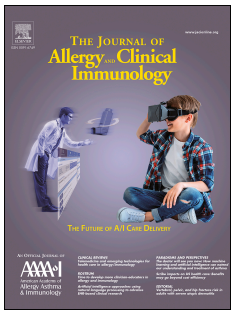
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Differential Induction of Allergen-specific IgA Responses following Timothy Grass Subcutaneous and Sublingual Immunotherapy

Mohamed H. Shamji, PhD, FAAAAI, David Larson, PhD, Aarif Eifan, MD, Guy W. Scadding, MD, PhD, Tielin Qin, PhD, Kaitie Lawson, MS, Michelle L. Sever, MSPH, PhD, Ellen Macfarlane, BSc, Janice A. Layhadi, MSc, PhD, Peter A. Würtzen, PhD, Rebecca V. Parkin, BSc, Srinath Sanda, MD, Kristina M. Harris, PhD, Gerald T. Nepom, MD, PhD, Alkis Togias, MD, Stephen R. Durham, MD, FRCP

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TITLE PAGE

Original Article

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Mohamed H. Shamji, PhD, FAAAAI[a], David Larson, PhD[b], Aarif Eifan, MD[a], Guy W. Scadding, MD, PhD[a], Tielin Qin, PhD[b], Kaitie Lawson, MS[c], Michelle L. Sever, MSPH, PhD[c], Ellen Macfarlane, BSc[a], Janice A. Layhadi, MSc, PhD[a], Peter A. Würtzen[d], PhD, Rebecca V. Parkin, BSc[a], Srinath Sanda, MD[e], Kristina M. Harris, PhD[b], Gerald T. Nepom MD, PhD[b, f], Alkis Togias MD[g], Stephen R. Durham, MD, FRCP[a, b]

[a]National Heart and Lung Institute, Allergy and Clinical Immunology, Imperial College NIHR Biomedical Research Centre, Asthma UK Centre in Allergic Mechanisms of Asthma, London, United Kingdom; [b]Immune Tolerance Network, Bethesda, MD, USA; [c]Rho Federal Systems Division, Durham, NC, USA; [d]Translational research, ALK, Hørsholm; [e]Immune Tolerance Network, San Francisco, CA, USA [f]Benaroya Research Institute at Virginia Mason, Seattle, WA, USA; [g]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA.

Corresponding author:
Dr Mohamed H. Shamji
National Heart and lung Institute,
Sir Alexander Fleming Building, Imperial College London,
Imperial College London, SW7 2AZ
London, SW7 2AZ, United Kingdom.

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28 Tel: +44 20 7594 3476, E-mail: m.shamji@imperial.ac.uk.

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Abbreviations:

AIT: Allergen Immunotherapy

TGP: Timothy Grass pollen

Ig: Immunoglobulins

IgE-FAB: IgE facilitated allergen binding

ISAC: Immuno Solid Allergen Chip

NAC: Nasal Allergen Challenge

NF: Nasal Fluid

Phl p: Phleum pratense

TNSS: Total nasal symptom score

SCIT: Subcutaneous Immunotherapy

sIgE: Specific IgE

sIgG4: Specific IgG4

sIgA1: Specific IgA1

sIgA2: Specific IgA2

SLIT: Sublingual Immunotherapy

Th1: Type 1 helper T cells

Th2: Type 2 helper T cells

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Introduction

Allergen immunotherapy (AIT) is currently the only disease-modifying treatment for allergic rhinitis, an IgE-mediated disease affecting up to 20-30% of adults and up to 40% of children.[1] Two routes of administration of AIT used clinically are subcutaneous (SCIT) and sublingual (SLIT). Whilst SCIT involves weekly up-dosing injections which are then followed by monthly maintenance injections for at least three years[2], SLIT is self-administered and involves daily drops or tablets placed under the tongue for the same duration of three years.[3] SCIT[4,5] and SLIT[6,7] are both disease-modifying and effective treatments in reducing clinical symptoms during the Timothy grass pollen (TGP) season and the overall need of rescue medications.

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

AIT confers its clinical effect through several mechanisms including immune-deviation of type 2 T helper (Th2) cell response towards a type 1 T helper (Th1) cell response, suppression or deletion of Th2 cells[10-14], induction of regulatory T and B cells[2, 15, 16] and induction of IgG blocking antibodies, in particular IgG4.[15, 17, 18] However, a detailed examination of the effect of SCIT and SLIT on a wider spectrum of humoral responses, such as nasal and systemic IgA_1, IgA_2, IgG and IgG_4 to AIT

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

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

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

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

TGP-specific IgA_1, IgA_2, IgG, IgG_4 and IgE were measured in nasal fluid collected at out-of-season nasal allergen challenge (NAC) visits (prior to the challenge) by an in-house optimized and validated Enzyme Linked Immunosorbent Assay (ELISA) (Methods section in the Online Repository). Specific IgE and IgG_4 to TGP and TGP components (Phl p 1, 2, 4, 5b, 6, 7, 11, 12) as well as IgA_1 and IgA_2 to TGP extract were measured in pre-challenge out-of-season sera by the ImmunoCAP system (Phadia US Inc., MI, USA) according to manufacturer's instructions and ELISA, respectively. Inhibition of IgE-facilitated allergen binding to B cells was measured as previously described.[15, 17] Further details can be found in the Methods section of the Online Repository.

Statistical Analysis

All immunologic data were assessed in the per-protocol (PP) population using a linear mixed model adjusted for baseline values. The PP population included participants who remained in the study at least three years and were compliant with study medications, (defined as taking 50% or more of their study medication for the duration of the study), and who had an evaluable primary endpoint. Missing baseline serum (8 out of 84) IgE and IgG_4 antibodies to TGP were imputed based on corresponding antibody levels to TGP components. Serum antibodies to TGP and TGP components (Phl p1, Phl p2, Phl p4, Phl p5b, Phl p6, Phl p7, Phl p11, Phl p12)

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

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Within the three treatment groups, only SLIT participants had increased levels of TGP-specific IgA_1 and IgA_2 in nasal fluid at years 2 (3.35- and 2.72-fold over baseline, respectively, $P < .001$) and 3 (3.37- and 2.77-fold over baseline, respectively; $P < .001$). As a result, levels of TGP-specific IgA_1 and IgA_2 were significantly higher in nasal fluids from the SLIT group compared to the placebo or SCIT groups at years 2 (all, $P < .01$) and 3 (all, $P < .01$) (Fig 2, A-B, Table E1 (https://www.itntrialshare.org/GRASS_antibody_fig2.url). Year 1 specimens were not assayed due to low sample volumes.

Serum TGP-specific IgA_1 levels were increased in SLIT-treated group at years 1, 2, and 3 compared to baseline (4.96-, 7.02-, and 2.89-fold respectively, all $P < .001$) (Fig 2, C, Table E1). However, serum IgA_1 levels declined in the SLIT-treated group at year 3 compared to year 2 ($P < .001$). Serum TGP-specific IgA_1 levels were not increased in the SCIT-treated group at years 1, 2 or 3, compared to baseline. Similarly, no changes in serum IgA_1 levels were observed in the placebo group. The SLIT group serum TGP-specific IgA_1 levels were higher than the placebo and SCIT-treated groups at year 1 (all, $P < .001$), year 2 (all, $P < .001$) and year 3 (all, $P < .001$) (Fig 2, C). Serum TGP- specific IgA_2 levels were increased in the SCIT and SLIT-treated groups at year 1 (all, $P < .01$) and year 2 (all, $P < .05$) compared to baseline. No significant change in serum IgA_2 levels from baseline were observed in placebo-treated participants. The SLIT group was not statistically different from placebo or from SCIT at any of the 3 study years. SCIT resulted in significantly higher serum IgA_2 compared to placebo, but only in year 1 (Fig 2, D).

We examined whether the clinical effect of AIT on nasal allergen-induced symptoms was associated with changes in nasal fluid or serum IgA. Figure 3 presents the relationships between AIT-induced log10-fold change in nasal TGP-specific IgA_1 and log10-fold changes in nasal allergen challenge-induced TNSS from baseline to year 2. We found a significant, albeit modest, correlation between nasal fluid IgA_1 and TNSS after SLIT, but not SCIT or placebo, (Pearson r = -0.52, P = .006). No significant relationships between changes in TNSS and serum IgA_1 (Fig 3, A-B, https://www.itntrialshare.org/GRASS_antibody_fig3.url) or serum IgA2 or nasal fluid IgA_2 were observed (Fig E2). We further confirmed this observation by applying a linear mixed-effect model, with TNSS AUC as the dependent variable. When only one biomarker was included at a time within the model along with visit, treatment, and visit treatment interaction, nasal IgA1 and IgA2 was found significant, indicating the level of nasal IgA1 and IgA2 had an effect on TNSS AUC in the expected direction, confirming our earlier observations (Table E2).

Nasal fluid and serum IgG, IgG_4 and IgE to grass pollen following SLIT, SCIT or placebo

TGP-specific IgG in nasal fluid and serum was elicited by both SCIT and SLIT. The levels in nasal fluid were increased from baseline at year 2 in both SCIT and SLIT group (P < .05) and at year 3 in the SLIT group (P < .05) (Fig 4, A). Similarly, serum TGP-specific IgG levels were increased from baseline at years 2 (P < .001) and 3 (P < .001) in both SCIT and SLIT groups (Fig 4, B). Levels of GP-specific IgG_4 in nasal fluids increased from baseline for the SLIT and SCIT groups at years 2 and 3 (P < .001), (Fig 4, C, Table E3). Similarly, serum IgG_4 to TGP increased from baseline in both SLIT and SCIT groups (Fig 4, D, Table E3). A decline in IgG (87%, SCIT, P <

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

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

TGP-specific nasal fluid IgE was higher in SLIT compared to SCIT (2.76-fold, P = .04) and placebo (3.07-fold, P = .02) at year 2, but not year 3 (Fig 4, E, Table E3). This differential treatment effect on IgE levels in nasal fluids was paralleled in serum (Fig 4, F, Table E3 https://www.itntrialshare.org/GRASS_antibody_fig4.url). Moreover, longitudinal changes in serum IgG4 and IgE to Phl p 1, 2, 4, 5b, 6, 7, 11 and 12 following SLIT, SCIT or placebo were observed (Fig E3 and E4, Table E4 and E5). Further details on this observation can be found in the results section of the Online Repository.

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

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

placebo, but not in SCIT (P = .21). Serum from both SCIT- and SLIT-treated groups blocked allergen-IgE complexes from binding to B cells at years 2 and 3, when compared to placebo, as previously reported[11] (Fig 5, B, Table E6 https://www.itntrialshare.org/GRASS_antibody_fig5.url).

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

We next explored whether relationships between clinical outcomes and biomarkers were present. We found that biomarkers after 1 year of AIT treatment were related to the 2-year treatment outcomes. We performed hierarchical clustering using the log10-fold changes in variables from baseline to year 1 and an agglomerative approach (bottom-up). We first used a set of already published biomarkers from the same study that included early and late phase skin reactions[9], BAT[12], serum IgE-FAB data, tetramer data[12] and serum IgE and IgG4 data. Hierarchical clustering of those biomarkers allowed for relatively good distinction between placebo and active treatment, but less distinction between SCIT and SLIT (Fig 6, A). When serum IgA data and serum component IgE and IgG4 data were added to the analysis, the ability to cluster study participants by treatment and response to treatment was greatly improved (Fig 6, B). The Figure 6B dendrogram shows that study participants could be separated into 3 clusters. One cluster contained all 7 participants who had <10% TNSS reduction from baseline to year 2. In general, these participants produced less serum IgA to grass extract, and less IgG4 and IgE to grass components from baseline to year 1. 14/18 of the members of this cluster belonged to the placebo arm. In contrast, the other two clusters contained the majority of participants who had >40% TNSS reduction from baseline to year 2. These two clusters had increased

359 serum IgA to grass extract, and more IgG_4 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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Discussion

Here we report a detailed longitudinal assessment of nasal and systemic antibody responses during sublingual and subcutaneous grass pollen immunotherapy. IgA_1 and IgA_2 antibody induction was higher in SLIT, compared to SCIT and placebo. This effect was most prominent in nasal fluids. In contrast, IgG and IgG_4 antibodies both in nasal fluids and in the circulation were greater in SCIT, compared to SLIT and to placebo. Moreover, adding serum IgA, IgG_4, and IgE measurements, taken one year after initiation of AIT, to an unbiased biomarker cluster analysis enabled participants to be relatively well stratified based on the treatment they received and also identified subjects who did not respond to treatment.

AIT treatment has been previously associated with the induction of a serum allergen-specific IgA_2 antibody response.[20] Increases in salivary concentrations of grass pollen-specific total IgA have also been observed in children following 3 years grass pollen SLIT that persisted for two years after treatment cessation [21]. Our study extends these observations by directly comparing local nasal and systemic IgA_1 and IgA_2 concentrations following grass pollen SCIT and SLIT. Elevated IgA in SLIT was observed during immunotherapy treatment and persisted even after discontinuation of treatment. Changes in allergen-induced symptoms and changes in the level of nasal fluid IgA_1 showed a significant inverse correlation in the SLIT arm, but not in SCIT or placebo, suggesting that IgA induced by SLIT may be related to clinical improvements observed during therapy. However, this correlation was relatively modest and, in year 3, after discontinuation of treatment, nasal fluid IgA_1 and IgA_2 remained elevated, whereas allergen-induced nasal symptoms did not differ between SLIT and placebo.

IgA produced during grass pollen immunotherapy may participate in blocking allergen from binding IgE receptors. The blocking capacity of IgA antibody has been previously reported in a study where ragweed-specific IgA from nasal secretions was shown to inhibit basophil histamine release.[22] Here we show that, compared to baseline, nasal fluids collected after 2 years of treatment had increased blocking activity against IgE-allergen complex binding to B cells. It is notable that the magnitude of this effect was equal between SLIT and SCIT. However, SLIT, compared to SCIT, had lower levels of nasal fluid IgG_4, the conventional "blocking antibody" subclass, and higher levels of IgA_1 and IgA_2. This is compatible with a hypothesis that IgA may also play the role of a "blocking antibody" in nasal fluids. This notion is further supported by the experiments with serum where the differences between SLIT and SCIT-induced IgA_1 vs IgG_4 were even more striking, but the blocking activity of serum against IgE-allergen complex binding to B cells was no different. Our data underscore that SCIT and SLIT likely involve differences in the mechanism of action with IgG_4 playing a predominant role in SCIT and IgA in SLIT.

The addition of serum IgA, as well as serum grass component IgE and IgG_4, to various other parameters, as part of an exploratory cluster analysis of the changes from baseline to year 1, yielded 3 clusters that largely separated participants according to the treatment they received (placebo, SLIT, SCIT). This suggests that differences in the production of IgA, IgE, and IgG_4 antibodies are defining characteristics of SCIT and SLIT treatment. Including the baseline to year 1 changes in these datasets (https://www.itntrialshare.org/GRASS_antibody.url) also allowed for the identification of participants who responded to therapy at year 2 and

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

Local antibody responses are reflective of immunological and clinical responses to individual allergen components. However, due to limitation in the amount of nasal fluid that was obtained in this study, only IgE, IgG, IgG_4, IgA_1 and IgA_2 measurements to the allergen extract, and not to individual grass pollen components, were assessed in this study. Whilst it is possible to utilize an assay such as ImmunoCAP solid-phase allergen chip technology (ISAC) to measure reactivity against individual allergen components in nasal fluids, studies have shown that allergen-specific IgG_4 may compete with specific IgE for allergen-binding within the assay. This may therefore underestimate the levels of sIgE obtained by ISAC.[23] Further to this, the baseline nasal fluid and serum samples measured in this study were collected at the end of the grass pollen season. For this reason, it would be challenging to derive any observations on the blunting of seasonal IgE increases in the local target organ or within the circulation.

During the GRASS trial, peak pollen count was found within study year 2. Despite this peak, each treatment group was exposed to similar levels of pollen as study 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 IgA_1, IgA_2,
450 IgG_4 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 study[24] 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 IgG_4 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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https://www.itntrialshare.org/GRASS_antibody_fig3.url

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

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

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

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

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

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

biomarker assays or (B) with the addition of serum IgA data and serum component IgE and IgG_4 data. Yellow color indicates large log10 increases from baseline to year 1 and blue color indicates large log10 decreases from baseline to year 1. Inclusion of IgE/IgG_4 component data and serum IgA data leads to improved clustering by treatment group (SLIT = green, n = 15; placebo = blue, n = 14; SCIT = red, n=9) and response to treatment (Non Responder: < 10% reduction in TNSS from baseline to year 2 = black, Partial Responder: 10 – 40 % reduction in TNSS from baseline to year 2 = white, Responder: > 40% reduction in TNSS from baseline to year 2 = purple).

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

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Mohamed H. Shamji, PhD, FAAAAI[a], David Larson, PhD[b], Aarif Eifan, MD[a], Guy W. Scadding, MD, PhD[a], Tielin Qin, PhD[b], Kaitie Lawson, MS[c], Michelle L. Sever, MSPH, PhD[c], Ellen Macfarlane, BSc[a], Janice A. Layhadi, MSc, PhD[a], Peter Würtzen[d], PhD, Rebecca V. Parkin, BSc[a], Srinath Sanda, MD[e], Kristina M. Harris, PhD[b], Gerald T. Nepom MD, PhD[b,f], Alkis Togias MD[g], Stephen R. Durham, MD, FRCP[a,b]

[a]National Heart and Lung Institute, Allergy and Clinical Immunology, Imperial College NIHR Biomedical Research Centre, Asthma UK Centre in Allergic Mechanisms of Asthma, London, United Kingdom; [b]Immune Tolerance Network, Bethesda, MD, USA; [c]Rho Federal Systems Division, Durham, NC, USA; [d]Immunology ALK-Abello, Hørsholm; [e]Immune Tolerance Network, San Francisco, CA, USA [f]Benaroya Research Institute at Virginia Mason, Seattle, WA, USA; [g]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA.

Corresponding author:

Dr Mohamed H. Shamji

National Heart and lung Institute,

Sir Alexander Fleming Building, Imperial College London,

Imperial College London, SW7 2AZ

London, SW7 2AZ, United Kingdom.

Tel: +44 20 7594 3476, E-mail: m.shamji@imperial.ac.uk

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Measurements (unit)

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Table E1.Levels of specific IgA_1 and IgA_2 to grass pollen in nasal fluid and serum

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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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Phl p5b
(kU/L)

Journal Pre-proof

Journal Pre-proof

Year 3 30

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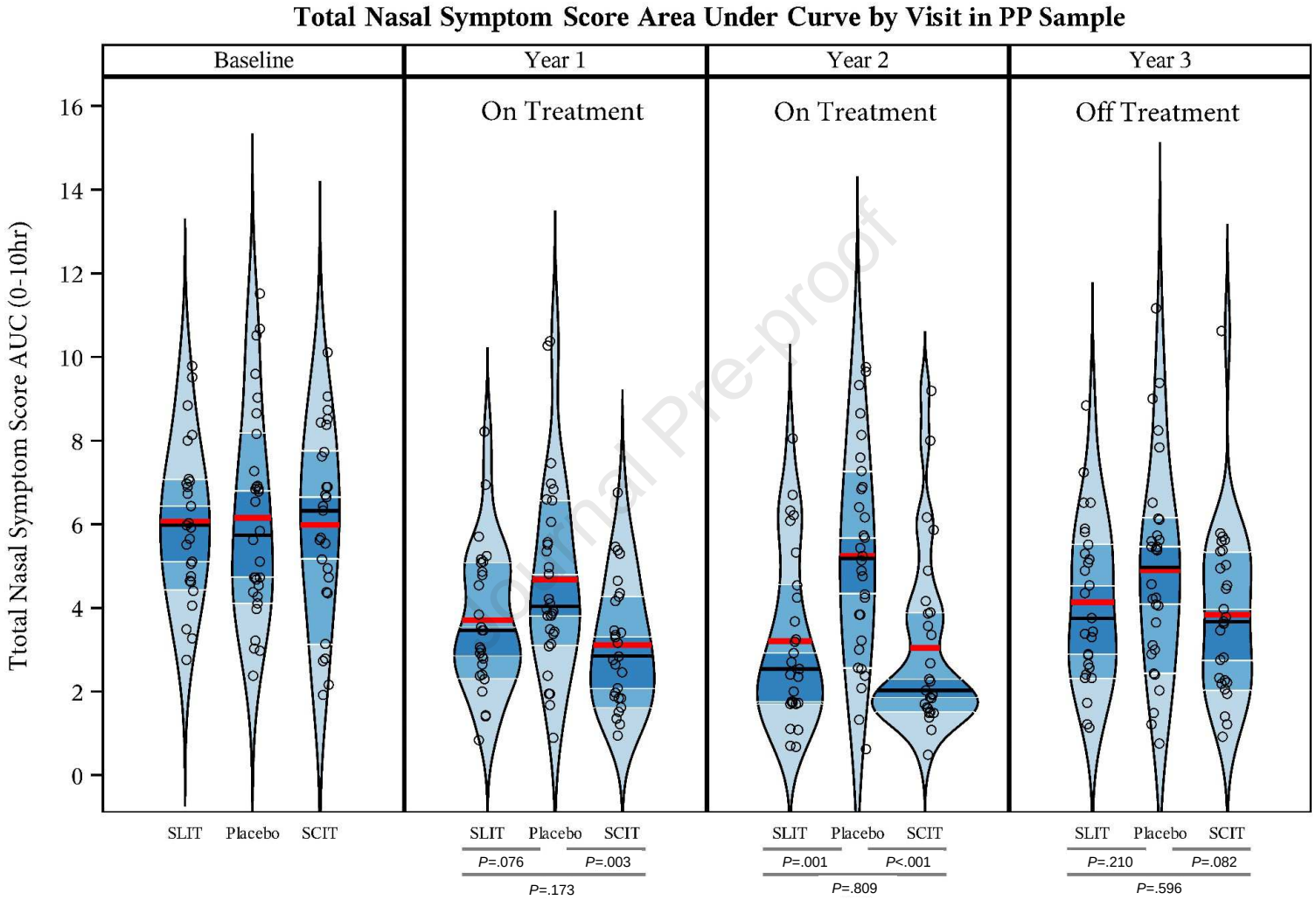
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Figure 4

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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

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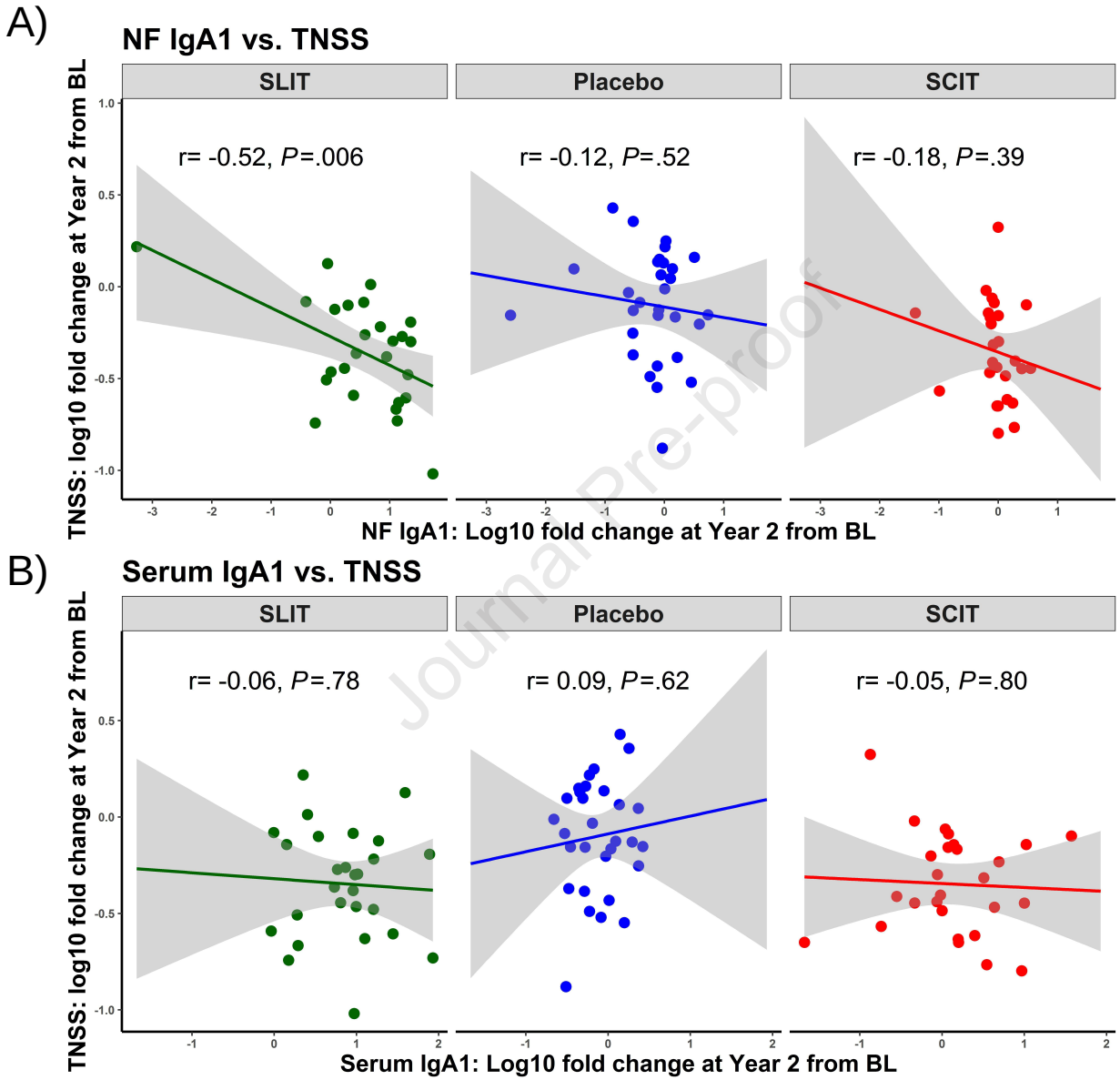


Figure 3

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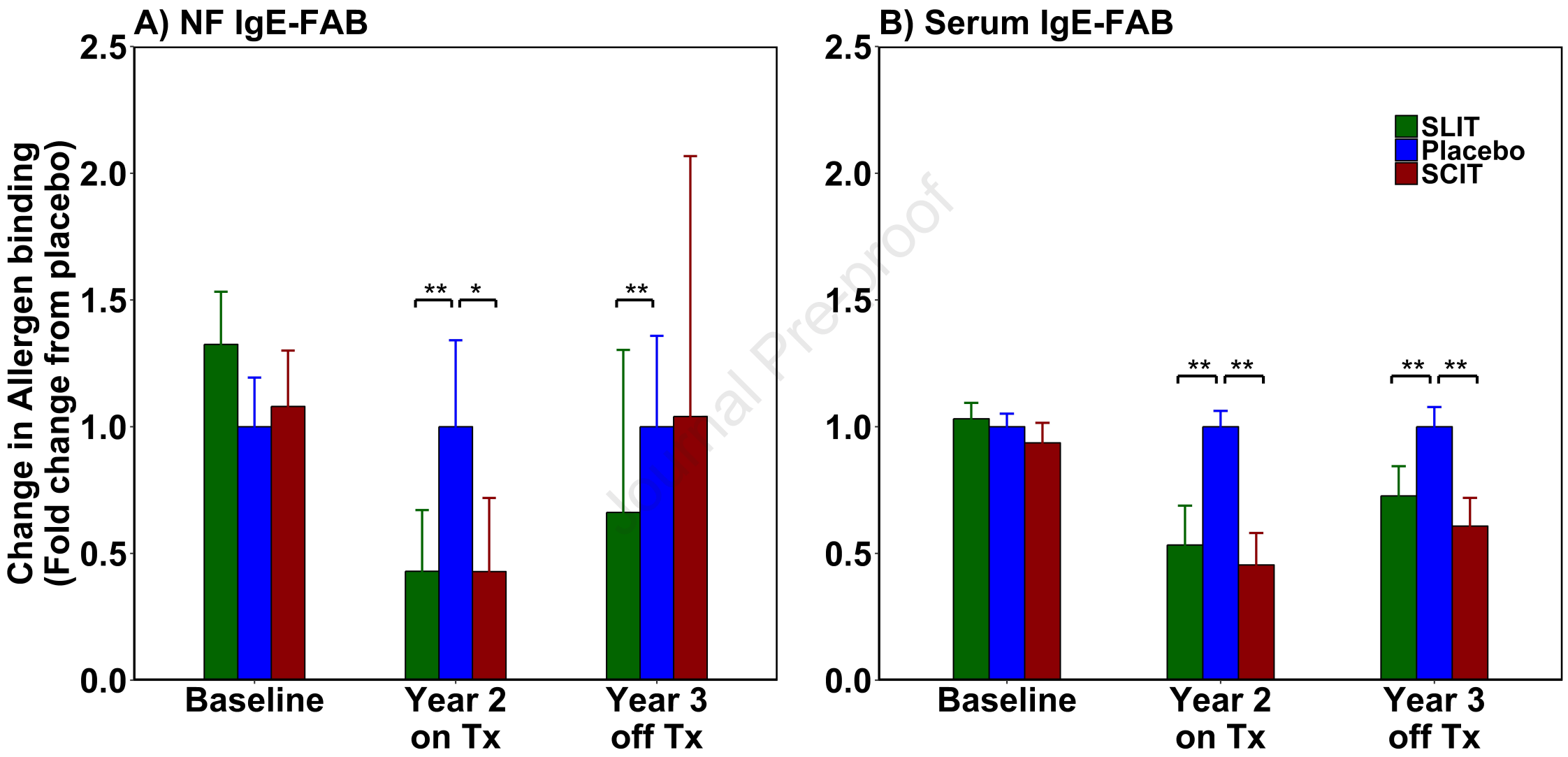


Figure 5

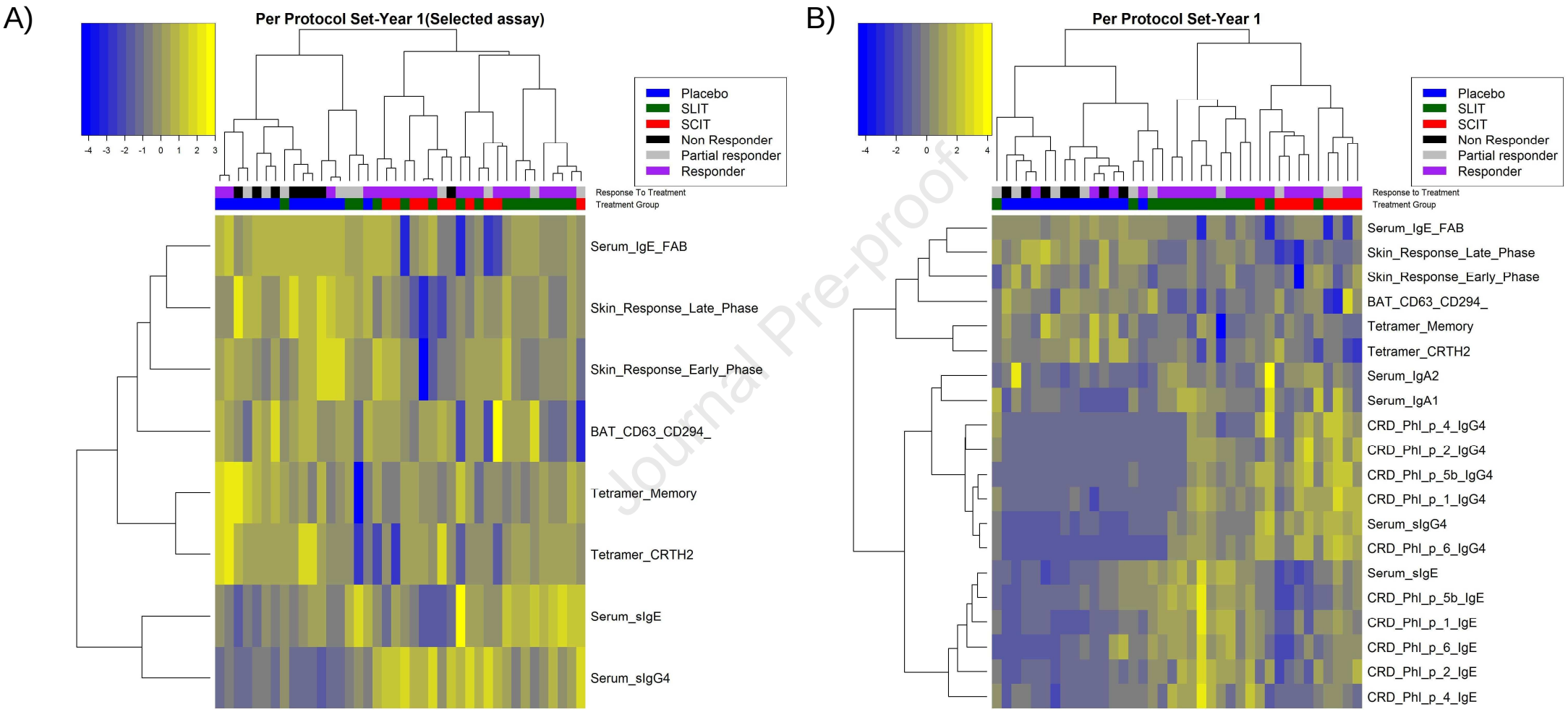
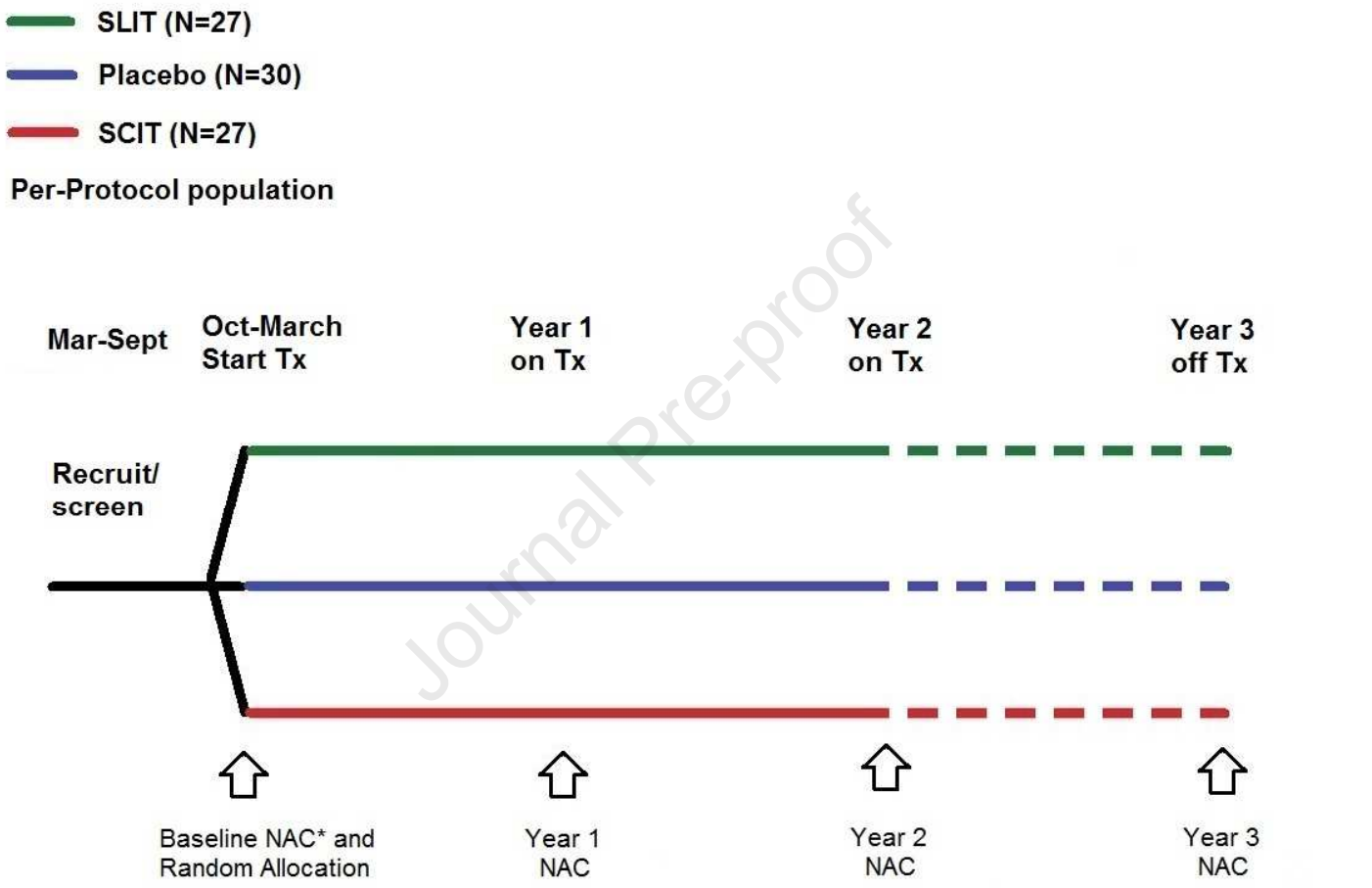

A)
Per Protocol Set-Year 1(Selected assay)
-4 -3 -2 -1 0 1 2 3
Placebo
SLIT
SCIT
Non Responder
Partial responder
Responder
Response To Treatment
Treatment Group
Serum_IgE_FAB
Skin_Response_Late_Phase
Skin_Response_Early_Phase
BAT_CD63_CD294_
Tetramer_Memory
Tetramer_CRTH2
Serum_sIgE
Serum_sIgG4
B)
Per Protocol Set-Year 1
-4 -2 0 2 4
Placebo
SLIT
SCIT
Non Responder
Partial responder
Responder
Response to Treatment
Treatment Group
Serum_IgE_FAB
Skin_Response_Late_Phase
Skin_Response_Early_Phase
BAT_CD63_CD294_
Tetramer_Memory
Tetramer_CRTH2
Serum_IgA2
Serum_IgA1
CRD_Phl_p_4_IgG4
CRD_Phl_p_2_IgG4
CRD_Phl_p_5b_IgG4
CRD_Phl_p_1_IgG4
Serum_sIgG4
CRD_Phl_p_6_IgG4
Serum_sIgE
CRD_Phl_p_5b_IgE
CRD_Phl_p_1_IgE
CRD_Phl_p_6_IgE
CRD_Phl_p_2_IgE
CRD_Phl_p_4_IgE

Figure 6

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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

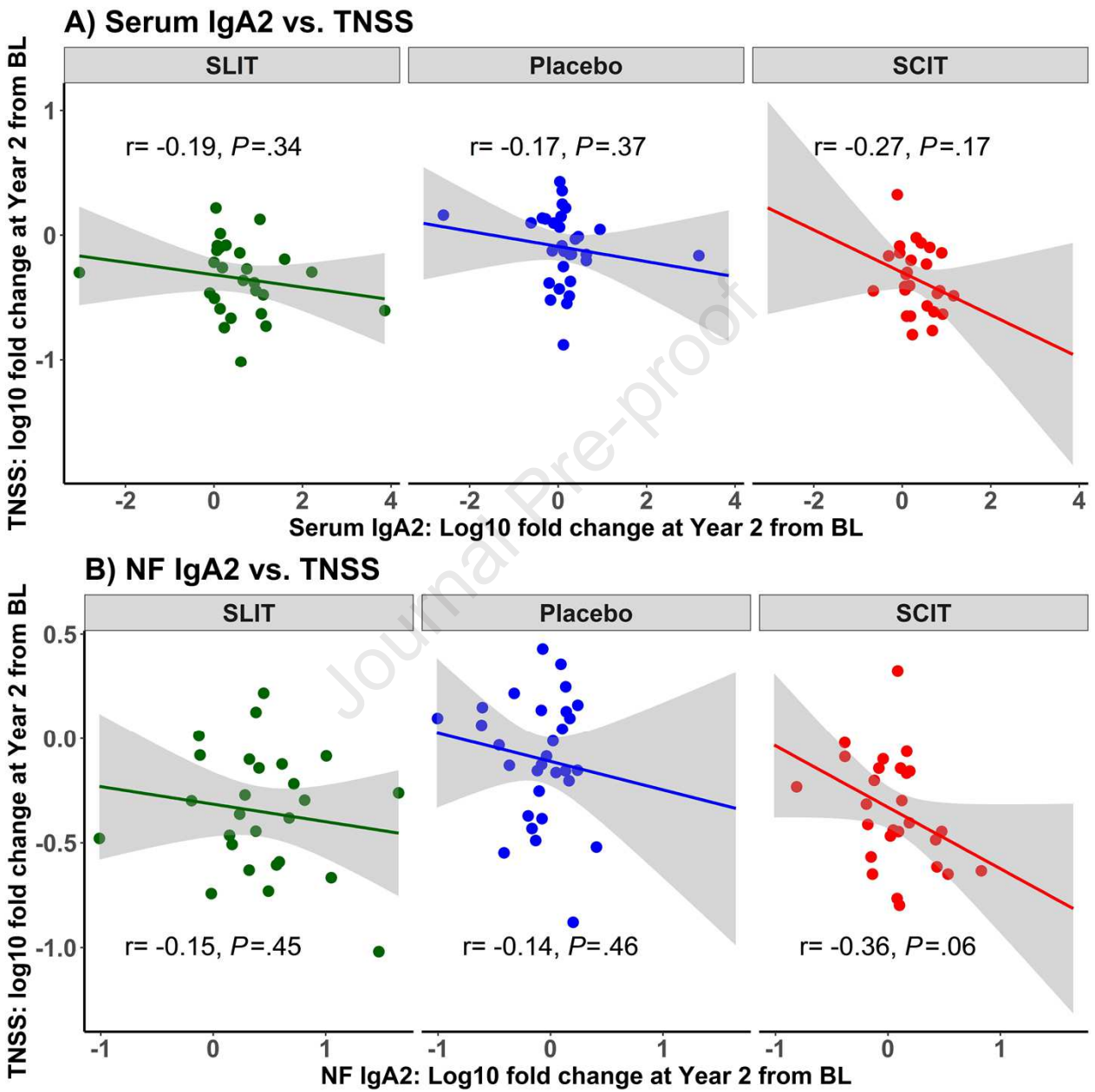

A) Serum IgA2 vs. TNSS
SLIT
Placebo
SCIT
r= -0.19, P=.34
r= -0.17, P=.37
r= -0.27, P=.17
TNSS: log10 fold change at Year 2 from BL
Serum IgA2: Log10 fold change at Year 2 from BL
B) NF IgA2 vs. TNSS
r= -0.15, P=.45
r= -0.14, P=.46
r= -0.36, P=.06
NF IgA2: Log10 fold change at Year 2 from BL

Figure E2

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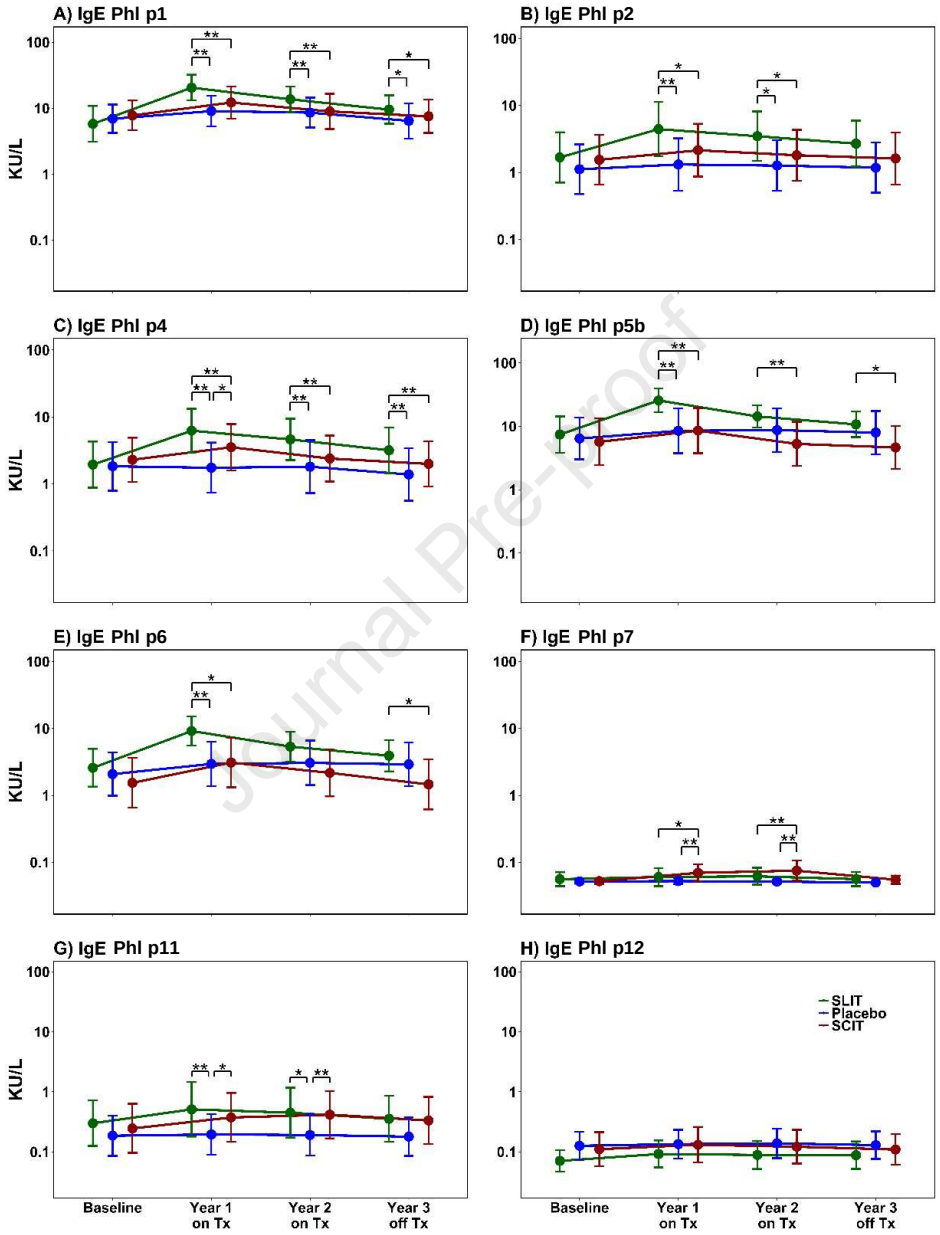


Figure E3

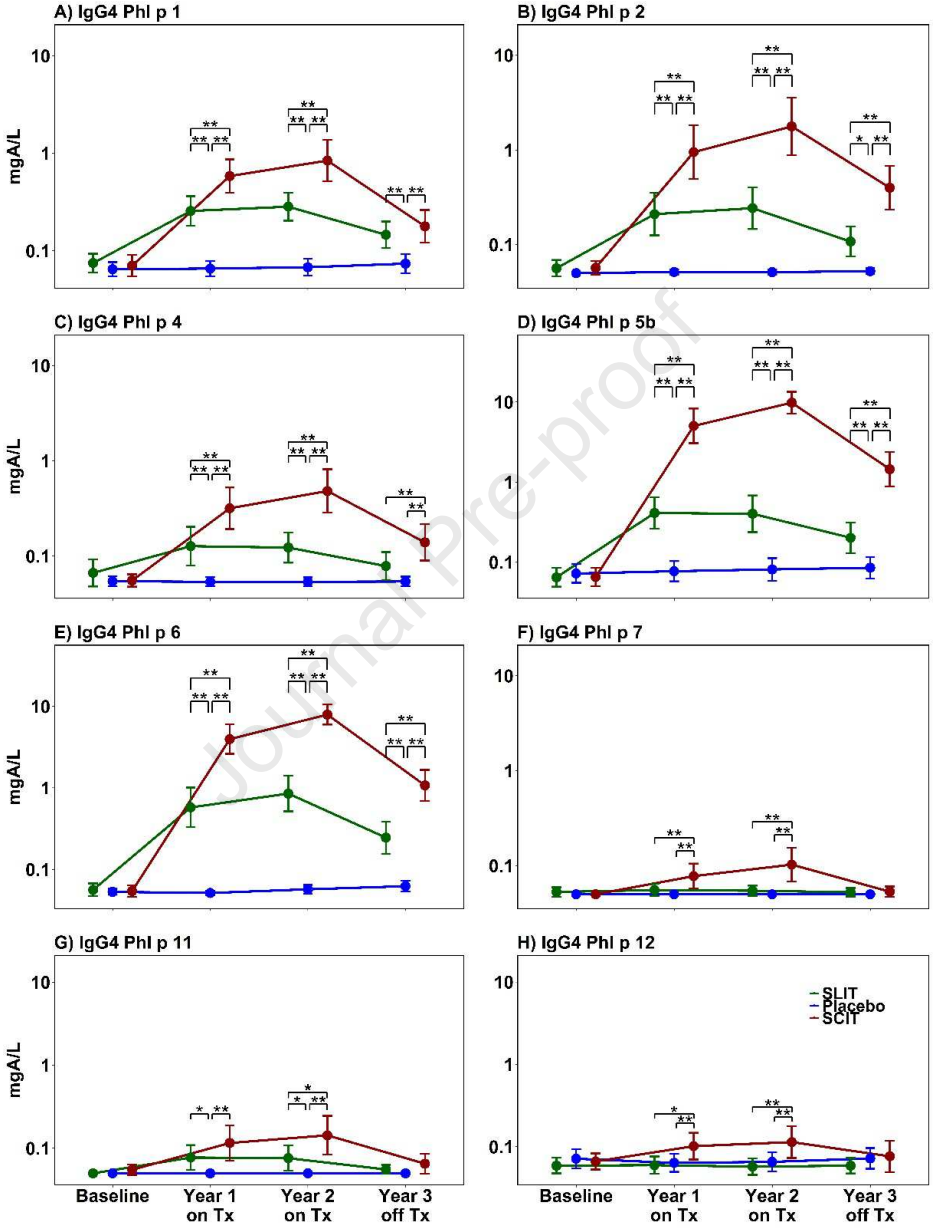


Figure E4

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ONLINE REPOSITORY TEXT

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

Mohamed H. Shamji, PhD, FAAAAI[a], David Larson, PhD[b], Aarif Eifan, MD[a], Guy W. Scadding, MD, PhD[a], Tielin Qin, PhD[b], Kaitie Lawson, MS[c], Michelle L. Sever, MSPH, PhD[c], Ellen Macfarlane, BSc[a], Janice A. Layhadi, MSc, PhD[a], Peter Würtzen[d], PhD, Rebecca V. Parkin, BSc[a], Srinath Sanda, MD[e], Kristina M. Harris, PhD[b], Gerald T. Nepom MD, PhD[b, f], Alkis Togias MD[g], Stephen R. Durham, MD, FRCP[a, b]

[a]National Heart and Lung Institute, Allergy and Clinical Immunology, Imperial College NIHR Biomedical Research Centre, Asthma UK Centre in Allergic Mechanisms of Asthma, London, United Kingdom; [b]Immune Tolerance Network, Bethesda, MD, USA; [c]Rho Federal Systems Division, Durham, NC, USA; [d]Immunology ALK-Abello, Hørsholm; [e]Immune Tolerance Network, San Francisco, CA, USA [f]Benaroya Research Institute at Virginia Mason, Seattle, WA, USA; [g]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA.

Corresponding author:
Dr Mohamed H. Shamji
National Heart and lung Institute,
Sir Alexander Fleming Building, Imperial College London,
Imperial College London, SW7 2AZ
London, SW7 2AZ, United Kingdom.
Tel: +44 20 7594 3476, E-mail: m.shamji@imperial.ac.uk.

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Methods

Measurement of IgA_1/IgA_2 in Nasal Fluid and Serum using ELISA

Plates were coated with 50 µL of of Phl p (5 µg/mL) for 1 hour at 37°C. Wells were washed three times using 0.05% Tween 20 in PBS, dried and blocked using 1% Bovine Serum Albumin (BSA) in PBS at 37°C for 1 hour. Plates were washed with wash buffer and dried completely. Standards and serum samples were added into corresponding wells and incubated overnight at 4°C. Following overnight incubation at 4°C, the plates were washed and incubated with monoclonal antibodies (mAbs) to IgA_1 or IgA_2 (Abcam) for 30 minutes. Plates were washed, dried and 100 µL of HRP-conjugated Streptavidin (Biolegend) was added to each well and incubated on a shaker for 30 minutes. After washing, TMB substrate was added in the dark for 8 minutes and 50 µL stopping solution (H_2SO_4) was added. Plates were read at OD_{450} using an ELISA microplate reader (Molecular Probes, Eugene, OR, USA). The anti-IgA1 antibody binds to the Fc portion of the heavy chain of human IgA_1. Meanwhile, anti-IgA_2 antibody binds to the Fc portion of the heavy chain of human IgA_2 and can detect both monomeric and dimeric IgA_2.

Measurement of nasal IgG, nasal IgG_4 and serum IgG by ELISA

Plates were coated with 50 µL Phl p (5 µg/mL) diluted in bicarbonate coating buffer at 37°C for 1 hour. Wells were washed three times with 0.05% Tween 20 in PBS, dried and blocked using 1% Bovine Serum Albumin (BSA) (Sigma, Gillingham, United Kingdom) in PBS at 37°C for 1 hour. Plates were washed, dried, and SCIT serum added in serial dilutions (1:1) with PBS to standard wells. Samples were added 1:1000 (for IgG) or 1:3.3 (for IgG_4) dilution with PBS to corresponding wells. The concentration of the standard serum was determined using ImmunoCAP as per

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

References:

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

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147 measured by ImmunoCAP. Data are presented as mean □ 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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