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Differential Induction of Allergen-specific IgA Responses following Timothy Grass Subcutaneous and Sublingual Immunotherapy

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1 TITLE PAGE

2 Original Article

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109 **Abbreviations:**

- 110 AIT: Allergen Immunotherapy
- 111 TGP: Timothy Grass pollen
- 112 Ig: Immunoglobulins
- 113 IgE-FAB: IgE facilitated allergen binding
- 114 ISAC: Immuno Solid Allergen Chip
- 115 NAC: Nasal Allergen Challenge
- 116 NF: Nasal Fluid
- 117 Phl p: Phleum pratense
- 118 TNSS: Total nasal symptom score
- 119 SCIT: Subcutaneous Immunotherapy
- 120 sIgE: Specific IgE
- 121 sIgG4: Specific IgG4
- 122 sIgA1: Specific IgA1
- 123 sIgA2: Specific IgA2
- 124 SLIT: Sublingual Immunotherapy
- 125 Th1: Type 1 helper T cells
- 126 Th2: Type 2 helper T cells

127 **Introduction**

128 Allergen immunotherapy (AIT) is currently the only disease-modifying treatment for
129 allergic rhinitis, an IgE-mediated disease affecting up to 20-30% of adults and up to
130 40% of children.¹ Two routes of administration of AIT used clinically are
131 subcutaneous (SCIT) and sublingual (SLIT). Whilst SCIT involves weekly up-dosing
132 injections which are then followed by monthly maintenance injections for at least
133 three years², SLIT is self-administered and involves daily drops or tablets placed
134 under the tongue for the same duration of three years.³ SCIT^{4,5} and SLIT^{6,7} are both
135 disease-modifying and effective treatments in reducing clinical symptoms during the
136 Timothy grass pollen (TGP) season and the overall need of rescue medications.

137

138 After a 3-year course of AIT ends, long-term clinical benefit persists for at least 2-3
139 years.^{5,6} However, we recently reported that, in a 2-year AIT study of both SCIT and
140 SLIT, the Gauging Response in Allergic Rhinitis to Sublingual and Subcutaneous
141 Immunotherapy (GRASS) trial, no difference in the nasal response to allergen
142 challenge was observed in either SCIT or SLIT-treated groups compared to the
143 placebo group, 1 year after cessation of treatment, indicating that 2 years of
144 immunotherapy was not sufficient to induce sustained clinical tolerance.⁹

145

146 AIT confers its clinical effect through several mechanisms including immune-
147 deviation of type 2 T helper (Th2) cell response towards a type 1 T helper (Th1) cell
148 response, suppression or deletion of Th2 cells¹⁰⁻¹⁴, induction of regulatory T and B
149 cells^{2, 15, 16} and induction of IgG blocking antibodies, in particular IgG4^{15, 17, 18}
150 However, a detailed examination of the effect of SCIT and SLIT on a wider spectrum
151 of humoral responses, such as nasal and systemic IgA1, IgA2, IgG and IgG4 to AIT

152 allergens and their molecular components has not been conducted. Assessing the
153 effect of AIT on IgA is of interest because IgA is a mucosal antibody likely to play a
154 central role in binding allergens upon environmental exposure; as such, it could
155 reduce allergen contact with mucosal and submucosal mast cell-bound IgE.¹⁹

156

157 Using samples from the GRASS trial, we herein report for the first time, differential
158 induction of allergen-specific IgA responses following SLIT, compared to SCIT in
159 serum and nasal fluids. Moreover, we conducted an unsupervised cluster analysis of
160 biomarkers to identify whether immunoglobulins measured after one year of
161 treatment, in conjunction with other immunologic parameters, could stratify subjects
162 according to treatment group and treatment response at year 2.

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191 March each year), on the day of the nasal allergen challenge (NAC). Serum was
192 collected prior to NAC while nasal fluid was collected during NAC.

193

194 Immunoglobulin measurements and inhibition of CD23-mediated IgE-facilitated
195 allergen binding

196 TGP-specific IgA1, IgA2, IgG, IgG4 and IgE were measured in nasal fluid collected at
197 out-of-season nasal allergen challenge (NAC) visits (prior to the challenge) by an in-
198 house optimized and validated Enzyme Linked Immunosorbent Assay (ELISA)
199 (Methods section in the Online Repository). Specific IgE and IgG4 to TGP and TGP
200 components (Phl p 1, 2, 4, 5b, 6, 7, 11, 12) as well as IgA1 and IgA2 to TGP extract
201 were measured in pre-challenge out-of-season sera by the ImmunoCAP system
202 (Phadia US Inc., MI, USA) according to manufacturer's instructions and ELISA,
203 respectively. Inhibition of IgE-facilitated allergen binding to B cells was measured as
204 previously described.^{15, 17} Further details can be found in the Methods section of the
205 Online Repository.

206

207 Statistical Analysis

208 All immunologic data were assessed in the per-protocol (PP) population using a
209 linear mixed model adjusted for baseline values. The PP population included
210 participants who remained in the study at least three years and were compliant with
211 study medications, (defined as taking 50% or more of their study medication for the
212 duration of the study), and who had an evaluable primary endpoint. Missing baseline
213 serum (8 out of 84) IgE and IgG4 antibodies to TGP were imputed based on
214 corresponding antibody levels to TGP components. Serum antibodies to TGP and
215 TGP components (Phl p1, Phl p2, Phl p4, Phl p5b, Phl p6, Phl p7, Phl p11, Phl p12)

216 have a joint multivariate normal distribution. Therefore, a Markov Chain Monte Carlo
217 (MCMC) method was used to impute missing baseline serum TGP-specific IgE and
218 IgG4 antibody data by drawing on the joint multivariate normal distribution. Nasal
219 challenge-induced Total Nasal Symptom Score area under curve (AUC) data were
220 re-analyzed in the PP population using an ANCOVA model adjusted for baseline
221 AUC. To determine how many clusters were present in the study cohort, a complete
222 lineage hierarchical clustering method was performed using an agglomerative
223 approach (bottom-up). Participants were categorized as “Non Responders”, “Partial
224 Responders”, or “Responders” based on changes in TNSS from baseline to year 2.
225 Non Responders had < 10% reduction in TNSS from baseline to year 2, Partial
226 Responders had 10 – 40% reduction in TNSS from baseline to year 2, and
227 Responder had > 40% reduction in TNSS from baseline to year 2. The threshold for
228 significance was $P < .05$ (two-sided). Since all analyses were considered
229 exploratory, P-values were not adjusted for multiple comparisons. All analyses were
230 performed with SAS Version 9.4 (SAS Institute Inc., Cary, NC) and R version 3.2.4
231 (R Foundation for Statistical Computing). Data are accessible through TrialShare
232 (the Immune Tolerance Network repository) at
233 (https://www.itntrialshare.org/GRASS_antibody.url).

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260 Within the three treatment groups, only SLIT participants had increased levels of
261 TGP-specific IgA₁ and IgA₂ in nasal fluid at years 2 (3.35- and 2.72-fold over
262 baseline, respectively, $P < .001$) and 3 (3.37- and 2.77-fold over baseline,
263 respectively; $P < .001$). As a result, levels of TGP-specific IgA₁ and IgA₂ were
264 significantly higher in nasal fluids from the SLIT group compared to the placebo or
265 SCIT groups at years 2 (all, $P < .01$) and 3 (all, $P < .01$) (Fig 2, A-B, Table E1
266 (https://www.itntrialshare.org/GRASS_antibody_fig2.url). Year 1 specimens were not
267 assayed due to low sample volumes.

268
269 Serum TGP-specific IgA₁ levels were increased in SLIT-treated group at years 1, 2,
270 and 3 compared to baseline (4.96-, 7.02-, and 2.89-fold respectively, all $P < .001$)
271 (Fig 2, C, Table E1). However, serum IgA₁ levels declined in the SLIT-treated group
272 at year 3 compared to year 2 ($P < .001$). Serum TGP-specific IgA₁ levels were not
273 increased in the SCIT-treated group at years 1, 2 or 3, compared to baseline.
274 Similarly, no changes in serum IgA₁ levels were observed in the placebo group. The
275 SLIT group serum TGP-specific IgA₁ levels were higher than the placebo and SCIT-
276 treated groups at year 1 (all, $P < .001$), year 2 (all, $P < .001$) and year 3 (all, $P <$
277 $.001$) (Fig 2, C). Serum TGP- specific IgA₂ levels were increased in the SCIT and
278 SLIT-treated groups at year 1 (all, $P < .01$) and year 2 (all, $P < .05$) compared to
279 baseline. No significant change in serum IgA₂ levels from baseline were observed in
280 placebo-treated participants. The SLIT group was not statistically different from
281 placebo or from SCIT at any of the 3 study years. SCIT resulted in significantly
282 higher serum IgA₂ compared to placebo, but only in year 1 (Fig 2, D).

283

284 We examined whether the clinical effect of AIT on nasal allergen-induced symptoms
285 was associated with changes in nasal fluid or serum IgA. Figure 3 presents the
286 relationships between AIT-induced log₁₀-fold change in nasal TGP-specific IgA₁ and
287 log₁₀-fold changes in nasal allergen challenge-induced TNSS from baseline to year
288 2. We found a significant, albeit modest, correlation between nasal fluid IgA₁ and
289 TNSS after SLIT, but not SCIT or placebo, (Pearson $r = -0.52$, $P = .006$). No
290 significant relationships between changes in TNSS and serum IgA₁ (Fig 3, A-B,
291 https://www.itntrialsshare.org/GRASS_antibody_fig3.url) or serum IgA₂ or nasal fluid
292 IgA₂ were observed (Fig E2). We further confirmed this observation by applying a
293 linear mixed-effect model, with TNSS AUC as the dependent variable. When only
294 one biomarker was included at a time within the model along with visit, treatment,
295 and visit treatment interaction, nasal IgA₁ and IgA₂ was found significant, indicating
296 the level of nasal IgA₁ and IgA₂ had an effect on TNSS AUC in the expected
297 direction, confirming our earlier observations (Table E2).

298

299 Nasal fluid and serum IgG, IgG₄ and IgE to grass pollen following SLIT, SCIT or
300 placebo

301 TGP-specific IgG in nasal fluid and serum was elicited by both SCIT and SLIT. The
302 levels in nasal fluid were increased from baseline at year 2 in both SCIT and SLIT
303 group ($P < .05$) and at year 3 in the SLIT group ($P < .05$) (Fig 4, A). Similarly, serum
304 TGP-specific IgG levels were increased from baseline at years 2 ($P < .001$) and 3 (P
305 $< .001$) in both SCIT and SLIT groups (Fig 4, B). Levels of GP-specific IgG₄ in nasal
306 fluids increased from baseline for the SLIT and SCIT groups at years 2 and 3 ($P <$
307 $.001$), (Fig 4, C, Table E3). Similarly, serum IgG₄ to TGP increased from baseline in
308 both SLIT and SCIT groups (Fig 4, D, Table E3). A decline in IgG (87%, SCIT, $P <$

309 .001; 16%, SLIT, $P=.7$) and IgG4 (84%, SCIT, $P = .001$; 49%, SLIT, $P = .16$) was
310 observed in nasal fluids at year 3 compared to year 2. The observations in nasal
311 fluids were paralleled by serum IgG (68%, SCIT, $P < .001$; 45%, SLIT, $P = 0.01$) and
312 IgG4 (92%, SCIT, $P < .001$; 57%, SLIT, $P < .001$) at year 3 compared to year 2.

313 In nasal fluids, levels of IgG4 to timothy grass were significantly higher in the SCIT
314 group at year 2 compared to the SLIT group ($P < .05$). In serum, levels of both IgG
315 and IgG4 were significantly higher in the SCIT group compared to the SLIT group at
316 year 2 ($P < .01$). This differential treatment effect persisted for IgG4 in serum at year
317 3.

318 TGP-specific nasal fluid IgE was higher in SLIT compared to SCIT (2.76-fold, $P =$
319 .04) and placebo (3.07-fold, $P = .02$) at year 2, but not year 3 (Fig 4, E, Table E3).

320 This differential treatment effect on IgE levels in nasal fluids was paralleled in serum
321 (Fig 4, F, Table E3 https://www.itntrialshare.org/GRASS_antibody_fig4.url).

322 Moreover, longitudinal changes in serum IgG4 and IgE to Phl p 1, 2, 4, 5b, 6, 7, 11
323 and 12 following SLIT, SCIT or placebo were observed (Fig E3 and E4, Table E4
324 and E5). Further details on this observation can be found in the results section of the
325 Online Repository.

326

327 Inhibitory activity against in vitro allergen-IgE complex binding to B cells

328 The allergen-IgE binding to B cells as measured by the IgE-FAB assay using nasal
329 fluid was similar at baseline in SLIT, SCIT and placebo-treated patients. At year 2,
330 allergen-IgE complex binding to B cells was lower in SLIT (62.9%, $P < .01$) and SCIT
331 (56.5%, $P < .05$) compared to placebo (Fig 5, A) indicating treatment-induced,
332 blocking antibody activity in nasal fluids. At year 3, the reduction in allergen-IgE
333 complexes binding to B cells persisted in SLIT (61.2%, $P < .01$) compared to

334 placebo, but not in SCIT ($P = .21$). Serum from both SCIT- and SLIT-treated groups
335 blocked allergen-IgE complexes from binding to B cells at years 2 and 3, when
336 compared to placebo, as previously reported¹¹ (Fig 5, B, Table E6
337 https://www.itntrials.org/GRASS_antibody_fig5.url).

338

339 Relationship between local and systemic grass pollen-specific antibody
340 responses to clinical response

341 We next explored whether relationships between clinical outcomes and biomarkers
342 were present. We found that biomarkers after 1 year of AIT treatment were related to
343 the 2-year treatment outcomes. We performed hierarchical clustering using the
344 log₁₀-fold changes in variables from baseline to year 1 and an agglomerative
345 approach (bottom-up). We first used a set of already published biomarkers from the
346 same study that included early and late phase skin reactions⁹, BAT¹², serum IgE-
347 FAB data, tetramer data¹² and serum IgE and IgG4 data. Hierarchical clustering of
348 those biomarkers allowed for relatively good distinction between placebo and active
349 treatment, but less distinction between SCIT and SLIT (Fig 6, A). When serum IgA
350 data and serum component IgE and IgG4 data were added to the analysis, the ability
351 to cluster study participants by treatment and response to treatment was greatly
352 improved (Fig 6, B). The Figure 6B dendrogram shows that study participants could
353 be separated into 3 clusters. One cluster contained all 7 participants who had <10%
354 TNSS reduction from baseline to year 2. In general, these participants produced
355 less serum IgA to grass extract, and less IgG4 and IgE to grass components from
356 baseline to year 1. 14/18 of the members of this cluster belonged to the placebo arm.
357 In contrast, the other two clusters contained the majority of participants who had
358 >40% TNSS reduction from baseline to year 2. These two clusters had increased

359 serum IgA to grass extract, and more IgG4 and IgE to grass components from
360 baseline to year 1 (Fig 6, A-B,
361 https://www.itntrialshare.org/GRASS_antibody_fig6.url). Of all the participants in the
362 second cluster, 0 were SCIT recipients, while 9 of the 11 participants in the third
363 cluster received SCIT. All of the participants in the second cluster (9/9), but only 2 of
364 the 11 participants in the third cluster received SLIT.

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366 Discussion

367 Here we report a detailed longitudinal assessment of nasal and systemic antibody
368 responses during sublingual and subcutaneous grass pollen immunotherapy. IgA1
369 and IgA2 antibody induction was higher in SLIT, compared to SCIT and placebo. This
370 effect was most prominent in nasal fluids. In contrast, IgG and IgG4 antibodies both
371 in nasal fluids and in the circulation were greater in SCIT, compared to SLIT and to
372 placebo. Moreover, adding serum IgA, IgG4, and IgE measurements, taken one year
373 after initiation of AIT, to an unbiased biomarker cluster analysis enabled participants
374 to be relatively well stratified based on the treatment they received and also
375 identified subjects who did not respond to treatment.

376

377 AIT treatment has been previously associated with the induction of a serum allergen-
378 specific IgA2 antibody response.²⁰ Increases in salivary concentrations of grass
379 pollen-specific total IgA have also been observed in children following 3 years grass
380 pollen SLIT that persisted for two years after treatment cessation²¹. Our study
381 extends these observations by directly comparing local nasal and systemic IgA1 and
382 IgA2 concentrations following grass pollen SCIT and SLIT. Elevated IgA in SLIT was
383 observed during immunotherapy treatment and persisted even after discontinuation
384 of treatment. Changes in allergen-induced symptoms and changes in the level of
385 nasal fluid IgA1 showed a significant inverse correlation in the SLIT arm, but not in
386 SCIT or placebo, suggesting that IgA induced by SLIT may be related to clinical
387 improvements observed during therapy. However, this correlation was relatively
388 modest and, in year 3, after discontinuation of treatment, nasal fluid IgA1 and IgA2
389 remained elevated, whereas allergen-induced nasal symptoms did not differ between
390 SLIT and placebo.

391

392 IgA produced during grass pollen immunotherapy may participate in blocking
393 allergen from binding IgE receptors. The blocking capacity of IgA antibody has been
394 previously reported in a study where ragweed-specific IgA from nasal secretions was
395 shown to inhibit basophil histamine release.²² Here we show that, compared to
396 baseline, nasal fluids collected after 2 years of treatment had increased blocking
397 activity against IgE-allergen complex binding to B cells. It is notable that the
398 magnitude of this effect was equal between SLIT and SCIT. However, SLIT,
399 compared to SCIT, had lower levels of nasal fluid IgG4, the conventional “blocking
400 antibody” subclass, and higher levels of IgA1 and IgA2. This is compatible with a
401 hypothesis that IgA may also play the role of a “blocking antibody” in nasal fluids.
402 This notion is further supported by the experiments with serum where the differences
403 between SLIT and SCIT-induced IgA1 vs IgG4 were even more striking, but the
404 blocking activity of serum against IgE-allergen complex binding to B cells was no
405 different. Our data underscore that SCIT and SLIT likely involve differences in the
406 mechanism of action with IgG4 playing a predominant role in SCIT and IgA in SLIT.

407

408 The addition of serum IgA, as well as serum grass component IgE and IgG4 to
409 various other parameters, as part of an exploratory cluster analysis of the changes
410 from baseline to year 1, yielded 3 clusters that largely separated participants
411 according to the treatment they received (placebo, SLIT, SCIT). This suggests that
412 differences in the production of IgA, IgE, and IgG4 antibodies are defining
413 characteristics of SCIT and SLIT treatment. Including the baseline to year 1
414 changes in these datasets (https://www.itntrialshare.org/GRASS_antibody.url) also
415 allowed for the identification of participants who responded to therapy at year 2 and

416 clustered all participants who did not respond to therapy into a separate group,
417 suggesting that treatment efficacy may involve various aspects of the immune
418 response and that it may be possible to identify patients early in treatment who might
419 benefit from adjunct therapy. Furthermore, since SLIT and SCIT may act by distinct
420 mechanisms, this raises the question whether it may be helpful to combine the two
421 therapies in treatment-resistant cases. This would require testing in an adequately
422 powered controlled comparative clinical trial'

423

424 Local antibody responses are reflective of immunological and clinical responses to
425 individual allergen components. However, due to limitation in the amount of nasal
426 fluid that was obtained in this study, only IgE, IgG, IgG4, IgA1 and IgA2
427 measurements to the allergen extract, and not to individual grass pollen
428 components, were assessed in this study. Whilst it is possible to utilize an assay
429 such as ImmunoCAP solid-phase allergen chip technology (ISAC) to measure
430 reactivity against individual allergen components in nasal fluids, studies have shown
431 that allergen-specific IgG4 may compete with specific IgE for allergen-binding within
432 the assay. This may therefore underestimate the levels of sIgE obtained by ISAC.²³
433 Further to this, the baseline nasal fluid and serum samples measured in this study
434 were collected at the end of the grass pollen season. For this reason, it would be
435 challenging to derive any observations on the blunting of seasonal IgE increases in
436 the local target organ or within the circulation.

437

438 During the GRASS trial, peak pollen count was found within study year 2. Despite
439 this peak, each treatment group was exposed to similar levels of pollen as study
440 participants were randomized into one of three treatment arms at the start of the

441 study, indicating that differences in antibody levels between treatment groups are not
442 due to differences to pollen exposure. Additionally, when looking at antibody trends
443 within the placebo group, there were no consistent increases between year 1 and 2,
444 and no consistent decrease between years 2 and 3, highlighting that exposure to
445 pollen have minimal priming effect on the out-of-season time points used for
446 antibody measurements.

447

448 Despite our speculation that IgA may act as a blocking antibody, we have no direct
449 evidence of this. Our data suggest that there is an interaction between IgA1, IgA2
450 IgG4 and IgE, but the nature of this interaction is not clear. Shared repertoires
451 between IgG and IgE have been illustrated previously in a murine study²⁴ and more
452 recently, in a human study involving allergic and non-allergic subjects.^{25, 26} Similar
453 analyses for IgA may be worth pursuing to help determine whether IgA induced by
454 SLIT is capable of inhibiting IgE-mediated immune responses. However, this was
455 beyond the scope of this study.

456

457 In conclusion, we have identified that production of IgA is a major biological
458 difference between SLIT and SCIT. Whereas, as expected, SCIT induced higher
459 specific IgG4 levels than SLIT, SLIT led to higher IgA levels, both in serum and nasal
460 fluid. Furthermore, the levels of IgA1 in nasal fluids correlated with SLIT's
461 suppression of nasal symptoms during NAC. Specific IgA antibody production may
462 therefore represent a distinct mechanism through which SLIT exerts its therapeutic
463 effects that needs to be further investigated.

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*Total IgE measurements were performed in samples collected during the grass pollen season.

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586 https://www.itntrialshare.org/GRASS_antibody_fig3.url

587

588

589 Figure 4. Longitudinal changes in IgG, IgG4 and IgE in nasal lining fluid and
590 serum following SLIT, SCIT or placebo.

591 Nasal and serum (A,B) specific IgG (AU/mL), (C,D) IgG4 (mg/mL) and (E,F) IgE
592 (kU/L) to grass pollen allergen. Nasal specific (A) IgG, (C) IgG4 (E) IgE and serum
593 (B) IgG were measured by ELISA. Serum (D) IgG4 and (F) IgE were measured by
594 ImmunoCAP in SCIT-, SLIT- and placebo-treated groups. Note: different y-axis scale
595 for panels E and F. Data are presented as mean \pm 95% CI. *P < .05, **P < .01, A
596 linear mixed model was used with adjustment for baseline value.

597 https://www.itntrialshare.org/GRASS_antibody_fig4.url

598

599

600 Figure 5. Time course of allergen-neutralizing blocking antibody responses in
601 nasal fluid and peripheral blood in SLIT, SCIT and Placebo. (A) The co-operative
602 allergen-IgE binding to B cells and inhibitory activity for IgE-FAB was measured in
603 nasal fluid and (B) serum obtained from SLIT, SCIT and Placebo-treated patients.
604 Data are presented as mean \pm 95%. *P < .05, **P < .01, A linear mixed model was
605 used with adjustment for baseline value.

606 https://www.itntrialshare.org/GRASS_antibody_fig5.url

607

608

609 Figure 6. Linkage hierarchical clustering analysis using immune monitoring
610 biomarker assays with or without IgE, IgG4 to grass pollen components and
611 IgA to grass pollen. Log10 fold changes in variables from baseline to year 1 were
612 used to perform complete linkage hierarchical clustering using (A) selected

613 biomarker assays or (B) with the addition of serum IgA data and serum component
614 IgE and IgG4 data. Yellow color indicates large log10 increases from baseline to year
615 1 and blue color indicates large log10 decreases from baseline to year 1. Inclusion
616 of IgE/IgG4 component data and serum IgA data leads to improved clustering by
617 treatment group (SLIT = green, n = 15; placebo = blue, n = 14; SCIT = red, n=9) and
618 response to treatment (Non Responder: < 10% reduction in TNSS from baseline to
619 year 2 = black, Partial Responder: 10 – 40 % reduction in TNSS from baseline to
620 year 2 = white, Responder: > 40% reduction in TNSS from baseline to year 2 =
621 purple).

622 https://www.itntrialshare.org/GRASS_antibody_fig6.url

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TITLE: Differential Induction of Allergen-specific IgA Responses following Timothy Grass Subcutaneous and Sublingual Immunotherapy

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Measurements (unit)

Table E1. Levels of specific IgA₁ and IgA₂ to grass pollen in nasal fluid and serum

Table E2. Linear mixed effect model with one bioassay covariate in the model.

Effect

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Table E3. Levels of specific IgE, IgG and IgG4 to grass pollen in nasal fluid and serum

Measurements (unit)

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Table E4. Levels of specific IgE to grass pollen components

Grass pollen specific IgE

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Phi p5b
(kU/L)

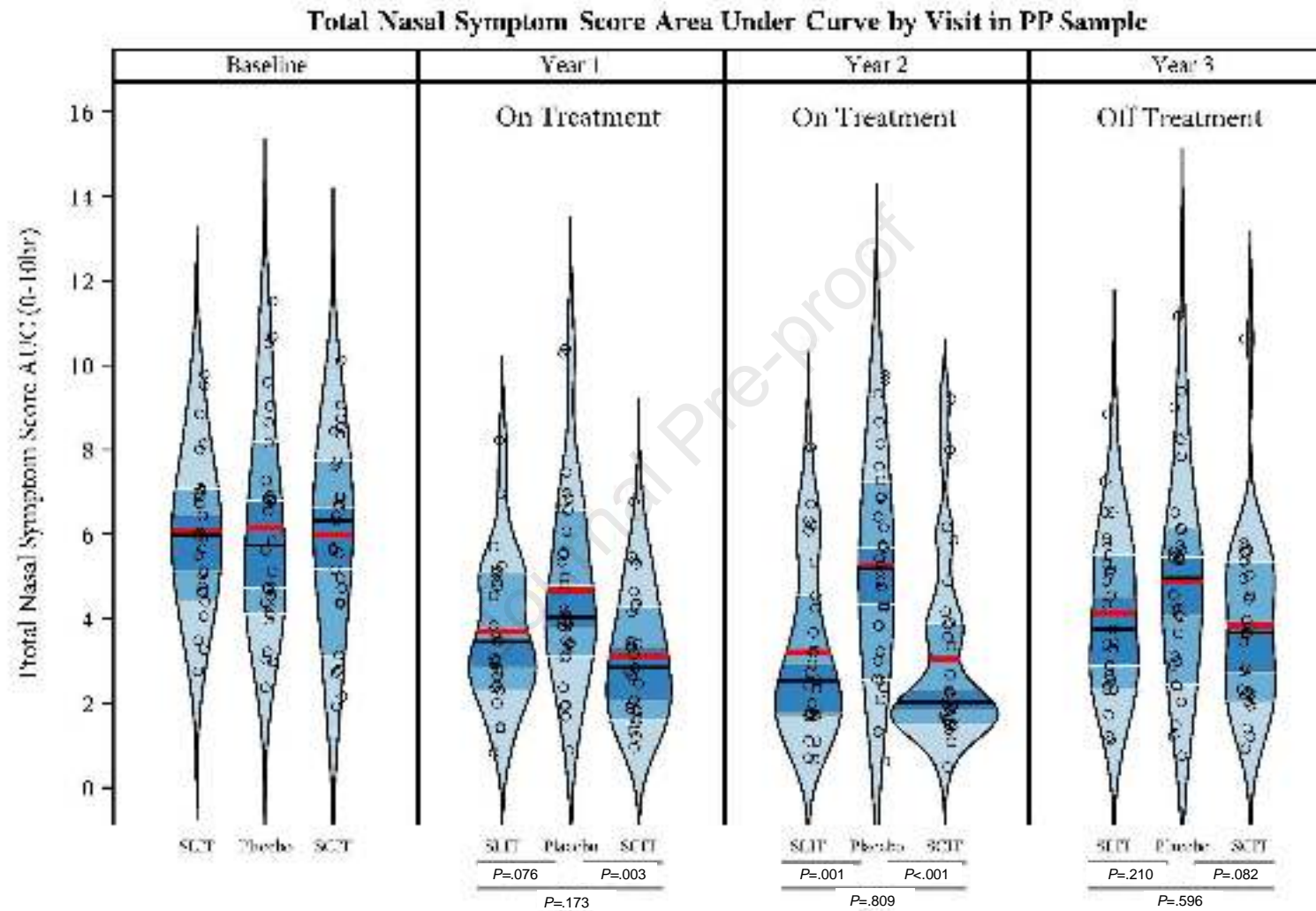
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Figure 4



Multicolored bands represent quintiles, red lines represent means, and black lines represent medians.
P-values calculated using an ANCOVA model adjusted for baseline AUC.

Figure 1

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Figure 2

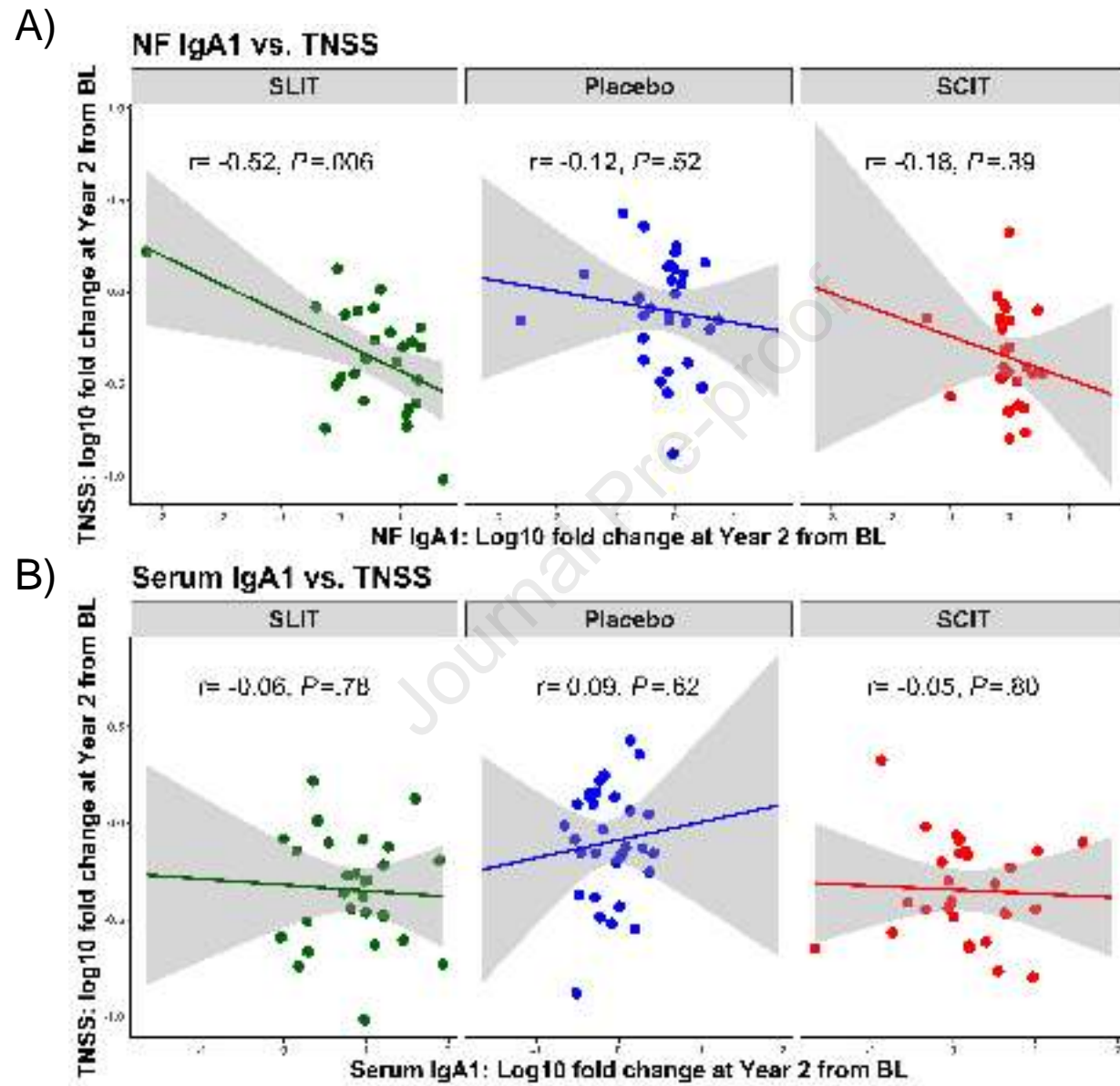


Figure 3

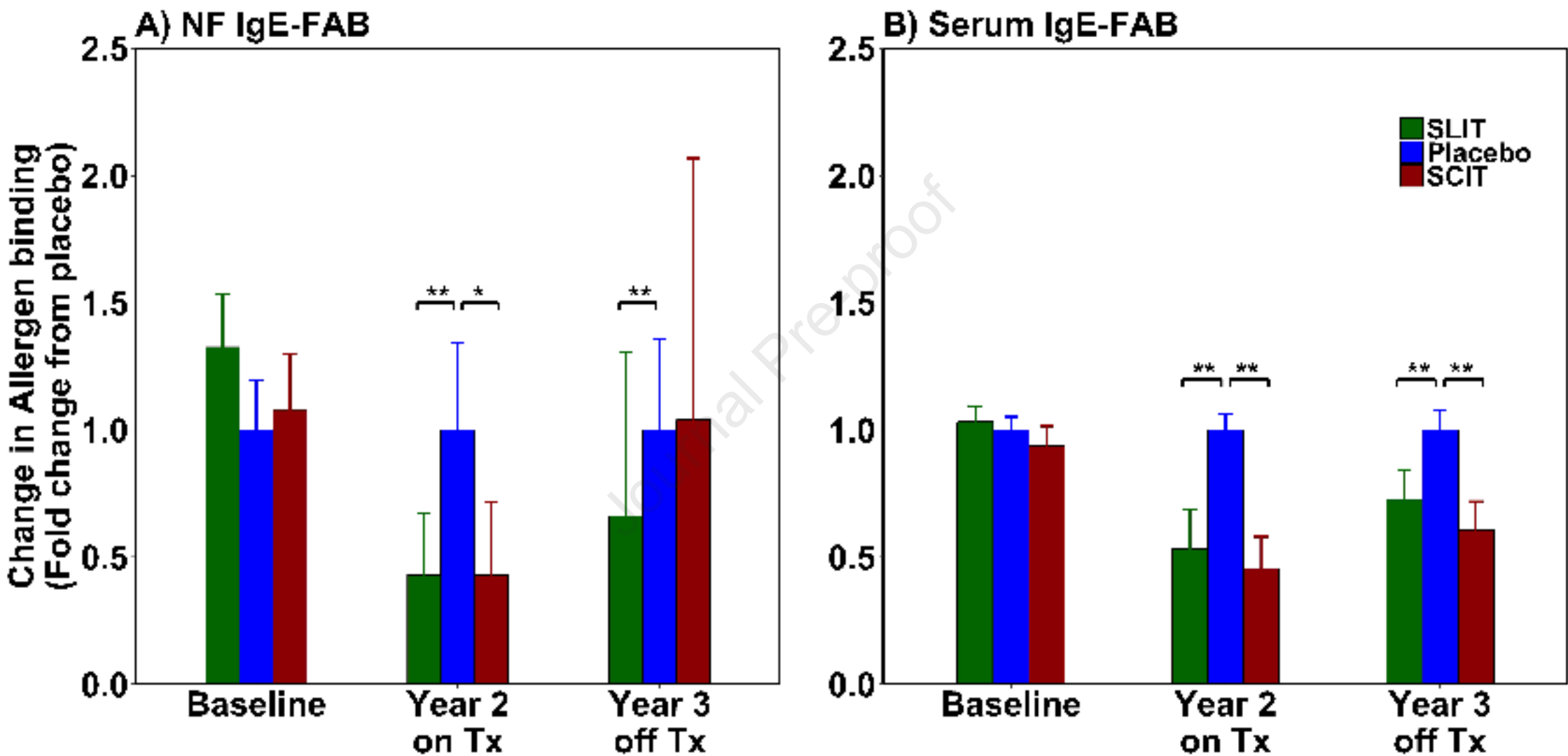


Figure 5

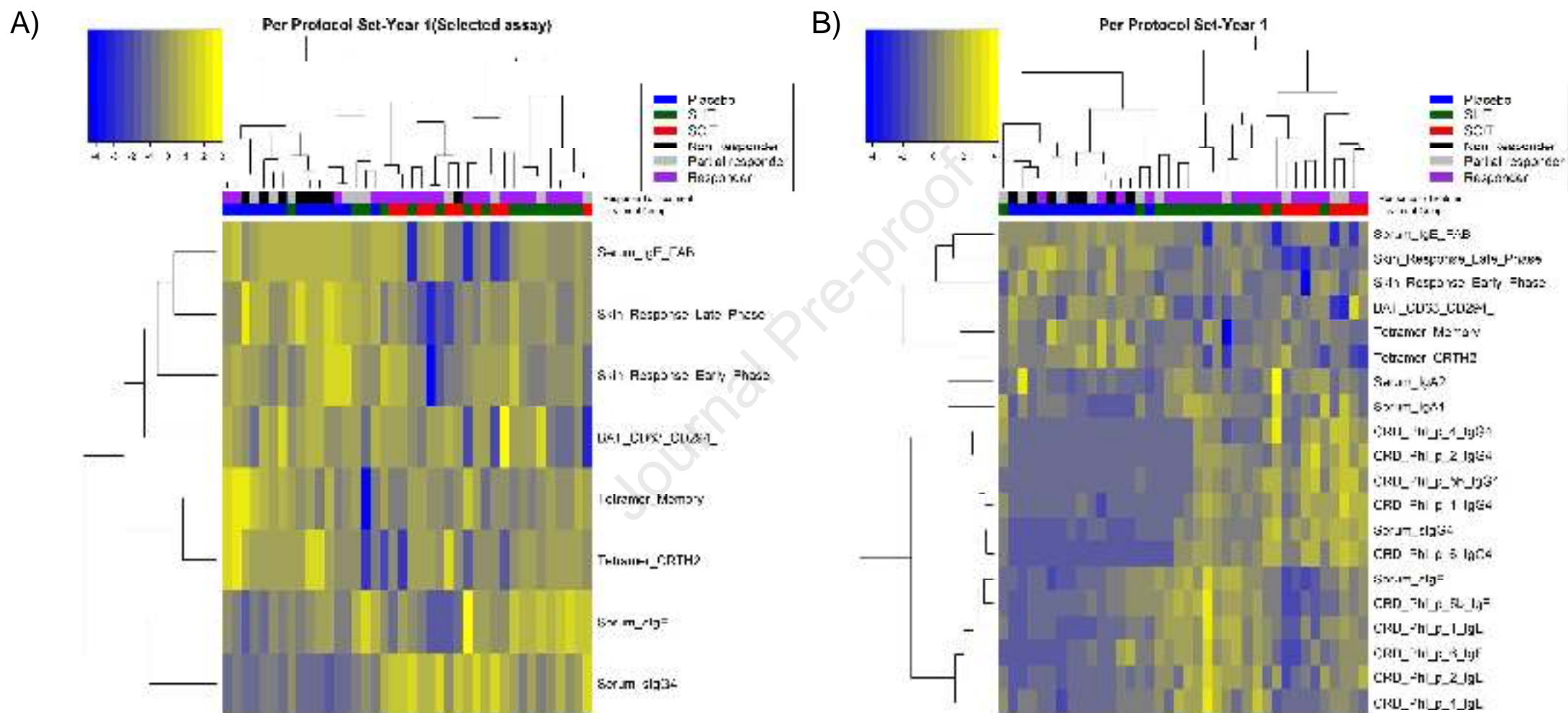
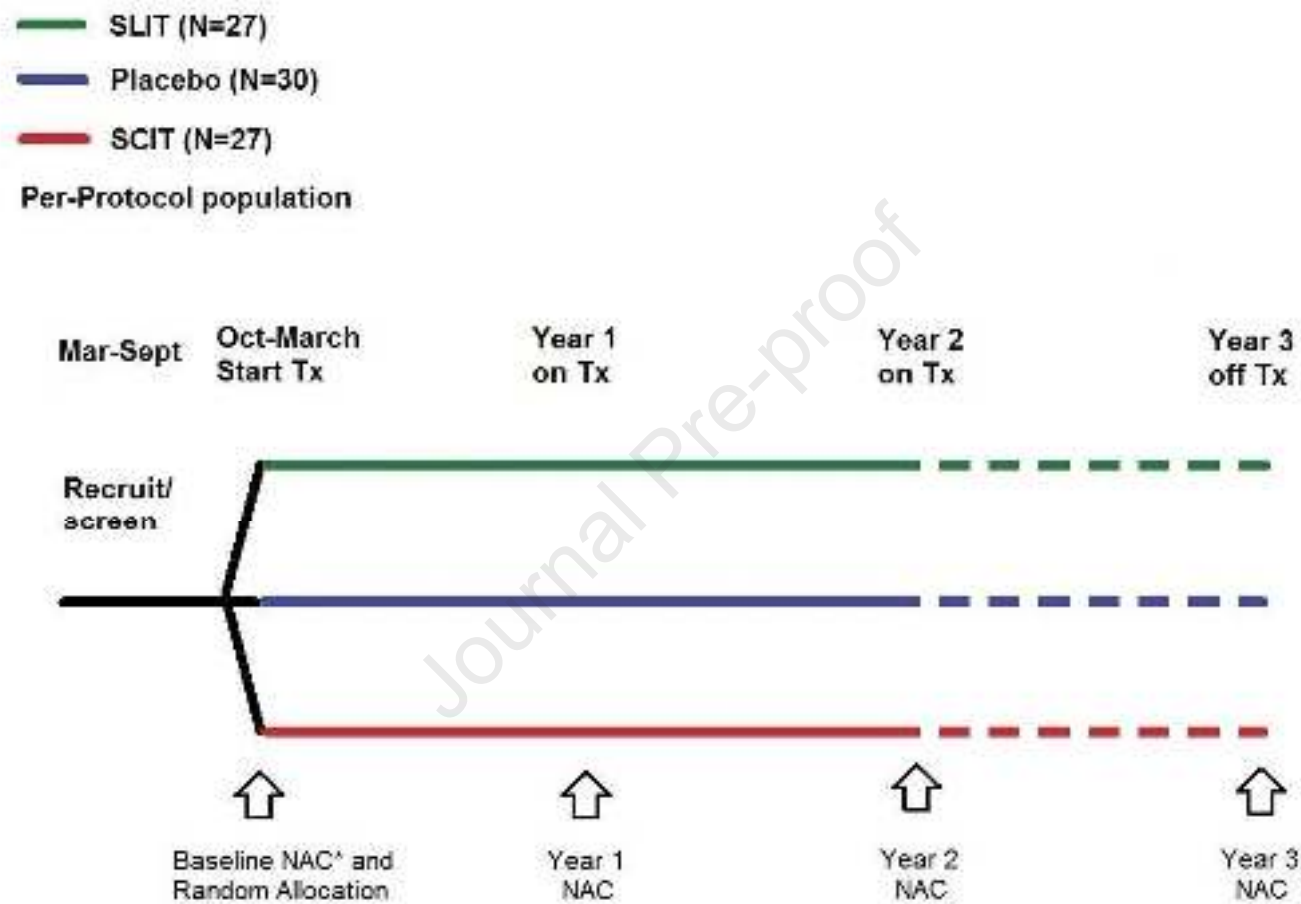


Figure 6



Sera and nasal fluids for antibody measurements were obtained at NAC visits, which occurred between October and March each year.

*: Baseline NAC was performed prior to treatment.

Figure E1

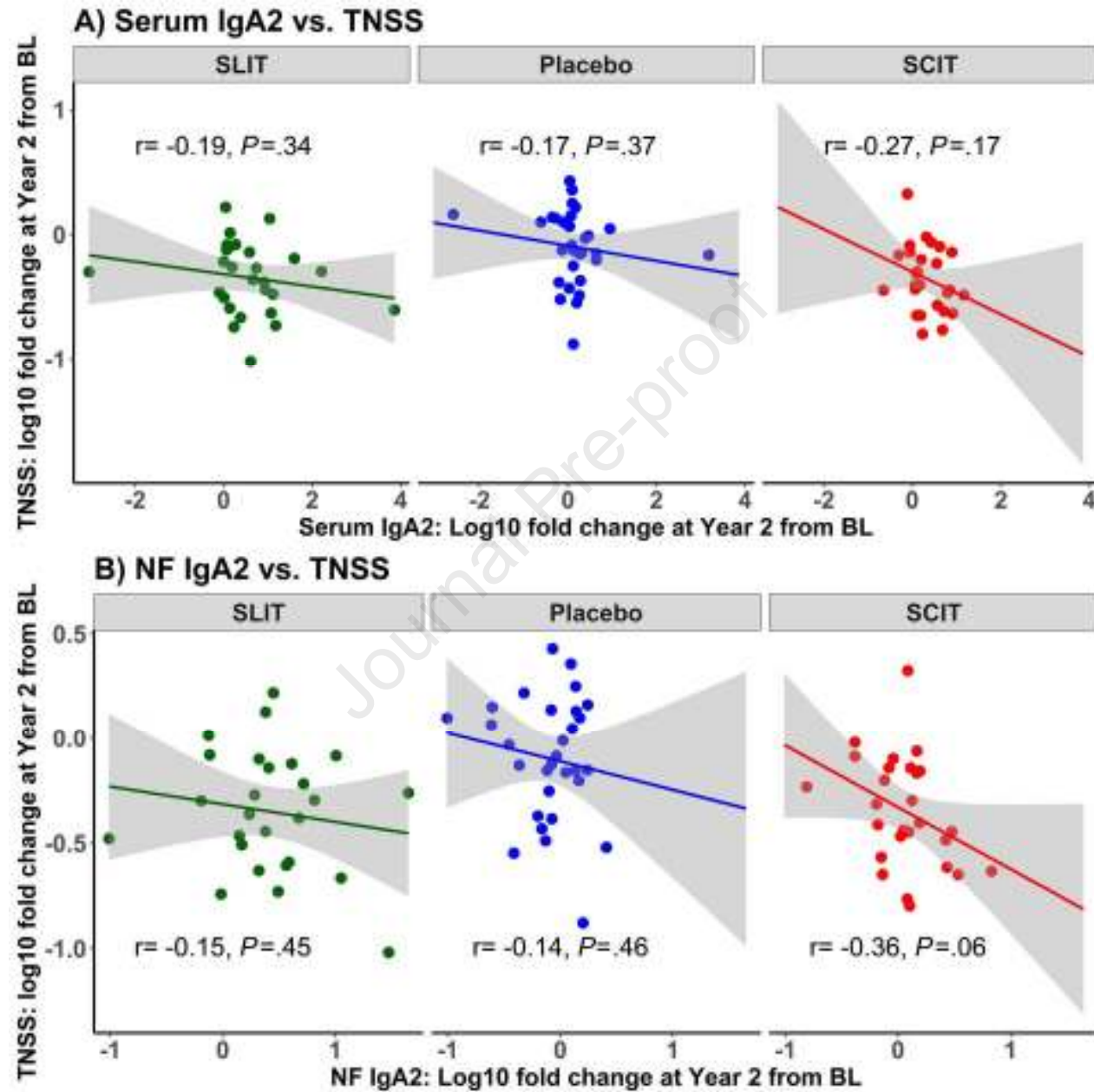


Figure E 2

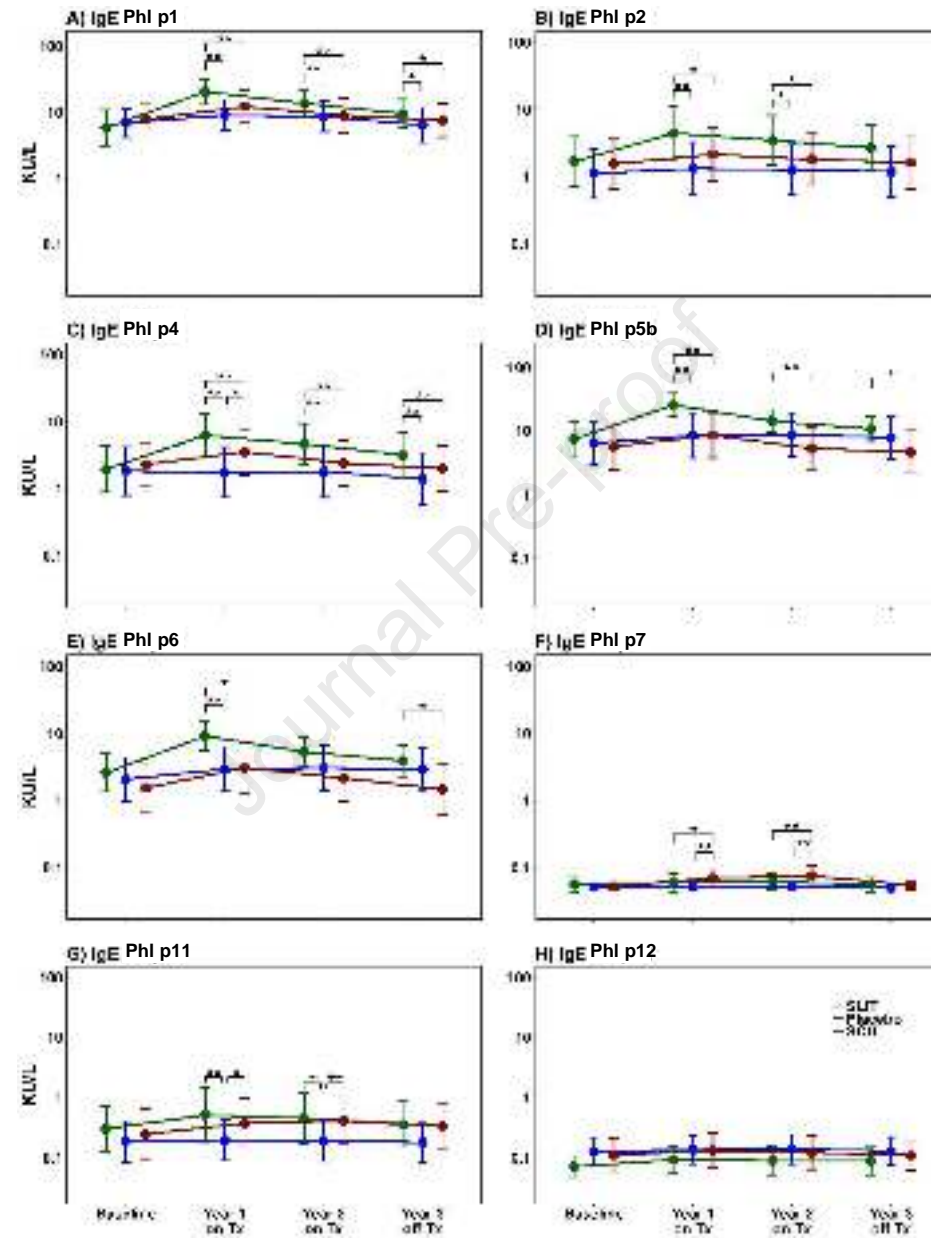


Figure E3

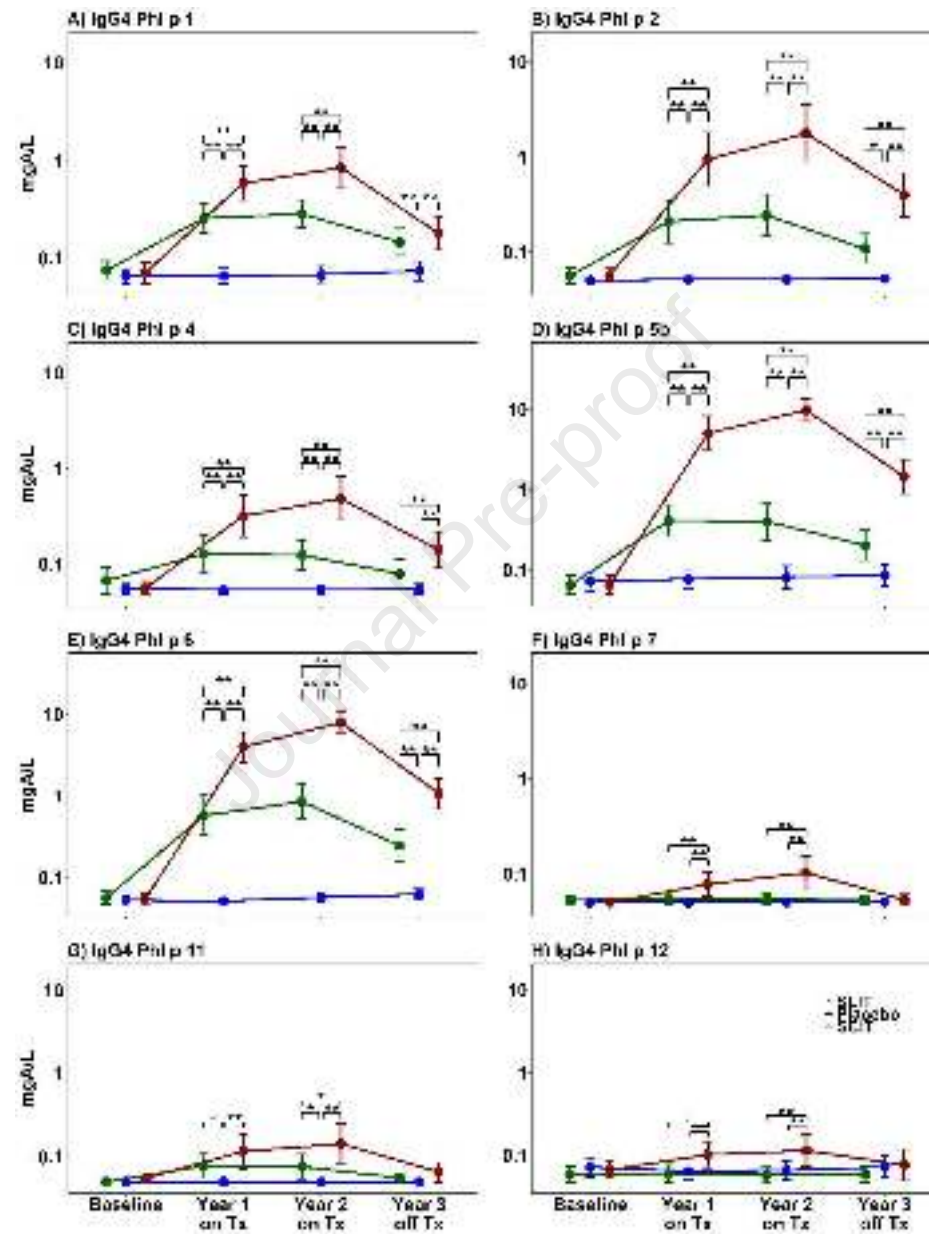


Figure E4

1 ONLINE REPOSITORY TEXT

2 TITLE: Differential Induction of Allergen-specific IgA Responses following
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27 **Methods**

28 Measurement of IgA₁/IgA₂ in Nasal Fluid and Serum using ELISA

29 Plates were coated with 50 µL of Phl p (5 µg/mL) for 1 hour at 37°C. Wells were
30 washed three times using 0.05% Tween 20 in PBS, dried and blocked using 1%
31 Bovine Serum Albumin (BSA) in PBS at 37°C for 1 hour. Plates were washed with
32 wash buffer and dried completely. Standards and serum samples were added into
33 corresponding wells and incubated overnight at 4°C. Following overnight incubation
34 at 4°C, the plates were washed and incubated with monoclonal antibodies (mAbs) to
35 IgA₁ or IgA₂ (Abcam) for 30 minutes. Plates were washed, dried and 100 µL of HRP-
36 conjugated Streptavidin (Biolegend) was added to each well and incubated on a
37 shaker for 30 minutes. After washing, TMB substrate was added in the dark for 8
38 minutes and 50 µL stopping solution (H₂SO₄) was added. Plates were read at OD₄₅₀
39 using an ELISA microplate reader (Molecular Probes, Eugene, OR, USA). The anti-
40 IgA₁ antibody binds to the Fc portion of the heavy chain of human IgA₁. Meanwhile,
41 anti-IgA₂ antibody binds to the Fc portion of the heavy chain of human IgA₂ and can
42 detect both monomeric and dimeric IgA₂.

43

44 Measurement of nasal IgG, nasal IgG₄ and serum IgG by ELISA

45 Plates were coated with 50 µL Phl p (5 µg/mL) diluted in bicarbonate coating buffer
46 at 37°C for 1 hour. Wells were washed three times with 0.05% Tween 20 in PBS,
47 dried and blocked using 1% Bovine Serum Albumin (BSA) (Sigma, Gillingham,
48 United Kingdom) in PBS at 37°C for 1 hour. Plates were washed, dried, and SCIT
49 serum added in serial dilutions (1:1) with PBS to standard wells. Samples were
50 added 1:1000 (for IgG) or 1:3.3 (for IgG₄) dilution with PBS to corresponding wells.
51 The concentration of the standard serum was determined using ImmunoCAP as per

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102 However, these differences did not persist off treatment at year 3 (Fig E2, F-H).
103 Consistent with the differential treatment effect observed for IgG4 to GP extract in
104 serum, the SCIT group exhibited superior IgG4 responses compared to SLIT group
105 to all grass pollen components in serum at years 1 and 2 ($P < .01$ for Phl p 1, 2, 4,
106 5b, 6, 7 and $P < .05$ for Phl p 11 (year 2 only) and 12), and these differences
107 persisted at year 3 ($P < .01$) with the exception of IgG4 to Phl p 1, 7, 11, and 12. In
108 contrast, the SLIT group displayed increased levels of IgE to Phl p 1, 2, and 4 at
109 years 1 and 2 compared to SCIT and placebo groups (Fig E2) that paralleled the
110 differences in serum IgE to grass pollen extract between treatment groups (Fig 4, F).

111

112

113 References:

114

- 115 1. Shamji MH, Wilcock LK, Wachholz PA, Dearman RJ, Kimber I, Wurtzen PA, et al. The
116 IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric
117 based method for the detection of inhibitory antibody responses. *J Immunol*
118 *Methods* 2006; 317:71-9.

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147 measured by ImmunoCAP. Data are presented as mean \pm 95%CI. *P < .05, **P <

148 .01, A linear mixed model was used with adjustment for baseline value.

149 https://www.itntrialshare.org/GRASS_antibody_figS4.url

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