving peanut SLIT were able to consume 5 g peanut protein or at least 10-fold more peanut powder than their baseline threshold compared with 3 (15 %) of 20 subjects receiving placebo ($p < 0.001$) [31]. However, most patients discontinued therapy by the end of year 3, and only 10.8 % (4/37) SLIT-treated subjects achieved SU after secondary elimination of peanut for 8 weeks [32].

A head to head peanut SLIT vs OIT study [33] involving 21 children (aged 7–13 years) with challenge-confirmed peanut allergy randomised subjects to receive either SLIT (3.7 mg/day maintenance) with placebo OIT or OIT (2 g/day maintenance) with placebo SLIT for 12 months. A 5-g peanut

challenge was then administered to evaluate for desensitization. One out of seven (14.3 %) of active OIT-treated subjects passed the challenge vs none (0 %) of the nine active SLIT-treated subjects. At this point, subjects were unblinded. Three active OIT subjects continued on active OIT (without SLIT), three active OIT subjects continued on active OIT with add-on active SLIT and nine active SLIT subjects continued on active SLIT with add-on active OIT for 6 months. Three out of three active OIT subjects passed the second desensitization challenge and two of them (66.7 %) passed the SU challenge after peanut avoidance for 4 weeks. One out of three active OIT with add-on SLIT subjects passed the second desensitization, but this subject failed the SU challenge. Five out of seven active SLIT with add-on OIT subjects passed the second desensitization challenge, and only one (14.3 %) of them passed the SU challenge. These findings suggest that OIT may be more effective at inducing both desensitization and SU than SLIT. However, the size of the study and its study design potentially dilute larger effect differences that may have been observed if active therapy with either active OIT or active SLIT was continued for a longer duration.

Epicutaneous Immunotherapy

There is evidence suggesting that epicutaneous exposure to allergens elicits immunologic responses. Early animal models evaluating the delivery of allergens topically and epicutaneously through intact skin conferred beneficial immunomodulatory effects leading to more recent studies of epicutaneous immunotherapy in humans.

A multicentre DBPC phase II-b trial [34] involving 221 challenge-proven peanut-allergic subjects (aged 6–55 years) randomised subjects to receive Viaskin Peanut (VP), at different doses (50, 100 and 250 mg peanut protein) or placebo for 1 year. The best response was seen in the group that received VP250, with 50 % of actively treated subjects responding (defined as an increase in post treatment cumulative reactive dose ≥ 10 -fold of basal reactive dose or post treatment cumulative reactive dose reaching ≥ 1000 mg peanut protein) compared to 25 % in the placebo group, $p = 0.0108$. Effects were greatest in children with limited benefit in adolescents and adults. These preliminary findings suggest that epicutaneous immunotherapy (EPIT) may be effective at inducing desensitization and improve subjects' ability to tolerate low doses of peanut following treatment. A follow study to assess induction of SU after an additional 12-month therapy is ongoing.

Conclusion

There is a need for disease-modifying treatment options for food allergy. Amongst the various novel approaches being

explored in the research setting, allergen-specific immunotherapy delivered orally offers the most promising option. There is emerging evidence to suggest that co-administration of OIT with immune-modifying adjuvants such as probiotics may further enhance efficacy and tolerability. Without deeper understanding about long-term and sustained effects, adverse effects and impact on quality of life, it is still premature to offer novel treatments such as OIT in the routine clinical setting. Elucidating underlying immunologic mechanisms driving the clinical effects of allergen immunotherapy may help identify biomarkers to optimise selection of patients who are most likely to benefit from such treatment.

Compliance with Ethical Standards

Conflict of Interest Kuang-Chih Hsiao and Mimi L.K. Tang declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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Articles

Long-term clinical and immunological effects of probiotic and peanut oral immunotherapy after treatment cessation: 4-year follow-up of a randomised, double-blind, placebo-controlled trial

Kuang-Chih Hsiao, Anne-Louise Ponsonby, Christine Axelrad, Sigrid Pitkin, Mimi L K Tang, on behalf of the PPOIT Study Team*

Summary

Background Oral immunotherapy has attracted much interest as a potential treatment for food allergy, yet little is known about its long-term effects. We aimed to assess long-term outcomes in participants who completed a randomised, double-blind, placebo-controlled trial of combined probiotic and peanut oral immunotherapy (PPOIT), which was previously shown to induce desensitisation and 2-week sustained unresponsiveness.

Methods All participants who completed the PPOIT randomised trial were eligible to participate in this follow-up study 4 years after treatment cessation. Peanut intake and adverse reactions to peanut in the 4 years after treatment cessation were systematically documented with a structured questionnaire administered by allergy nurses. Additionally, participants were invited to undergo peanut skin prick tests, measurement of peanut sIgE and sIgG4 concentrations, and double-blind placebo-controlled peanut challenge to assess 8-week sustained unresponsiveness.

Findings 48 (86%) of 56 eligible participants were enrolled in the follow-up study. Mean time since stopping treatment was 4.2 years in both PPOIT (SD 0.6) and placebo (SD 0.7) participants. Participants from the PPOIT group were significantly more likely than those from the placebo group to have continued eating peanut (16 [67%] of 24 vs one [4%] of 24; absolute difference 63% [95% CI 42–83], $p=0.001$; number needed to treat 1.6 [95% CI 1.2–2.4]). Four PPOIT-treated participants and six placebo participants reported allergic reactions to peanut after intentional or accidental intake since stopping treatment, but none had anaphylaxis. PPOIT-treated participants had smaller wheals in peanut skin prick test (mean 8.1 mm [SD 7.7] vs 13.3 mm [7.6]; absolute difference –5.2 mm [95% CI –10.3 to 0.0]; age-adjusted and sex-adjusted $p=0.035$) and significantly higher peanut sIgG4:sIgE ratios than placebo participants (geometric mean 67.3 [95% CI 10.3–440.0] vs 5.2 [1.2–21.8]; $p=0.031$). Seven (58%) of 12 participants from the PPOIT group attained 8-week sustained unresponsiveness, compared with one (7%) of 15 participants from the placebo group (absolute difference 52% [95% CI 21–82], $p=0.012$; number needed to treat 1.9 [95% CI 1.2–4.8]).

Interpretation PPOIT provides long-lasting clinical benefit and persistent suppression of the allergic immune response to peanut.

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Introduction

The prevalence of food allergy has risen substantially in the past 20 years.^{1–3} Whereas allergies to egg, milk, wheat, and soy generally resolve during childhood, nut and seafood allergies often persist throughout life. Management includes allergen avoidance and education in the emergency management of allergic reactions. The constant vigilance required to avoid allergens substantially impairs quality of life of both food-allergic children and their families.^{4,5} The quality of life of children with food allergies is worse than that of children with diabetes.^{4,7} Accidental exposure to food allergens is common, with an annual incidence of 12–15% in children with peanut allergy.^{8,9} Although most allergic reactions to food can be managed successfully, fatalities still occur, with an incidence of roughly three per million person-years in children aged 0–19 years.¹⁰ A curative treatment could potentially improve quality of life and prevent allergy-related deaths.

There is intense interest in oral immunotherapy as a food allergy treatment to induce desensitisation (defined as being able to tolerate the allergen while on treatment)^{11–17} or sustained unresponsiveness (defined as being able to tolerate the allergen weeks or months after stopping treatment).^{18,19} Results of randomised placebo-controlled trials suggest that oral immunotherapy can induce desensitisation in around two-thirds and sustained unresponsiveness of several weeks' duration in a small subset of children.²⁰ Whether desensitisation or sustained unresponsiveness persists long term after oral immunotherapy remains unknown.²¹ Long-term effects after oral immunotherapy have been reported in participants with egg or milk allergies, for which natural resolution can be expected in a substantial proportion of people. In the only follow-up study²² of a randomised trial of oral immunotherapy, no difference in frequency of egg ingestion was reported between people in the egg



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Research in context

Evidence before this study

We searched PubMed with the medical subject heading terms “food allergy”, “peanut allergy”, “oral immunotherapy”, and “long term” for articles published in English on or before May 12, 2017. We found four publications about long-term outcomes after cessation of oral immunotherapy, one in children with egg allergies and three in children with milk allergies. However, we identified no studies of similar outcomes in patients who received peanut oral immunotherapy. Unlike most allergies to milk or eggs, peanut allergies tend to persist into adulthood, so long-term outcomes after egg or milk oral immunotherapy cannot be generalised to peanut oral immunotherapy. Furthermore, we identified no studies in which challenge-proven sustained unresponsiveness was assessed years after stopping oral immunotherapy.

Added value of this study

Ours is the first report, to our knowledge, of long-term outcomes several years after cessation of peanut oral

immunotherapy and the first study to incorporate challenge-proven sustained unresponsiveness as an outcome measure. Our findings show that combined probiotic and peanut oral immunotherapy provides long-lasting clinical benefit compared with placebo, with two-thirds of treated participants symptom free after peanut ingestion 4 years after completing treatment. The treatment resulted in symptom-free peanut ingestion, food-challenge-proven sustained unresponsiveness, and persistent suppression of the allergic immune response to peanut 4 years after treatment cessation.

Implications of all the available evidence

Long-term clinical benefits after standard oral immunotherapy remain uncertain. Combined administration of probiotic and peanut oral immunotherapy provided long-term clinical benefits, and the findings of this study are a step towards identification of an effective treatment to address the food allergy problem in developed countries.

oral immunotherapy group and those in the placebo group at least 12 months after treatment.

No data for long-term outcomes after peanut oral immunotherapy have been published. The natural history of peanut allergy is different from that of egg or milk allergy, so long-term outcomes for egg or milk oral immunotherapy cannot be extrapolated to peanut oral immunotherapy. We previously reported that combined probiotic and peanut oral immunotherapy (PPOIT) was effective at inducing desensitisation and sustained unresponsiveness after 2 weeks of secondary allergen information in children with peanut allergy (number needed to treat 1.27 [95% CI 1.06–1.59]).²³ We selected the probiotic *Lactobacillus rhamnosus* CGMCC 1.3724 (NCC4007), which is genetically indistinguishable from *L. rhamnosus* ATCC 53103, for use in PPOIT on the basis of its ability to induce regulatory T cells, antigen-specific IgA, and regulatory and T helper 1 cytokine responses.^{24–28} We postulated that administration of this probiotic with peanut oral immunotherapy would support redirection of the peanut-specific allergic response towards tolerance by providing a tolerogenic milieu at the time of antigen uptake and processing by antigen-presenting cells. In this study, we aimed to examine whether the previously reported clinical and immunological benefits of PPOIT were maintained 4 years after treatment.

Methods

Study background

Participants who completed the PPOIT randomised trial (the parent study) were assessed 4 years after treatment cessation (between January, 2015, and April, 2016). The parent study design and outcomes have been reported previously.²³ Briefly, 62 children with peanut allergy were

randomly assigned (1:1) to receive PPOIT (2×10^{10} colony-forming units of *L. rhamnosus* CGMCC 1.3724 and 2 g of peanut protein) or placebo (two formulations of maltodextrin) once daily for 18 months. During the study, all participants and their parents were reminded at each scheduled study visit to avoid taking probiotics and to remain on a peanut elimination diet as stipulated in the study protocol. At 18 months, all participants underwent a double-blind, placebo-controlled food challenge to assess for desensitisation. Participants who passed the desensitisation food challenge completed a second challenge 2–6 weeks after discontinuation of study treatment to assess for sustained unresponsiveness.

Participants who passed the sustained unresponsiveness challenge at the end of the parent study were instructed to continue intake of peanut ad libitum (ie, as part of their normal diet, without specific instructions on ingestion frequency or amount). Participants who did not attain sustained unresponsiveness were instructed to continue with strict peanut avoidance. Participants probably did not continue to take the probiotic at treatment doses because *L. rhamnosus* CGMCC 1.3724 is not known to the general public by that name, and participants were advised that the dose they were taking was equivalent to 20 tubs of yogurt each day. The parent study's primary outcome was the proportion of participants who attained sustained unresponsiveness.

Data collection and procedures

All participants who completed the parent study (n=56) were eligible for this follow-up study; consent was given at enrolment into the follow-up study (ie, 4 years after treatment). A structured peanut intake questionnaire and a validated food allergy quality-of-life questionnaire (Food

Allergy Quality of Life Questionnaire [FAQLQ]-parent form if participants were younger than 13 years, FAQLQ-teenager form if they were aged 13 years or older)³⁰ were administered to participants or their parents by allergy research nurses. The peanut intake questionnaire systematically recorded participants' peanut intake history, including the average amount ingested, ingestion frequency, and adverse reactions to peanuts after accidental or intentional ingestion since stopping study treatment. We also invited participants to give additional, optional consent for peanut skin prick tests, blood sample measurements of peanut specific IgE (sIgE) and specific IgG4 (sIgG4), and double-blind placebo-controlled food challenge (cumulative dose 4 g peanut protein after peanut elimination for 8 weeks; appendix) to assess for sustained unresponsiveness.

Peanut skin prick tests (peanut extract from Hollister-Stier, Spokane, WA, USA; Greer Pick from Greer Laboratories, Lenoir, NC, USA) were done by allergy research nurses. Serum concentrations of peanut sIgE and sIgG4 were measured by ImmunoCAP (Phadia AB, Uppsala, Sweden). The peanut sIgG4:sIgE ratio was calculated by converting peanut sIgG4 concentrations from µg/L to ng/mL and peanut sIgE concentrations from kU/L to ng/mL and then using the formula:³⁰

$$\frac{\text{IgG4}}{\text{IgE} \times 2.4}$$

Ethics approval for this study, including the study protocol and an a-priori statistical analysis plan, was obtained from the Royal Children's Hospital Human Research Ethics Committee (HREC 27086Q).

Statistical analysis

Allowing for 30% loss to follow-up, we calculated that a sample size of 19 participants in each intervention arm would provide 90% power to detect a difference between 12% sustained unresponsiveness in the placebo group (3% spontaneously outgrowing peanut allergy per year) and 60% sustained unresponsiveness in the PPOIT group. Clinical outcomes at 4-year follow-up were analysed by intention to treat. The effects of treatment were estimated in terms of absolute differences, numbers needed to treat, and risk ratios (RRs) with 95% CIs. We tested the hypothesis of no difference in the effect of treatment between intervention groups with the test of proportions with Fisher's exact tests (with double the one-tailed exact probability), and with unadjusted and adjusted (by age, sex, or time since treatment cessation) generalised linear models. We addressed missing data (ie, participants lost to follow-up) with two approaches: worst-case-scenario models for peanut ingestion outcomes and weighted inverse probability models for sustained unresponsiveness outcomes. Detailed description of these approaches and the formula for calculating reaction rates per 10 person-years are in the appendix. Peanut skin prick

test wheal sizes were reported as mean (SD), and between-group comparisons were analysed by *t* test. Other immune markers, including peanut sIgE, sIgG4, and the sIgG4:sIgE ratio had skewed distributions, and were summarised as geometric means (95% CI) and assessed by regression models. Study data were gathered and managed with REDCap³¹ electronic data-capture tools hosted at Murdoch Childrens Research Institute and analysed with Stata (version 14.2).

Role of the funding source

The study sponsors had no role in study design; data collection, analysis, or interpretation; or writing of the Article. K-CH, CA, and MLKT had access to all study data and final responsibility for the decision to submit for publication.

See Online for appendix

Results

48 (86%) of 56 eligible participants were enrolled, 24 from the PPOIT group and 24 from the placebo group (figure). Mean time from treatment cessation to entry into this study was 4.2 years (SD 0.6) in the PPOIT group and 4.2 years (SD 0.7) in the placebo group. Baseline characteristics were similar between groups (table 1). Of the four PPOIT-treated participants who declined to participate in the follow-up study (figure), one was peanut desensitised and three had sustained unresponsiveness at the end of the parent study. All four placebo-treated participants who declined to participate were peanut

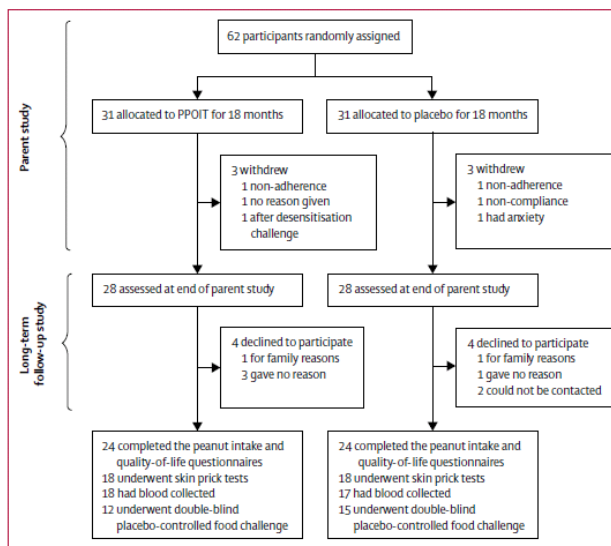


Figure: Participant flow
PPOIT=probiotic and peanut oral immunotherapy.

	PPOIT group (n=24)	Placebo group (n=24)
At entry to parent study		
Male sex	15 (63%)	16 (67%)
Had one or more siblings	22 (92%)	21 (88%)
Physician-diagnosed asthma	13 (54%)	9 (38%)
Physician-diagnosed eczema	19 (79%)	19 (79%)
At entry to 4-year follow-up study		
Age, years	12.1 (2.4)	11.7 (2.9)
Time since commencing study treatment, years	5.8 (0.6)	5.8 (0.7)
Time since completing study treatment, years	4.2 (0.6)	4.2 (0.7)

Data are mean (SD) or n (%). PPOIT=probiotic and peanut oral immunotherapy.

Table 1: Baseline characteristics

	PPOIT group	Placebo group
Peanut ingestion		
Ingesting peanut	16/24 (67%)	1/24 (4%)
Not ingesting peanut	8/24 (33%)	23/24 (96%)
Sensitivity analysis		
Scenario 1*	16/28 (57%)	1/28 (4%)
Scenario 2†	16/28 (57%)	5/28 (18%)
Frequency of ingestion		
Never	8 (33%)	23 (96%)
Less than once a week	5 (21%)	0 (0%)
Once or twice a week	7 (29%)	0 (0%)
Three or more times a week	4 (17%)	1 (4%)
Amount of peanut ingested‡		
None	7 (30%)	23 (96%)
<2 g	4 (17%)	0 (0%)
2 g to <4 g	6 (26%)	1 (4%)
≥4 g	6 (26%)	0 (0%)
≥2 g (ie, moderate-to-large amount)	12 (52%)	1 (4%)
Peanut skin prick test wheal size (mm)		
n	18	18
Mean (SD)	8.1 (7.7)	13.3 (7.6)
Median (IQR)	6.3 (1.0 to 14.0)	14.0 (9.0 to 18.5)

Data are n/N (%) or n (%), unless otherwise specified. PPOIT=probiotic and peanut oral immunotherapy. *All who declined to participate were recorded as not ingesting. †All participants in the PPOIT group who declined to participate were recorded as not ingesting; all placebo participants who declined to participate were recorded as ingesting. ‡n=23 for the PPOIT group because one participant did not provide a response about the amount of peanut they ingested.

Table 2: Peanut ingestion and skin prick test

allergic at the end of the parent study. We noted no between-group differences at entry to the parent study for participants who declined long-term follow-up, but significant differences in skin prick test results ($p=0.001$ at end of study; $p=0.009$ 3 months later). All other demographic features were similar between groups for participants who declined follow-up.

16 (67%) of 24 participants in the PPOIT group were still eating peanut 4 years after stopping study treatment, compared with one (4%) of 24 in the placebo group (absolute difference 63% [95% CI 42–83], $p=0.001$; number needed to treat 1.6 [95% CI 1.2–2.4]; unadjusted RR 16.0 [95% CI 2.3–113.6]; table 2). Adjustment for age, sex, or time since treatment cessation as separate models resulted in RRs of 15.6–16.4 ($p=0.006$). 12 (52%) of 23 PPOIT-treated participants (one person did not provide information about peanut ingestion) were ingesting moderate-to-large amounts of peanut (ie, ≥ 2 g peanut protein) per ingestion episode, and 11 (46%) were ingesting peanut at least once a week (table 2), suggesting ingestion ad libitum. More participants in the PPOIT group were ingesting more than 2 g of peanut protein than in the placebo group (absolute difference 48% [95% CI 26–70], $p=0.001$; unadjusted RR 12.5 [95% CI 1.7–90.6]). The RR was 11.7 (1.5–90.6, $p=0.019$) after adjusting for age, sex, and time since completion of treatment as combined model.

16 (80%) of 20 PPOIT-treated participants who achieved 2-week sustained unresponsiveness at the end of the parent study were regularly ingesting peanuts at 4-year follow-up. Of the other four participants, one ceased all peanut intake shortly after the end of the parent study because they disliked the taste of peanut, two reported symptoms after peanut ingestion within 24 months of treatment cessation, and one maintained regular peanut ingestion for more than 1 year after stopping study treatment but then discontinued peanut intake for around 6 months because of disruptive social circumstances before notifying the study team. The latter three participants subsequently failed formal peanut challenges up to 3 years after stopping treatment and were instructed by the study team to discontinue peanut intake. The one participant in the placebo group who attained sustained unresponsiveness at the end of the parent study continued to ingest peanuts at 4-year follow-up, but did not undergo the 8-week food challenge. The other 23 placebo-treated participants were all peanut allergic at the end of the parent study and had been advised to avoid peanut. None intentionally ingested peanut to test their allergic status.

In the first sensitivity analysis, assuming that all participants who declined to participate in the follow-up study were abstaining from peanuts, the absolute difference between groups was 54% (95% CI 34–73, $p=0.001$), with an unadjusted RR of 16.0 (95% CI 2.2–114.6). The RR was 15.7 (2.1–117.8, $p=0.008$) after adjustment for age, sex, and time since completion of treatment as a combined model. In the second sensitivity analysis, we assumed that all participants from the PPOIT group who declined follow-up had ceased peanut intake and that placebo-treated participants who declined follow-up had acquired the ability to eat peanut. The absolute difference between groups was 39% (95% CI 16–62, $p=0.006$), with an unadjusted RR of 3.2 (1.3 to 7.6) and an adjusted RR of 3.1 (1.3–7.7, $p=0.013$).

	PPOIT group (n=24)	Placebo group (n=24)
Patients who had one or more reactions	4 (17%)	6 (25%)
Reactions per patient		
0	20 (83%)	18 (75%)
1	2 (8%)	3 (13%)
2	1 (4%)	3 (13%)
7	1 (4%)	0 (0%)
Reactions by timepoint		
Total number of reactions	11	9
Within 3 months of stopping treatment	0 (0%)	0 (0%)
Between 3 and 12 months of stopping treatment	2 (18%)	1 (11%)
1–5 years after stopping treatment	9 (82%)	8 (89%)
Reactions by class*		
Anaphylaxis	0 (0%)	0 (0%)
Urticaria	0 (0%)	2 (22%)†
Urticaria plus abdominal pain with or without vomiting	0 (0%)	1 (11%)‡
Urticaria plus transient cough	7 (64%)‡	0 (0%)
Urticaria plus persistent cough	0 (0%)	0 (0%)
Oropharyngeal pruritus	2 (18%)‡	3 (33%)†
Abdominal pain with or without vomiting	2 (18%)†	1 (11%)‡
Abdominal pain plus transient cough plus oropharyngeal pruritus	0 (0%)	1 (11%)‡
Information not available	0 (0%)	1 (11%)‡
Total person-years followed up	99.8	102.0
Estimated number of events per 10 person-years	1.1	0.9

Data are n (%), unless otherwise specified. PPOIT=probiotic and peanut oral immunotherapy. *Two participants in the placebo group had more than one class of reaction. †Two participants. ‡One participant.

Table 3: Reported reactions from completion of study treatment to time of long-term follow-up study

Mean wheal size after skin prick test was similar in both groups before treatment (16.9 mm [SD 6.7] in the PPOIT group vs 16.9 mm [SD 6.9]).²³ At 4-year follow-up, wheals in PPOIT-treated participants (n=18) were smaller than those in placebo-treated participants (n=18; absolute difference -5.2 [95% CI -10.3 to 0.0]; unadjusted p=0.050; age-adjusted and sex-adjusted p=0.035).

20 (83%) of 24 PPOIT-treated participants reported no allergic reactions after peanut ingestion since stopping treatment (table 3). 11 reactions occurred after intentional peanut ingestion in four participants who attained 2-week sustained unresponsiveness in the parent study (table 3). Six placebo-treated participants reported nine allergic reactions after accidental ingestion of peanut (table 3). In both groups, all reactions were minor, none were anaphylaxis, and none necessitated adrenaline treatment (appendix). The number of reactions to peanut was 1.1 per 10 person-years in the PPOIT group and 0.9 per 10 person-years in the placebo group.

	PPOIT group	Placebo group	p value*
Peanut sIgE (kU/L)			
n	18	17	..
Median (IQR)	2.9 (0.7 to 8.8)	9.8 (1.6 to 100.0)	0.057
Geometric mean (95% CI)	2.8 (1.0 to 8.2)	10.7 (3.2 to 36.1)	0.089
Median difference in sIgE since time of entry to parent study (IQR)	-10.9 (-44.9 to -1.0)	-0.2 (-20.3 to 1.6)	0.086
Peanut sIgG4 (mg/L)			
n	15	14	..
Median (IQR)	0.7 (0.1 to 1.6)	0.2 (0.1 to 0.5)	0.150
Geometric mean (95% CI)	0.4 (0.1 to 1.5)	0.2 (0.1 to 0.5)†	0.409
Median difference in sIgG4 since time of entry to parent study (IQR)	0.3 (-0.1 to 1.4)	-0.1 (-0.3 to 0.0)	0.085
Peanut sIgG4:sIgE ratio			
n	15	14	..
Median (IQR)	91.4 (21.4 to 298.1)	2.4 (0.5 to 61.9)	0.018
Geometric mean (95% CI)	67.3 (10.3 to 440.0)	5.2 (1.2 to 21.8)†	0.031
Median difference in ratio since time of entry to parent study (IQR)	35.8 (-0.1 to 296.6)	-1.2 (-3.1 to 6.5)	0.013

PPOIT=probiotic and peanut oral immunotherapy. sIgE=specific IgE. sIgG4=specific IgG4. *The Wilcoxon rank sum (Mann-Whitney) test was applied for data expressed as medians (IQRs). The t test on the log scale was applied for data presented as geometric means (95% CIs). †n=13.

Table 4: Peanut sIgE and sIgG4 concentrations, and sIgG4:sIgE ratio 4 years after completion of study treatment

4 years after treatment cessation, PPOIT-treated participants had numerically lower peanut sIgE concentrations (geometric mean 2.8 kU/L [95% CI 1.0–8.2] vs 10.7 kU/L [95% CI 3.2–36.1]; p=0.089) and higher peanut sIgG4 concentrations (geometric mean 0.4 mg/L [95% CI 0.1–1.5] vs 0.2 mg/L [95% CI 0.1–0.5]; p=0.409) than placebo-treated participants, although these differences were not significant (table 4). Peanut sIgG4:sIgE ratios were significantly higher in PPOIT-treated than in placebo-treated participants (table 4).

27 participants (12 from the PPOIT and 15 from the placebo group) consented to double-blind placebo-controlled food challenge to assess 8-week sustained unresponsiveness. In the placebo group, participants who consented to food challenge were younger than those who declined (p=0.02; appendix), but no other differences—specifically, in peanut ingestion frequency, amount of peanut ingested, peanut skin prick test wheal size, peanut sIgE and sIgG4 concentrations, and status of peanut intake (ingesting or not ingesting) at the time of follow-up—were noted between participants who consented to food challenge and those who did not (appendix). Furthermore, among PPOIT-treated participants, those who achieved 2-week sustained unresponsiveness and maintained regular peanut ingestion afterwards, those who initially achieved sustained unresponsiveness but then discontinued peanut ingestion before the follow-up study, and those who did not achieve 2-week sustained unresponsiveness at the end of the parent study were equally distributed between

	PPOIT group (n=12)	Placebo group (n=15)
8-week sustained unresponsiveness at 4-year follow-up	7 (58%)	1 (7%)*
Achieved sustained unresponsiveness at end of parent study	10	0
Achieved sustained unresponsiveness at end of parent study and maintained it at 4-year follow-up	7	0

Data are n (%) or n. PPOIT=probiotic and peanut oral immunotherapy.
*One participant was on azathioprine at the time of food challenge; they were not ingesting peanut, had a peanut skin prick test result of 13 mm and a peanut specific IgE concentration of 5.43 kU/L, but they passed the 4 g food challenge.

Table 5: Long-term peanut challenge outcomes in participants who underwent sustained unresponsiveness food challenge after 8 weeks' peanut elimination

the participants who consented to food challenge and the participants who declined.

8-week sustained unresponsiveness as shown by double-blind placebo-controlled food challenge was significantly more common in the PPOIT group than in the placebo group (seven [58%] of 12 vs one [7%] of 15; absolute difference 52% [95% CI 21–82], $p=0.012$; unadjusted RR 8.8 [95% CI 1.2–64.0]; table 5). The RR was 8.7–9.9 ($p=0.023$ – 0.036) after adjustment for age or time since completion of treatment as separate models; the number needed to treat was 1.9 (95% CI 1.2–4.8). Seven (70%) of ten PPOIT-treated participants who had achieved 2-week sustained unresponsiveness at the end of the parent study showed challenge-proven 8-week sustained unresponsiveness 4 years after stopping treatment. The one placebo-treated participant who passed the follow-up food challenge was taking azathioprine for severe eczema (commenced 4 months before the follow-up study) and had a peanut skin prick test wheal size of 13 mm at the time of the challenge. Exclusion of this participant's outcomes strengthened the effect of PPOIT treatment on 8-week sustained unresponsiveness ($p=0.002$). The median cumulative doses tolerated during food challenge were 4000 mg (IQR 2438–4000) in the PPOIT group and 938 mg (188–1938) in the placebo group (Mann-Whitney test $p=0.003$).

The sensitivity analysis to account for non-participation, including backweighting for factors that could relate to the likelihood of failing the food challenge (ie, participant's sex, age at entry to parent study, history of doctor-diagnosed eczema or asthma at entry to parent study, and peanut allergy status [achieved sustained unresponsiveness vs did not achieve sustained unresponsiveness] at the end of the parent study) showed a beneficial effect for PPOIT as measured by challenge-proven 8-week sustained unresponsiveness (adjusted RR 10.0 [95% CI 1.3–78.5], $p=0.029$).

Characteristics of the five PPOIT-treated participants who did not have sustained unresponsiveness after food challenge are in the appendix. Two were peanut allergic

(ie, did not attain 2-week sustained unresponsiveness after the parent study), one attained sustained unresponsiveness at the end of the parent study but became resensitised 26 months after treatment cessation, and two reported regular peanut ingestion without reactions before the follow-up study, but reacted during food challenge at a cumulative dose of 4 g peanut protein. Two participants in the PPOIT group and four in the placebo group developed anaphylaxis during food challenge and required intramuscular adrenaline (appendix). Quality-of-life questionnaires were also completed as part of this study; findings will be reported separately.

Discussion

PPOIT was associated with long-lasting peanut tolerance 4 years after stopping treatment. Two-thirds of PPOIT-treated participants were able to continue regular peanut ingestion, and more than half were ingesting moderate-to-large amounts of peanut on a regular basis, compared with only one (4%) of 24 placebo-treated participants. Allergic reactions from intentional peanut ingestion were uncommon and all reactions were mild, suggesting that those who achieved PPOIT-induced sustained unresponsiveness can safely continue peanut ingestion. However, larger studies and meta-analyses of long-term safety outcomes are needed. 58% of PPOIT-treated participants who completed a double-blind placebo-controlled food challenge 4 years after stopping treatment attained 8-week sustained unresponsiveness, and 70% of participants who had attained sustained unresponsiveness at the end of the parent study maintained this status.

Strengths of our study include the multidimensional assessment of long-term efficacy, the standardised follow-up protocol, the high rate of participation (86%) with equal numbers from PPOIT and placebo groups, and the administration of double-blind, placebo-controlled food challenge after an extended 8-week period of secondary peanut elimination, with similar levels of participation from both groups. Furthermore, the inclusion of placebo-treated participants in this follow-up study allowed detection of effects attributable to the natural resolution of peanut allergy over time. In our study, excluding the participant who was taking a systemic immunosuppressant at the time of food challenge, none of the placebo-treated participants who were allergic to peanuts at the end of the parent study acquired an ability to ingest peanut or passed the food challenge 4 years later.

The major limitation of our study is low participation in the food challenge at 4-year follow-up. However, to our knowledge, this study is the first peanut oral immunotherapy study in which persistence of sustained unresponsiveness by double-blind, placebo-controlled food challenge years after treatment cessation was assessed. Furthermore, prolonged secondary peanut elimination for 8 weeks allowed a high level of confidence in outcome. We noted no differences in

clinical and immunological characteristics (including age at follow-up, peanut ingestion frequency, amount of peanut ingested, sustained unresponsiveness vs allergy status at end of parent study, peanut skin prick test wheal size, and sIgE and sIgG4 concentrations) between those who participated in the challenge and those who did not, suggesting that our findings are probably representative of the total sample of the parent study. Furthermore, we used inverse probability weighting to account for the potential effect of participants lost to follow-up, and a sensitivity analysis accounting for non-response, which still showed a favourable effect of PPOIT treatment 4 years after treatment. Nonetheless, we acknowledge that some caution is needed when generalising our challenge findings to those who declined to participate in the food challenge.

Other limitations include the retrospective collection of data for peanut ingestion and reactions, although this approach has been used in other oral immunotherapy follow-up studies,^{32,33} and the small sample size, which was similar to that in other published oral immunotherapy follow-up studies^{14,15,17–19,22} and reflects the proof-of-concept nature of the parent study. Generalisability of our findings might be partly limited by the single-centre study design. We also acknowledge the limitations of the parent study design, which we have previously discussed.²³ Briefly, the absence of a probiotic only group and an oral immunotherapy only group limit our ability to delineate the individual contributions of the two constituents of PPOIT. We note, however, that PPOIT did not modify skin prick test wheal sizes for other food allergens,²³ suggesting that probiotic alone probably did not produce the noted beneficial effects. Future randomised controlled trials comparing allergen oral immunotherapy, a combination of a probiotic and allergen oral immunotherapy, and placebo are needed to establish whether the probiotic acts synergistically with oral immunotherapy. The parent study also did not include a double-blind, placebo-controlled food challenge at study entry to confirm peanut allergy, and instead relied on objective reaction to peanut and 95% positive predictive value thresholds for sIgE concentrations and skin prick tests, in line with other oral immunotherapy trials at the time. However, randomisation would distribute any misclassifications evenly between groups, and placebo participants had a low frequency of sustained unresponsiveness, suggesting that misclassification of peanut allergy was unlikely.

Participants who achieved 2-week sustained unresponsiveness after 18 months of study treatment were instructed to incorporate peanut into their normal diet *ad libitum*, without predefined ingestion frequency or amounts, which is generally the advice given to patients who have naturally outgrown their food allergy. Consistent with the general view, we do not consider *ad libitum* peanut ingestion to be the same as continuing active peanut oral immunotherapy. The proportion of

PPOIT-treated participants who were ingesting peanuts 4 years after treatment compares favourably with those from other post-treatment follow-up studies of milk and egg oral immunotherapy.^{32,33} 80% of participants who attained 2-week sustained unresponsiveness after PPOIT treatment were ingesting peanut *ad libitum* 4 years after treatment, with 60% tolerating moderate-to-large amounts (>2 g peanut protein). Few studies of sustained unresponsiveness years after cessation of oral immunotherapy are available for comparison, but the proportion of patients achieving sustained unresponsiveness at 4 years is more than double that achieved immediately after oral immunotherapy alone in most studies, suggesting that addition of the probiotic could enhance the tolerance-inducing capacity of oral immunotherapy.

An important finding was that reactions to intentional peanut ingestion were uncommon in those who had attained sustained unresponsiveness at the end of the parent study and who had been instructed to ingest peanut freely. The frequency and severity of reactions in PPOIT-treated participants were similar to those after accidental ingestion in placebo-treated participants, suggesting that PPOIT-induced sustained unresponsiveness offers an equivalent safety profile to peanut avoidance. Furthermore, PPOIT-treated participants who maintained their sustained unresponsiveness status did not report any allergic reactions to peanut in the preceding 4 years. By comparison, 24 (75%) of 32 children who were desensitised after milk oral immunotherapy reported reactions in the preceding 12 months when assessed 4–5 years after treatment cessation, with anaphylaxis in six (25%).³² These findings suggest that sustained unresponsiveness is a preferred outcome for individuals with food allergy. Further studies are needed to establish the safety of continuing peanut ingestion in patients who initially achieve sustained unresponsiveness but subsequently have symptoms after ingestion. Investigation of long-term quality of life in people who achieve sustained unresponsiveness or desensitisation compared with those who do not achieve such outcomes is also important.

Reactions to accidental peanut ingestion in placebo participants were infrequent and reaction severity tended to be mild. This finding contrasts with published reports of retrospective studies,^{34–36} which describe accidental reactions in 50% of children with peanut allergy within 1–2 years, and 75% within 3–5 years, with roughly 50% of these reactions involving the respiratory system. In both the parent study and this follow-up study, we systematically gathered information about reactions and calculated the number and severity of reactions over a fixed period in all participants. This procedure probably increased the accuracy of information gathered but could also have modified participant behaviour, leading to improved vigilance and early management of allergic reactions. During the parent study, allergy nurses reviewed the symptoms and emergency management of

allergic reactions with each participant or their parents, or both, at each study visit (every 2 weeks for at least 8 months, then every month for up to 10 months). This intensive education probably provided trial participants and their caretakers with increased knowledge, confidence, and ability to avoid allergens and recognise and treat allergic symptoms, which could have led to fewer and less severe reactions. This intensive education is the most likely explanation for the low frequency of accidental peanut ingestion and mild reactions reported by participants in the placebo group, and consistent with the common observation that participation in a clinical trial leads to improved outcomes for placebo participants, highlighting the importance of including a placebo group. However, another plausible explanation is that published data for rates and severity of accidental reactions might not be generalisable to the wider population or to our select study population.

We did not establish whether the three PPOIT-treated participants who attained 2-week sustained unresponsiveness at the end of the parent study but did not achieve 8-week sustained unresponsiveness at 4 years had become resensitised or had never attained true sustained unresponsiveness in the first place. Perhaps these participants would not have passed an 8-week sustained unresponsiveness food challenge at the end of the parent study despite passing the 2-week challenge, because the likelihood of passing a sustained unresponsiveness food challenge reduces with increasing periods of secondary allergen elimination.^{35,37} Alternatively, initial sustained unresponsiveness might have been lost during the subsequent 4 years, a possibility that is supported by the finding that reactions to peanut occurred more than 12 months after stopping treatment despite continued peanut intake. Future studies of 8-week sustained unresponsiveness immediately after treatment and again years later will clarify this issue.

Probable reasons for participants to decline to participate in the placebo-controlled double-blind food challenge include potential inconvenience, risk of severe allergic reaction, and perceived risk of losing the ability to ingest peanut. Regular intake is known to maintain a desensitised state. Thus, many PPOIT-treated participants who had attained sustained unresponsiveness at the end of the parent study and were ingesting peanut declined to participate in the follow-up study food challenge (data not shown). Conversely, some participants welcomed the opportunity to undergo a food challenge to clarify whether they had maintained sustained unresponsiveness or were only desensitised. We suggest that sustained unresponsiveness as confirmed by placebo-controlled, double-blind food challenge should be used to monitor long-term clinical effectiveness in oral immunotherapy trials.

Although our results suggest long-lasting modulation of the peanut-specific immune response with reduction in typical allergy markers and increased sIgG4, these

changes have also been reported with desensitisation in the absence of tolerance,³⁸ emphasising the need to identify accurate and reliable biomarkers to distinguish desensitisation from sustained unresponsiveness.

To conclude, our results suggest that PPOIT is effective at inducing long-term sustained unresponsiveness that persists for up to 4 years after completing treatment and is safe. Furthermore, the finding that sustained unresponsiveness was maintained without the need to follow a regular prespecified ingestion schedule provides a compelling argument that PPOIT-induced immune tolerance. Ours is the first study to show prolonged 8-week sustained unresponsiveness several years after treatment has ceased, and suggests the possibility that tolerance is a realistic target for food allergy treatments. In a future study, microbial composition of stool samples will be analysed to examine effects of PPOIT therapy on the gut microbiome. Furthermore, a three-group, multicentre randomised controlled trial (ACTRN12616000322437) of PPOIT versus peanut oral immunotherapy versus placebo is underway to address the important and as-yet unanswered question of whether the addition of a probiotic confers greater benefit than oral immunotherapy alone.

Contributors

K-CH contributed to study conception and design; managed the study; did the clinical procedures, mechanistic work, and statistical analysis; drafted the Article; and contributed to the final version. A-LP contributed to study design and conduct, statistical analysis, data interpretation, and writing of the final report. CA and SP did the clinical procedures, and contributed to and reviewed the final report. MLKT conceived the parent PPOIT randomised trial, developed the follow-up study protocol, was responsible for oversight of study conduct and data analyses, and contributed to writing of the Article as senior author.

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Declaration of interests

K-CH reports grants from Murdoch Childrens Research Institute and the Australian Food Allergy Foundation. A-LP reports grants from Murdoch Childrens Research Institute and the Australian Food Allergy Foundation (previously the Ilhan Food Allergy Foundation), and personal fees from the National Health and Medical Research Council. MLKT reports grants from the Australian Food Allergy Foundation and Murdoch Childrens Research Institute, and personal fees from Nestlé Nutrition Institute, Danone Nutricia, GLG Consulting, Deerfield Consulting, Bayer, Prota Therapeutics, and Wiley. She also has a licensed patent method for inducing tolerance. CA and SP declare no competing interests.

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