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Longitudinal antibody responses to peanut following probiotic and peanut oral immunotherapy in children with peanut allergy

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Abstract

Introduction: Probiotic and Peanut Oral Immunotherapy (PPOIT) is effective at inducing sustained unresponsiveness (SU) at end-of-treatment and this effect persists up to 4 years post-treatment, referred to as persistent SU. We sought to evaluate (i) how PPOIT altered peanut-specific humoral immune indices, and (ii) how such longitudinal indices relate to persistent SU.

Methods: Longitudinal serum/plasma levels of whole peanut- and peanut component- (Ara-h1, -h2, -h3, -h8, -h9) specific-IgE (sIgE) and specific-IgG4 (sIgG4) antibodies were measured by ImmunoCAP and salivary peanut-specific-IgA (sIgA) by ELISA in children ($n = 62$) enrolled in the PPOIT-001 randomized trial from baseline (T0) to 4 years post-treatment (T5). Multivariate regression analyses of log-transformed values were used for point-in-time between group comparisons. Generalized estimating equations (GEE) were used for longitudinal comparisons between groups.

Results: Probiotic and Peanut Oral Immunotherapy was associated with changes in sIgE and sIgG4 over time. sIgE levels were significantly reduced post-treatment [T5, PPOIT vs. Placebo ratio of geometric mean (GM): Ara-h1 0.07, $p = .008$; Ara-h2 0.08, $p = .007$; Ara-h3 0.15, $p = .021$]. sIgG4 levels were significantly increased by end-of-treatment (T1, PPOIT vs. Placebo ratio of GM: Ara-h1 3.77, $p = .011$; Ara-h2 17.97, $p < .001$; Ara-h3 10.42, $p < .001$) but levels in PPOIT group decreased once treatment was stopped and returned to levels comparable with Placebo group by T5. Similarly, salivary peanut sIgA increased during treatment, as early as 4 months of treatment (PPOIT vs. Placebo, ratio of GM: 2.04, $p = .014$), then reduced post-treatment.

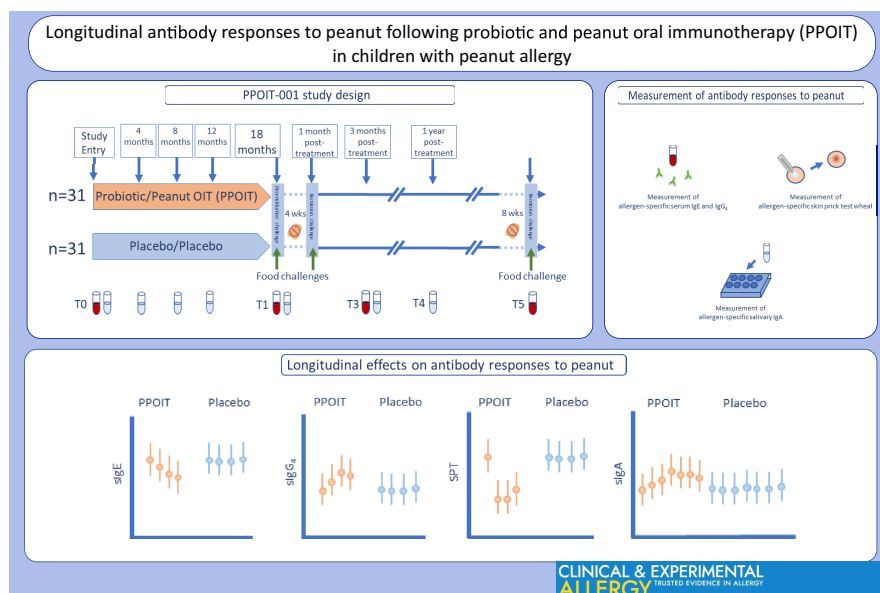
Conclusion: Probiotic and Peanut Oral Immunotherapy was associated with broad reduction in peanut-specific humoral responses which may mediate the clinical effects of SU that persists to 4 years post-treatment.

KEYWORDS

antibody responses, desensitization, oral immunotherapy, peanut allergy, probiotic, remission, sustained unresponsiveness

Sarah Ashley, Cassandra Yuen Yan Lee, and Lalita Jindal contributed equally.

[†]The PPOIT Study Team members are given in the Appendix.



GRAPHICAL ABSTRACT

Probiotic and Peanut Oral Immunotherapy (PPOIT) was associated with lasting changes in peanut and peanut component sIgE, with significant reductions persisting to 4 years post-treatment, consistent with durable redirection of the underlying peanut-specific allergic response. Peanut sIgG4 and salivary sIgA levels increased with treatment; however, the levels decreased rapidly upon treatment cessation, returning to Placebo group levels at 4 years post-treatment. This suggests that lasting changes in sIgG4 and sIgA may not be crucial for durable redirection of the allergic response.

1 | INTRODUCTION

A novel peanut oral immunotherapy treatment, combining probiotic with peanut oral immunotherapy (PPOIT), was shown to be effective at inducing clinical remission [2–6 weeks of sustained unresponsiveness (SU)] at end of treatment¹; and more importantly, persistent SU, after 4 years of *ad libitum* peanut intake.² Gradual exposure to allergen is postulated to suppress the allergic response (desensitization) through down-regulation of effector cells and may eventually redirect the allergen-specific immune response from allergy towards tolerance. Mechanistic studies during oral immunotherapy (OIT) have demonstrated treatment-associated reduction in allergen skin prick test (SPT) wheal size,^{1,3–6} which reflects suppression of allergen-specific mast cell activation; however reductions in circulating levels of allergen-specific IgE (sIgE) after around a year of treatment have not been achieved.^{3,5,7} A few studies have demonstrated reduced sIgE after 2 or more years of treatment,^{8,9} suggesting that the underlying allergic response may be modulated after a longer duration of OIT treatment. Allergen-specific OIT is consistently associated with up-regulation of allergen-specific IgG4 (sIgG4)^{3,6,7,10–12} and salivary sIgA¹³ during the treatment phase, however the roles of sIgG4 and salivary sIgA in tolerance acquisition following OIT remain uncertain.

We hypothesized that (1) PPOIT induces up-regulation of peanut and peanut component sIgG4 and peanut salivary sIgA levels during treatment, and (2) PPOIT induces long-term down-regulation of peanut and peanut component sIgE levels and (3) persistent SU after 4 years of *ad libitum* peanut intake, is associated with reduction in peanut and peanut component sIgE levels compared to those who did not achieve persistent SU.

Key Messages

- Probiotic and Peanut Oral Immunotherapy (PPOIT) induced long-term redirection of the peanut-specific allergic response
- Increased levels of peanut sIgG4 associated with oral immunotherapy reflect active allergen exposure
- Persistent sustained unresponsiveness (SU) is associated with lasting inhibition of peanut sIgE

2 | MATERIALS & METHODS

2.1 | Participants

This study describes *a priori* longitudinal serological outcomes from the PPOIT-001 randomized trial (ACTRN 12608000594325, 25/11/2008). Longitudinal cellular responses will be reported separately. The PPOIT-001 trial is a double-blind placebo-controlled randomized trial that randomized 62 children aged 1–10 years with peanut allergy to receive PPOIT (probiotic adjuvant *Lactobacillus rhamnosus* CGMCC 1.3724) at 2×10^{10} cfu/day and peanut oral immunotherapy maintenance dose 2 g/day peanut protein) or placebo/placebo for 18 months (Figure 1).¹

A double-blind placebo-controlled food challenge (DBPCFC) to peanut (cumulative dose 4 g peanut protein) was administered to all participants on the day after the final day of treatment (T1) to assess for peanut desensitization. Participants who passed this

desensitization challenge underwent a second DBPCFC 2–6 weeks post-treatment (T2) to assess for SU. An end-of-treatment visit was completed at 3 months post-treatment (T3). Participants who attained SU were advised to ingest peanut *ad libitum* (i.e. as part of their normal diet, without specific instructions on ingestion frequency or amount), whereas those who failed to attain SU were instructed to continue strict peanut avoidance as per standard of care. 56 participants completed the parent trial (PPOIT, $n = 28$; placebo, $n = 28$) and were eligible to enrol into 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 persistent SU at 4 years post-treatment (PPOIT, $n = 12$; Placebo, $n = 15$).²

2.2 | Study samples

Whole blood and saliva samples were collected at baseline (T0), end-of-treatment (T1), 3 months post-treatment (T3) and 4 years post-treatment (T5). Saliva samples were also collected at weeks 17, 33, 49 (4, 8, 12 months) during treatment, 2–6 weeks post-treatment

(T2) and 12 months post-treatment (T4) from eligible participants; no saliva sample was collected at T5. Blood samples were processed and cryopreserved at -80°C (serum, plasma) or in liquid nitrogen (peripheral blood mononuclear cells, PBMC). Serum/plasma was subsequently batch thawed and analysed.

2.3 | Whole peanut and peanut component sIgE and sIgG4 measurements

Levels of peanut and peanut component (Ara-h1, -h2, -h3, -h8 and -h9) sIgE and sIgG4 were measured by ImmunoCAP (Phadia AB) according to the manufacturer's instructions. Samples were centrifuged at 700 g for 5 min prior to aliquoting. Commercial and in-house quality control materials were used. For sIgE, data were reported as kUA/L and sIgG4 data were reported as mgA/L.

2.4 | Salivary peanut sIgA measurement

Salivary peanut sIgA was measured using an in-house ELISA assay adapted from a previously published protocol.¹³ Briefly, 96-well microtitre plates were coated with 100 μl /well of Crude Peanut Extract (11 mg/ml; kindly provided by Professor Wesley Burks,

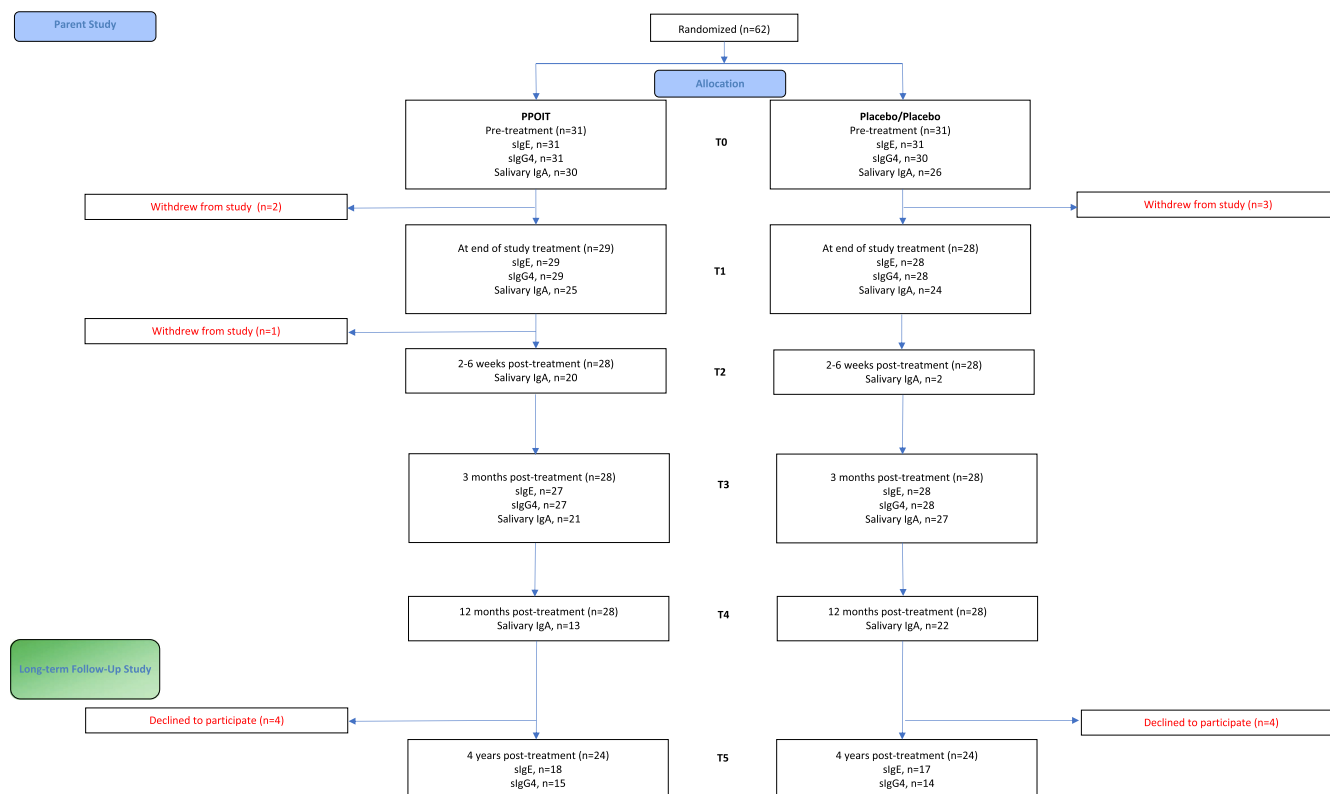


FIGURE 1 CONSORT diagram of participant flow in the PPOIT randomized controlled trial and the follow-on study of long-term outcome. T0: baseline; T1: end-of-treatment; T2: 2–6 weeks post-treatment assessment for sustained unresponsiveness (SU); T3: 3 months post-treatment; T4: 12 months post-treatment; T5: 4 years post-treatment assessment for persistent SU, after 4 years of *ad libitum* peanut intake. Salivary peanut sIgA was measured at additional timepoints 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

Duke University Medical Centre, USA). Saliva samples were diluted 1:2 in PBS containing 0.05% Tween 20 (PBS-T) and 2% foetal calf serum and incubated for 24 h at 4°C. Plates were washed four times with PBS-T and incubated (1 h, 37°C) with horseradish peroxidase-conjugated goat anti-human IgA (Southern Biotech) at 1:5000 dilution. After washing four times in both PBS-T and distilled water, separately, tetramethylbenzidine (TMB) substrate (KP;) was added at room temperature and the reaction stopped after 20 min with 1 M phosphoric acid (H_3PO_4). Plates were read within 30 min (BioTek plate reader) and results analysed using the KCjunior V1.40.3 software package (BioTek). Concentrations were measured in ELISA units (EU/ml).

2.5 | Statistical methods

Immune indices were examined for normality and linearity. Effects of age at baseline (T0) and sex as potential confounders on immunological outcome measures were examined using multiple logistic or linear regression models. There was a trend towards lower peanut sIgE at T5 being associated with older age (coefficient -0.807 , $p = .063$), however the effects of age and sex on immune indices reported in this paper did not reach statistical significance.

As per our registered study protocol (ACTRN 12608000594325, 25/11/2008), parametric immunological variables were analysed using standard t test with data summarized using arithmetic means and standard deviations (SD). Non-parametric immunological variables were analysed using standard t test on logarithmic scaled values with data summarized using geometric means and 95% confidence intervals (95% CI). Analyses, both without and with adjustment for potential confounders including age, sex and peanut ingestion where appropriate, were by complete case approach (where outcome data were available) as per the approach adopted in previous publications relating to this study. For evaluation of longitudinal trends from baseline (T0) to 4 years post-treatment (T5), we used generalized estimating equation (GEE) models which adjust both parameter estimates and standard errors to allow for clustering within participant because of repeated measures in longitudinal studies.^{14,15} Given that we were investigating a number of hypotheses and given the ongoing controversies surrounding whether or when multiplicity adjustments are warranted,^{16–19} we did not adjust p -values for multiple comparisons.

Data were analysed with Stata release 15 software (StataCorp). Graphs were generated in open-source programming language R (version 3.6.1).

2.6 | Ethics

Ethics approval was obtained from The Royal Children's Hospital Human Research Ethics Committee (HREC 27086). Informed consent was obtained from all participants or their legal guardians.

The trial, including the *a priori* plan to analyse immunological variables as secondary outcomes, was registered with the Australian New Zealand Clinical Trials Registry (ACTRN 12608000594325, 25/11/2008).

3 | RESULTS

3.1 | Participant flow

Figure 1 shows the participant flow. 62 children were randomized. As reported in the parent study, baseline characteristics including immune indices were comparable in PPOIT and placebo groups. Two PPOIT-treated and 3 Placebo-treated children withdrew during treatment and 1 PPOIT-treated child withdrew between T1 and T2. Of the 56 children who completed the parent study, 48 (PPOIT $n = 24$, Placebo $n = 24$) participated in this long-term follow-up study at 4 years post-treatment. Twenty-seven participants (12 PPOIT, 15 placebo) completed DBPCFC at 4 years post-treatment to evaluate for persistence of 8-week SU. Analyses were performed on the complete case population, representing the full ITT population where data were available. Peanut sIgE and sIgG4 measurements were available for over 98% of participants from baseline to 3 months post-treatment and for 73% and 60% of participants respectively at the 4-year post-treatment time point (T5) (Figure 1).

3.2 | Effects of PPOIT treatment on peanut and peanut component sIgE levels

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 (Figure 2), and a trend towards lower post-treatment peanut, Ara-h2 and Ara-h3 sIgE levels in the PPOIT group compared with the placebo group at earlier timepoints (T1 and T3). There were no between group differences for Ara-h8 and Ara-h9 sIgE levels at any timepoint. PPOIT treatment was associated with significant downward longitudinal trajectories in the levels of peanut, Ara-h1, Ara-h2 and Ara-h3 sIgE from baseline to 4 years post-treatment compared with placebo treatment (GEE, age- and sex-adjusted $p < .001$, $p = .003$, $p < .001$, $p = .040$ respectively) (Table S1a).

3.3 | 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 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 $p < .001$, $p = .011$, $p < .001$, $p < .001$ respectively; Figure 3). However, sIgG4 levels reduced rapidly after PPOIT treatment cessation and were comparable to the

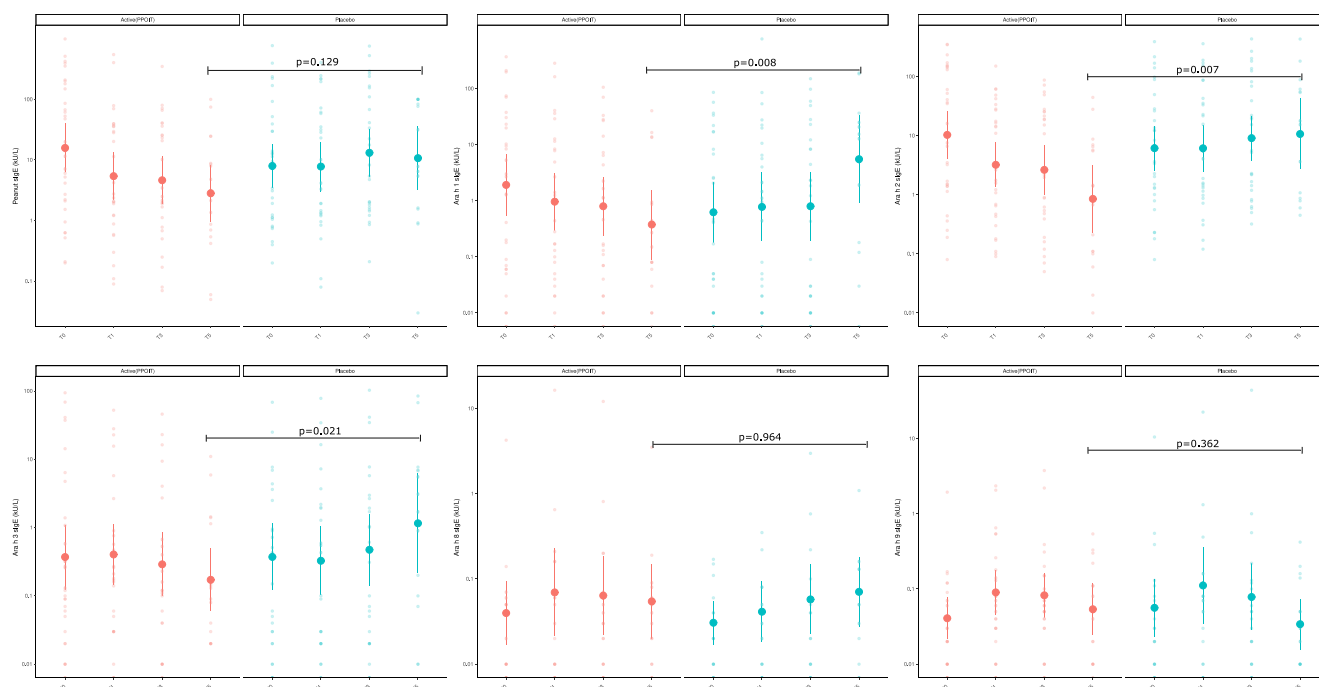


FIGURE 2 Dot plots of peanut and peanut component (Ara-h1, -h2, -h3, -h8, -h9) sIgE by treatment groups. Levels of peanut and peanut components (Ara-h1, -h2, -h3, -h8, -h9) sIgE were measured at baseline (T0), end-of-treatment (T1), 3 months post-treatment (T3) and 4 years post-treatment (T5) in both the active PPOIT (red) and Placebo (blue) groups. Geometric mean is illustrated with a circle and 95% CI as a solid line. sIgE levels are on the y-axis, presented on a log10 scale and units are in kU/L and visits are on the x-axis. The standard t test on logarithmic scaled values was used for comparisons between groups, adjusted for age and sex

placebo group by 4 years post-treatment (T5) (adjusted $p = .756$, $p = .305$, $p = .036$, $p = .526$ respectively; Table S1b).

3.4 | Effects of PPOIT on peanut SPT wheal size

The PPOIT group had significantly lower peanut SPT wheal size at end of treatment (T1; adjusted $p < .001$), 3 months post-treatment (T3; adjusted $p < .001$) and 4 years post-treatment (T5; adjusted $p = .039$) compared with the placebo group (Figure 4). GEE repeated measures analysis from baseline to 4 years post-treatment showed a significant downward trajectory in peanut SPT in the PPOIT group as compared to the placebo group (adjusted $p < .001$; Table S2).

3.5 | Effects of PPOIT on salivary peanut sIgA levels

Levels of salivary peanut sIgA increased early in the course of PPOIT treatment. Levels were significantly higher in the PPOIT group compared with the placebo group from as early as week 17 of treatment [geometric mean (95%CI) PPOIT 43.3 (30.2, 62.0) vs. Placebo 21.2 (14.8, 30.3); adjusted $p = .014$; Figure 5 and Table S3]. This increase in peanut sIgA was sustained to end-of-treatment (T1; adjusted $p = .016$). However, sIgA levels declined post-treatment and there was no between group difference by 12 months post-treatment.

3.6 | Peanut ingestion is associated with increased levels of peanut and peanut component sIgG4 at 4 years post-treatment

Given that peanut sIgG4 levels were only increased during the PPOIT treatment phase and returned to pre-treatment or baseline levels once PPOIT treatment was stopped, we hypothesized that the increase in peanut and peanut component sIgG4 levels reflected peanut ingestion (allergen exposure) *per se* rather than a fundamental redirection of immune responses towards tolerance. To investigate this, we performed post hoc subgroup analyses examining the relationship between peanut ingestion and sIgG4 levels in PPOIT-treated participants who attained 2- to 6-week SU at end-of-treatment and were permitted to ingest peanut *ad libitum*. As previously reported, 23 of 31 PPOIT-treated participants achieved SU at end-of-treatment in the parent study and were instructed to incorporate peanut into their diet *ad libitum*. At 4 years post-treatment, 12 (52%) participants reported ingesting moderate-to-large amounts of peanut (i.e. ≥ 2 g peanut protein) per ingestion episode, and 11 were ingesting peanut at least once a week, suggesting ingestion *ad libitum*. The post hoc analyses at T5 included all available measurements (peanut sIgG4 $n = 12$; peanut sIgE $n = 14$; SPT $n = 14$) from PPOIT-treated, end-of-treatment SU participants.²

Amongst participants in the PPOIT group who initially achieved 2- to 6-week SU (initial treatment responders), peanut sIgG4 levels at 4 years post-treatment were significantly higher in those who were ingesting peanut at T5 compared with those not-ingesting

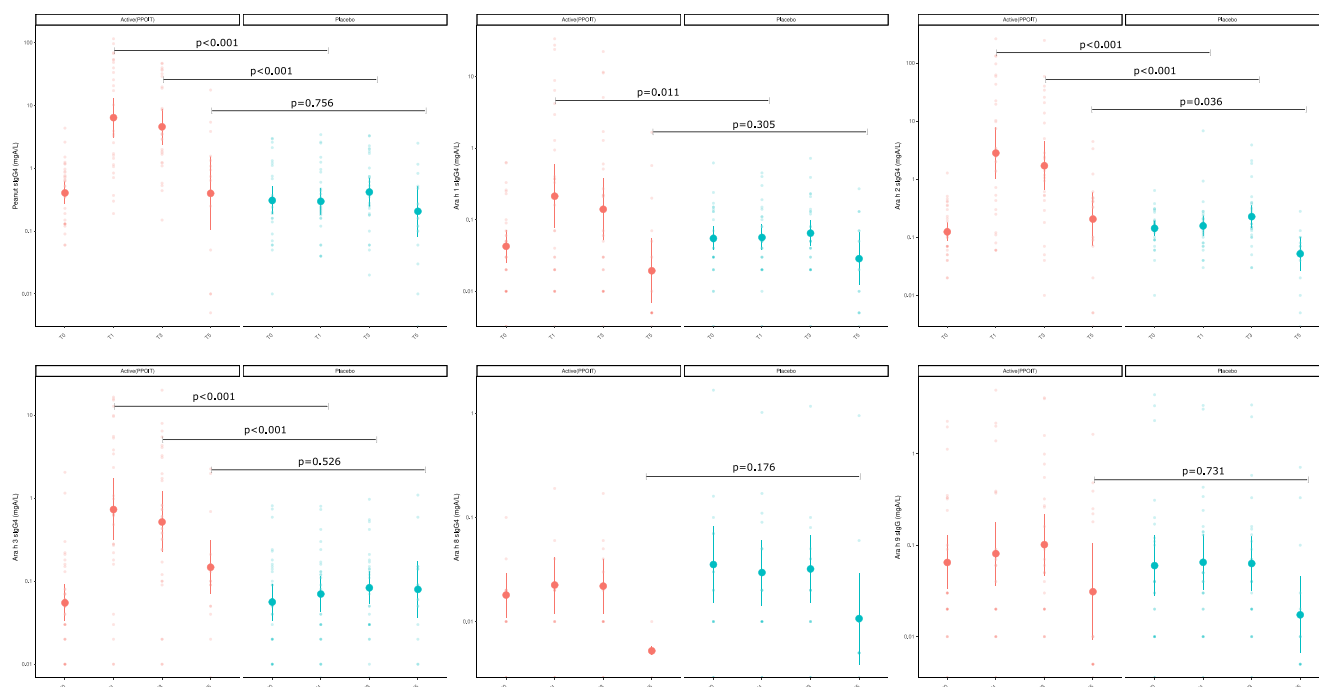


FIGURE 3 Dot plots of peanut and peanut component (Ara-h1, -h2, -h3, -h8, -h9) sIgG4 by treatment groups. Levels of peanut and peanut component (Ara-h1, -h2, -h3, -h8, -h9) sIgG4 were measured at baseline (T0), end-of-treatment (T1), 3 months post-treatment (T3) and 4 years post-treatment (T5) in both the active PPOIT (red) and Placebo (blue) groups. Geometric mean is illustrated with a circle and 95% CI as a solid line. sIgG4 levels are on the y-axis, presented on a log₁₀ scale and units are in mgA/L and visits are on the x-axis. The standard t test on logarithmic scaled values was used for comparisons between groups, adjusted for age and sex

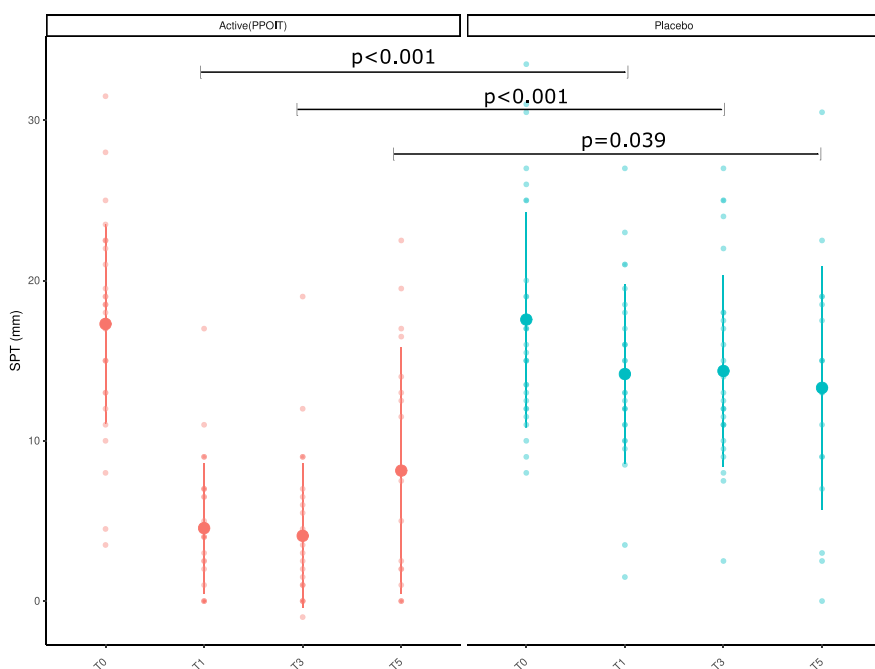


FIGURE 4 Dot plot of peanut skin prick test (SPT) wheal size by treatment groups. Peanut SPT wheal sizes were measured at baseline (T0), end-of-treatment (T1), 3 months post-treatment (T3) and 4 years post-treatment (T5) in both the active PPOIT (red) and Placebo (blue) groups. Arithmetic mean is illustrated with a circle and 95% confidence interval as a solid line. SPT levels are on the y-axis and units are wheal size in millimetres (mm) and visits are on the x-axis. The standard t test was used for comparisons between groups, adjusted for age and sex

peanut (adjusted $p = .026$). Furthermore, peanut sIgG4 levels were also higher in participants who were ingesting moderate or large amounts (≥ 2 g) compared with those ingesting none or only a small amount (age- and sex-adjusted $p = .015$). sIgG4 levels were not associated with ingestion frequency, although the small sample size does not allow confident exclusion of an effect (Table 1 and Table S4a).

3.7 | Peanut ingestion does not modify peanut and peanut component sIgE levels in PPOIT-treated participants with SU at 4 years post-treatment

In contrast to the findings for sIgG4, there were no differences in peanut and Ara-h1, -h2, -h3 sIgE levels between initial treatment

FIGURE 5 Dot plot of salivary peanut sIgA by treatment groups. Levels of salivary peanut-specific IgA (sIgA) were measured at baseline (T0), weeks 17, 33, 49 (4, 8, 12 months) during treatment, end-of-treatment (T1), 3 months post-treatment (T3) and 12 months post-treatment (T4) in both the active PPOIT (red) and Placebo (blue) groups. No saliva sample was collected at T5. Geometric mean is illustrated with a circle and 95% CI as a solid line. sIgA levels are on the y-axis, presented on a log10 scale and units are in EU/mL and visits are on the x-axis. The standard t test on logarithmic scaled values was used for comparisons between groups, adjusted for age and sex

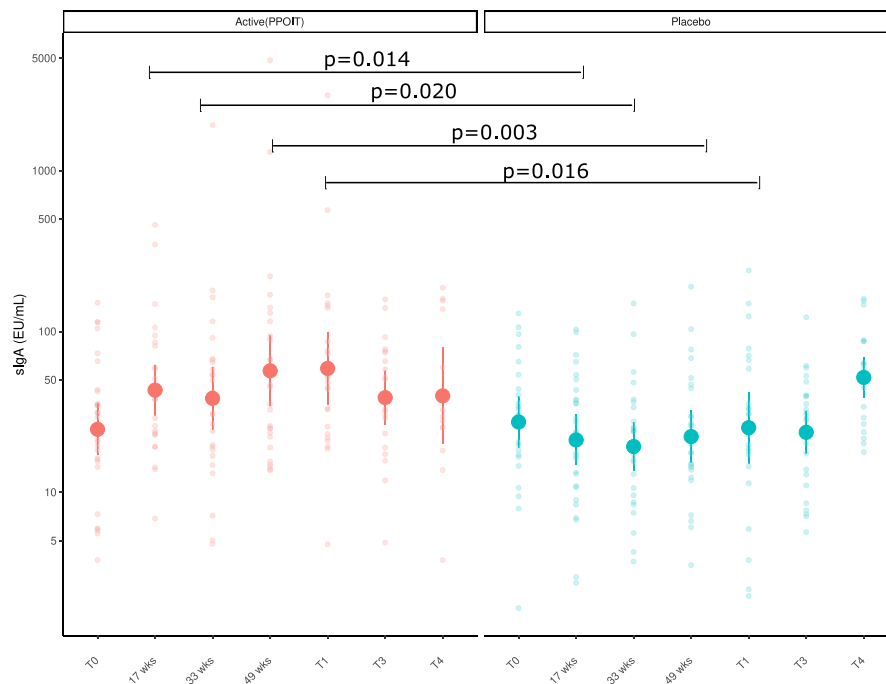


TABLE 1 Peanut sIgG4 and sIgE levels in PPOIT-treated participants who were or were not ingesting peanut at 4 years post-treatment

	Geometric mean (95% CI) of sIgG4 (mgA/L), n		Ratio of Geometric mean	Unadjusted p-value	Adjusted p-value*
	Ingesting vs. Not ingesting				
	Ingesting	Not ingesting			
Peanut sIgG4	1.48 (0.35, 6.33), 8	0.24 (0.01, 7.57), 4	6.08	.147	.026
	Ingesting once a week or more vs. Ingesting less than once a week				
	Once a week or more	Less than once a week			
Peanut sIgG4	1.81 (0.59, 5.61), 5	0.46 (0.05, 4.25), 7	3.98	.256	.215
	Ingesting moderate to large amount vs. Ingesting none or small amount				
	Moderate to large amount	None or small amount			
Peanut sIgG4	2.99 (0.95, 9.43), 6	0.22 (0.03, 1.55), 6	13.62	.014	.015
	Geometric mean (95% CI) of sIgE (kUA/L), n		Ratio of Geometric mean	Unadjusted p-value	Adjusted p-value*
	Ingesting vs. Not ingesting				
	Ingesting	Not ingesting			
Peanut sIgE	1.22 (0.26, 5.73), 10	3.35 (0.52, 21.73), 4	0.36	.402	.344
	Ingesting once a week or more vs. Ingesting less than once a week				
	Once a week or more	Less than once a week			
Peanut sIgE	1.28 (0.20, 8.10), 7	2.07 (0.32, 13.39), 7	0.62	.664	.182
	Ingesting moderate to large amount vs. Ingesting none or small amount				
	Moderate to large amount	None or small amount			
Peanut sIgE	1.26 (0.16, 9.72), 8	2.30 (0.73, 7.22), 6	0.55	.585	.462

*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.

Bold values denote when the adjust *p*-values are $< .05$.

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 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

TABLE 2 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), <i>n</i>	Mean difference	Unadjusted <i>p</i> -value	Adjusted <i>p</i> -value ^a	
	Ingesting vs. Not ingesting				
	Ingesting	Not ingesting			
Peanut SPT	2.65 (3.85), 10	15.88 (7.65), 4	-13.23	.001	.003
	Ingesting once a week or more vs. Ingesting less than once a week				
	Once a week or more	Less than once a week			
Peanut SPT	2.14 (4.23), 7	10.71 (8.61), 7	-8.57	.036	.031
	Ingesting moderate to large amount vs. Ingesting none or small amount				
	Moderate to large amount	None or small amount			
Peanut SPT	2.75 (4.36), 8	11.33 (9.20), 6	-8.58	.038	.032

^aAge- and sex-adjusted regression (of actual values).

numbers sample size does not allow confident exclusion of an effect (Table 1 and Table S4b).

3.8 | Peanut ingestion is associated with smaller peanut SPT wheal size in PPOIT-treated participants with SU at 4 years post-treatment

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 $p = .003$), 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 $p = .031$) 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 $p = .032$; Table 2).

3.9 | Peanut and peanut component sIgE and sIgG4 levels, and peanut SPT wheal size in PPOIT-treated participants with persistent SU after 4 years of *ad libitum* peanut intake and those without persistent SU

Next, we performed post hoc analyses to examine whether peanut SPT, peanut and peanut component sIgE and sIgG4 levels at baseline (T0) and at 4 years post-treatment (T5) were different in PPOIT-treated participants who attained persistent SU 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.

Participants with persistent SU had significantly lower pre-treatment levels of peanut, Ara-h1, -h2, -h3 sIgE compared with participants who did not have persistent SU (age-, sex- adjusted $p = .003$, $p = .012$, $p = .001$, $p = .004$; Table 3). Baseline peanut SPT wheal size was independent of persistent SU at 4 years post treatment ($p = .102$). At T5, peanut, Ara-h1, -h2, -h3 sIgE and peanut SPT,

were associated with persistent SU at 4 years post-treatment (age-, sex- adjusted $p = .001$, $p = .002$, $p < .001$, $p = .029$, $p < .001$ respectively; Table 3). Significance of these associations was maintained after adjusting for post-treatment peanut ingestion (age-, sex-, peanut ingestion-adjusted $p = .003$, $p = .004$, $p = .001$, $p = .001$, $p = .001$ respectively).

Surprisingly, persistent SU was also associated with significantly lower levels of peanut and peanut component sIgG4 at T5 [age-, sex-adjusted $p = .011$ (Ara-h1); age-, sex-, peanut ingestion-adjusted $p = .032$ (peanut), $p = .002$ (Ara-h1), $p = .018$ (Ara-h3); Table 3]. When examining the association between immune indices and persistence of SU after 4 years of *ad libitum* ingestion, we found no difference when we adjusted either for amount or frequency of peanut ingestion or for the composite variable of peanut ingestion frequency and amount.

4 | DISCUSSION

This is the first study to evaluate the long-term immunological effects of a combined probiotic and peanut oral immunotherapy (PPOIT) and their relationship to persistence of SU 4 years after discontinuation of OIT treatment. PPOIT treatment induced reduction in peanut and peanut component sIgE levels. sIgE levels reduced further over time such that by 4 years post-treatment, they were significantly lower than for participants in the placebo group, a post-treatment long-term finding that has not been reported in other peanut OIT trials to date. Conversely, peanut and peanut component sIgG4 and salivary peanut sIgA increased with PPOIT treatment but rapidly returned to baseline levels on treatment cessation. Of note, persistence of SU at 4 years post-treatment was associated with reduced levels of sIgE to peanut and peanut components at both baseline and at 4 years post-treatment, suggesting that subjects with lower peanut-specific IgE at baseline may be more likely to achieve durable persistence of SU. These findings are consistent with previous reports.²⁰⁻²²

Two novel findings were: first, that peanut and peanut component sIgG4 levels were directly correlated with the amount of

TABLE 3 Association between persistent SU at 4 years post-treatment and peanut and peanut component sIgE and sIgG4 levels and peanut SPT wheal size in PPOIT-treated participants

	Geometric mean (95% CI) of sIgE (kUA/L), n		Ratio of Geometric mean	Co-efficient [*]	p-Value [*]	Co-efficient ^{**}	p-Value ^{**}
	SU	No SU					
Peanut sIgE, T0	1.29 (0.26, 6.32), 7	85.34 (16.73, 435.41), 5	0.02	-0.366	0.003		
Peanut sIgE, T5	0.63 (0.12, 3.29), 7	12.79 (1.66, 98.27), 5	0.05	-0.564	0.001	-.525	.003
Ara h1 sIgE, T0	0.11 (0.00, 3.29), 5	27.20 (3.49, 212.10), 5	0.00	-0.266	0.012		
Ara h1 sIgE, T5	0.04 (0.01, 0.15), 6	4.11 (0.22, 75.22), 5	0.01	-0.342	0.002	-.319	.004
Ara h2 sIgE, T0	0.67 (0.15, 3.10), 7	38.91 (12.01, 126.02), 5	0.02	-0.394	0.001		
Ara h2 sIgE, T5	0.08 (0.02, 0.29), 7	4.87 (1.01, 23.56), 5	0.02	-0.431	<0.001	-.422	.001
Ara h3 sIgE, T0	0.03 (0.00, 0.16), 5	1.48 (0.03, 84.28), 5	0.02	-0.319	0.044		
Ara h3 sIgE, T5	0.06 (0.02, 0.14), 7	0.68 (0.02, 20.33), 5	0.08	-0.419	0.029	-.399	.023
	Geometric mean (95% CI) of sIgG4 (mgA/L), n		Ratio of Geometric mean	Co-efficient [*]	p-Value [*]	Co-efficient ^{**}	p-Value ^{**}
	SU	No SU					
Peanut sIgG4, T0	0.21 (0.11, 0.40), 7	0.62 (0.44, 0.87), 5	0.34	-1.056	.025		
Peanut sIgG4, T5	0.29 (0.03, 2.59), 6	2.05 (0.10, 43.35), 4	0.14	-0.176	.467	-.615	.032
Ara h1 sIgG4, T0	0.02 (0.01, 0.03), 5	0.13 (0.02, 0.94), 5	0.15	-0.617	.063		
Ara h1 sIgG4, T5	0.01 (0.00, 0.02), 7	0.12 (0.01, 2.21), 5	0.08	-0.465	.011	-.474	.002
Ara h2 sIgG4, T0	0.07 (0.05, 0.09), 7	0.18 (0.06, 0.54), 5	0.39	-0.948	.055		
Ara h2 sIgG4, T5	0.18 (0.02, 1.42), 6	0.72 (0.09, 5.80), 5	0.25	-0.272	.248	-.290	.084
Ara h3 sIgG4, T0	0.03 (0.02, 0.06), 7	0.08 (0.02, 0.24), 5	0.38	-0.608	.198		
Ara h3 sIgG4, T5	0.07 (0.03, 0.16), 7	0.27 (0.04, 1.65), 5	0.26	-0.468	.199	-.755	.018
	Mean (SD) of Peanut SPT (mm), n		Mean difference	Co-efficient [^]	p-value [^]	Co-efficient ^{^^}	p-value ^{^^}
	SU	No SU					
Peanut SPT, T0	12.07 (6.44), 7	19.00 (9.13), 5	-6.93	-0.034	.102		
Peanut SPT, T5	1.64 (1.84), 7	13.60 (4.72), 5	-11.96	-0.064	<.001	-0.084	.001

T0 = baseline/at randomization; T5 = 4 years post-treatment.

Bold values denote when the adjust *p*-values are < .05.

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

[^] Age-, sex-adjusted regression (of actual values);

^{^^}Age-, sex-, ingestion-adjusted regression (of actual values).

peanut ingestion post-treatment amongst subjects who attained SU post-treatment and introduced peanut *ad libitum* into their diet; and secondly, that peanut and peanut component sIgG4 levels were significantly lower in subjects with persistent SU compared to those without. Taken together, these observations suggest that increased levels of peanut sIgG4 during allergen immunotherapy may reflect allergen exposure per se and that sIgG4 antibody production might contribute to early desensitization effects but are not requisite for the persistence of SU. This is consistent with published reports of increased allergen-specific IgG4 amongst participants with desensitization who did not achieve SU.^{4,8}

Suppressed cutaneous mast cell reactivity to a food allergen accompanies desensitization during immunotherapy treatment but its role in tolerance induction remains unclear. Syed et al. found that while peanut OIT was associated with reduction in peanut SPT wheal size, this measure was not different between those who achieved

immune tolerance and those who did not.⁴ We hypothesize that allergen exposure during OIT leads to suppression of antigen-specific cutaneous mast cell reactivity, reflecting desensitization.

Early induction of IgG4 antibodies is one of the most consistent observations of immunotherapy. Although there are some data from OIT studies to suggest that IgG4 induction may confer reduction of allergic symptoms²³ 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 signalling,^{24,25} there has been no consistent correlation between sIgG4 levels and sustained clinical response to immunotherapy.²⁶ Moreover, there is currently no evidence of sIgG4 induction being the mechanism driving treatment-induced SU or tolerance acquisition in food allergy.

Data from pollen immunotherapy also 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 discontinuation²⁷ and there is no association between long-term clinical response and immunoreactive sIgG4 levels.²⁵ In contrast, long-term subcutaneous immunotherapy results in significantly reduced allergen sIgE,^{28,29} a phenomenon that is proposed to support environmental allergen tolerance acquisition.²⁶

Potentiation of memory B cells has been found to be critical in maintaining allergy in a murine model.³⁰ Signals that drive B-cell class switch to IgE, namely the production of Th2 cytokines IL-4, IL-13 along with ligation of CD40³¹ and activation of downstream signals including c-Rel,³² STAT6 and NF-kappa B,^{33,34} are the same signals that drive class switch to IgG4.^{35,36} These unified IL-4 induced signalling pathways are consistent with a synergistic relationship between IgG4 and IgE.³⁷ Evidence of sequential class switch of naïve B cells to IgE producing B cells through IgG(1) B cells^{38,39} 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,³⁰ further support the 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.^{40,41} Therefore, successful abrogation of both sIgE and sIgG responses through suppression of the overall allergen-specific memory B-cell response may be critical determinants of persistence of SU as demonstrated in our study. Deletion of allergen-specific memory B cells may be an effective strategy for treating allergic disease.

This study has a number of strengths. First, in the PPOIT randomized trial, SU was determined by stringent criteria of DBPCFC performed after a period of peanut elimination and persistent SU after 4 years of *ad libitum* peanut intake was assessed by DBPCFC following an 8-week period of secondary peanut elimination. Longitudinal bio-specimens were collected at multiple timepoints and correlated to clinical outcomes. The PPOIT RCT was the first to report clinical outcomes to 4 years post-treatment.² 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 persistent SU and immune response, acknowledging limitations of the analyses being performed post hoc on data available from only a small subset of participants.

We acknowledge other limitations in association with the study design of the parent trial as discussed previously.¹ 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 we are unable to attribute specific immune or clinical effects to the probiotic. Nevertheless, the current findings remain entirely relevant to the acquisition of persistent 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 immunological effects.

In this present study, immunoreactive sIgG4 was measured but not its function which might correlate with clinical response. There is emerging evidence suggesting that inhibitory function of sIgG4 plays an important role in mediating clinical effects of environmental allergen immunotherapy.²⁷ 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 allergy⁴² and sIgG4 response to OIT follows a similar trajectory as sIgG.³ Nevertheless, these other IgG subclasses and associated memory B cells³⁸ might be functionally and phenotypically distinct³⁷ and may also play a role in mediating clinical response to OIT. We have not evaluated antigen-specific IgE memory B cells given that ultrasensitive methods for tracking these cells have only been developed recently. Such investigations may provide important insight into mechanisms of tolerance induction. Another limitation is that we did not adjust for multiple comparisons because we were investigating a number of different hypotheses. It is recognized that adjusting p-values can sometimes be inappropriate and can increase the likelihood of type 2 statistical errors.^{16,17} Therefore, we have simply described the tests of significance performed to enable readers to reach reasonable conclusions independently without the help of adjustments.

5 | CONCLUSION

In summary, our findings provide compelling evidence that long-lasting suppression of allergen-specific IgE plays an important role in acquisition of SU and persistence of SU. 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 participants with persistent SU. Although up-regulation of antigen-specific IgG4 through antigen exposure may confer transient protection against clinical reactivity (i.e. desensitization), induction of sIgG4 does not appear to be required for persistence of SU. To the contrary, Persistent SU was associated with uniform reduction of circulating sIgE and sIgG4 levels suggesting that down-regulation of antigen-specific IgE and IgG4 producing B cells plays an important role in persistence of remission from allergy.

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CONFLICT OF INTEREST

KCH reports speaker and consultant fees from Danone Nutricia; membership of the Medical Advisory Board of Allergy New Zealand; membership of expert committees of the Australasian Society of Clinical Immunology and Allergy (ASCI). Mimi L.K. Tang (MLKT) reports consultant fees from Pfizer; inventorship on patents covering PPOIT; employee of and holding of share interest/options in Prota Therapeutics; membership of the Medical Advisory Board of Anaphylaxis & Anaphylaxis Australia and past membership of the

Board of Directors of the World Allergy Organisation (WAO, ended 2019); membership of expert committees of the American Academy of Allergy Asthma and Immunology, Asia Pacific Association of Allergy Asthma and Clinical Immunology, ASCIA, WAO (all current) and the International Union of Immunological Societies (ended 2019). The other authors report no conflict of interest.

AUTHOR CONTRIBUTIONS

Kuang-Chih Hsiao was involved in conceptualization (supporting), methodology, investigation (lead, equal), data curation, formal analysis (lead), writing—original draft (lead) and writing—reviewing and editing (lead, equal) of the manuscript. **Anne-Louise Ponsonby** carried out methodology, investigation, formal analysis, writing—reviewing and editing of the manuscript. **Sarah Ashley** carried out visualization, formal analysis (supporting) and writing—reviewing and editing of the manuscript. **Cassandra Yuen Yan Lee** was involved in methodology, investigation, formal analysis (supporting) and writing—reviewing and editing of the manuscript. **Lalita Jindal** carried out methodology, investigation, formal analysis (supporting) and writing—reviewing and editing of the manuscript. **Mimi L. K. Tang** carried out funding acquisition, conceptualization (lead), methodology, formal analysis, investigation (lead, equal) and writing—reviewing and editing (lead, equal) of the manuscript. PPOIT Study Team (group authorship) contribution: **Paxton Loke**, **Christine Axelrad**, **Sigrid Pitkin**, **Marnie Robinson**, **Dean Tey** and **Ee Lyn Su** carried out investigation (supporting) and writing—reviewing and editing of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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APPENDIX

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