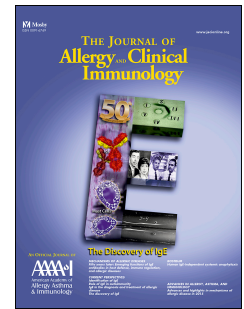


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The effects of house dust mite sublingual immunotherapy (SLIT)-tablet effects on immunological biomarkers and nasal allergen challenge symptoms

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Summary

SQ HDM SLIT-tablet significantly increased serum HDM-specific IgG₄ and IgE blocking factor, and significantly decreased early phase nasal symptoms after nasal allergen challenge. No significant effects on cytokines or gene expression in nasal mucosa were observed.

Key words: allergen immunotherapy, serum IgE blocking factors, early allergic reaction, IgE-dependent mast cell activation, nasosorption, type 2 inflammation

Abbreviations

AR, allergic rhinitis; HDM, house dust mite; IgE-BF, IgE blocking factor; MLF, mucosal lining fluid; NAC, nasal allergen challenge; PNIF, peak nasal inspiratory flow; SAM, synthetic absorptive membrane; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; TNSS, total nasal symptom score; VAS, visual analogue score

To the Editor:

House dust mite (HDM) is the most common cause of allergic sensitization in many countries worldwide¹ and allergic asthma is strongly associated with HDM sensitization.² Treatment with the SQ HDM sublingual immunotherapy (SLIT)-tablet (12 SQ-HDM) significantly improves allergic rhinoconjunctivitis symptoms, reduces the risk of moderate or severe asthma exacerbations, and decreases inhaled corticosteroid use in subjects with HDM allergic asthma.³⁻⁷ SLIT is likely to modify T and B lymphocyte populations resulting in an increase in local regulatory pathways, including the production of cytokines that inhibit allergic inflammation.⁸ SLIT increases non-IgE antibodies with the capacity to block the actions of allergen-specific IgE (IgE blocking factor [IgE-BF] effect), including IgG₄, which inversely correlates with symptoms in grass pollen immunotherapy.⁹ There have been difficulties in establishing accurate, reliable biomarkers to assess immunotherapy treatment response, and there has been little work surrounding the pharmacodynamics and identification of biomarkers for HDM allergy immunotherapy.

In this exploratory double-blind, randomized, parallel group trial (NCT01852825), adults with HDM allergic rhinitis received 12 SQ-HDM (MK-8237, Merck & Co., Inc., Kenilworth, NJ, USA/ALK, Hørsholm, Denmark; n=16) or placebo (n=7) daily for 12 weeks. Nasal allergen challenges (NAC) were conducted at 2 weeks pre-treatment (baseline) and weeks 8 and 12 (see **Figure E1** in the Online Repository). The co-primary endpoints were change in HDM-specific serum IgG₄ (against *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) and IgE-BF at week 12. Nasosorption and nasal curettage were used to assess nasal mucosal molecular

and cellular events that occur following NAC. Nasal congestion, rhinorrhea, itching, and sneezing were measured as a total nasal symptom score (TNSS) and peak nasal inspiratory flow (PNIF) rates were recorded at intervals up to 6 hours after NAC. TNSS and PNIF were measured as average time-weighted scores for early phase (1 hour pre NAC through 1 hour post NAC) and late phase (2 hours post NAC through 6 hours). Nasosorption for IL-5, IL-13 and TARC levels and nasal curettage for mucosal mRNA expression were performed before and after NAC. Further details of the study design, subject selection criteria, assessments, and statistical methods are described in this article's Online Repository.

A total of 23 subjects were randomized (n=16, 12 SQ-HDM; n=7, placebo) and 21 subjects completed the study. Demographics and baseline characteristics are shown in **Table EI** in the Online Repository.

This is the first study to explore early (week 2) changes in immunologic parameters after HDM SLIT-tablet treatment. Changes in serum IgG₄ against *D farinae* and *D pteronyssinus* were not apparent at week 2, but IgG₄ was significantly increased from baseline with 12 SQ-HDM versus placebo at week 8 and increased further by week 12 (**Figure 1A** and **Table EII** in the Online Repository). Similar results were observed for IgE-BF. Thus, the primary hypotheses were confirmed based on statistical significance at one-side $\alpha = 0.05$ after the multiplicity adjustment. The demonstrated induction of immunologic parameters with 12 SQ-HDM treatment is consistent with results found in large clinical trials, where immunologic changes were detected as early as week 4.^{3, 5}

NAC induced a rise in early phase TNSS, peaking at 15 minutes post NAC. Symptoms abated within the first hour and no late-phase symptoms were observed in either treatment group (**Figure 1B**). Treatment with 12 SQ-HDM reduced early phase NAC-induced symptoms by 43% at week 8 and 57% at week 12 (see **Figure E2** in the Online Repository). The difference between the 12 SQ-HDM group and placebo (90% CI) was -40.0 ($-81.4, 1.5$) at week 8 ($P=0.11$) and -61.1 ($-100.2, -22.0$) at week 12 ($P=0.01$). Similar results were observed for peak symptoms (15 minutes post NAC), with 12 SQ-HDM reducing symptoms by 36% at week 8 and 52% at week 12 (**Figure 1B**). The difference between the 12 SQ-HDM group and placebo (90% CI) for peak symptoms was -72.4 ($-149.2, 4.5$) at week 8 ($P=0.12$) and -114.4 ($-189.1, -39.6$) at week 12 ($P=0.02$). The reduction in early phase symptoms suggests an effect on mucosal mast cell activity, which could be caused by IgE-blocking antibodies.

There was no significant difference between the 12 SQ-HDM group and placebo in change from baseline for PNIF during any NAC challenge. The difference in change from baseline (90% CI) for time-weighted PNIF during the NAC early phase was -7.3 ($-32.1, 17.5$) at week 8 ($P=0.62$) and -0.6 ($-20.5, 19.3$) at week 12 ($P=0.96$).

Cytokines such as IL-5 and IL-13 are markers of type 2 inflammation during late-phase allergic inflammation. NAC increased levels of IL-5, IL-13, and TARC in the nasal mucosa from baseline to 6.5 hours post-challenge in most subjects (see **Figure E3** in the Online Repository). No significant differences in fold-changes from -1 hour pre NAC to 6.5 hours post NAC between 12 SQ-HDM and placebo were observed for IL-5 at any timepoint (**Figure 2A**). No significant differences from baseline NAC in nasal MLF IL-5, IL-13, or TARC concentrations were observed between 12 SQ-HDM and placebo at

weeks 8 or 12. Thus, this study failed to demonstrate a reduction with 12 SQ-HDM in type 2 inflammation induced in response to NAC.

There was a good correlation between absolute values for known mRNA markers of mast cells, IL-5 and IL-13, and eosinophils (see **Figure E4** in the Online Repository). A trend toward reduction in eosinophil, mast cell, and Th2 inflammation markers from baseline in 12-SQ HDM treated group versus placebo was observed, but this did not reach statistical significance (**Figure 2B**). Further mRNA analysis was restricted due to incomplete sample sets after processing. There were no consistent correlations found between the eosinophil, mast cell, and Th2 inflammation markers and VAS symptoms across all the NAC timepoints, either with placebo or 12 SQ-HDM. The current results are within the context of NAC conditions (as opposed to a natural setting) and are limited by the sample size. It is possible that treatment with 12 SQ-HDM for longer than 12 weeks is necessary to reduce Th2 mediators and mucosal eosinophil infiltration.

HDM NAC and treatment with 12 SQ-HDM was well tolerated. No serious AEs, systemic allergic events, or epinephrine administrations were reported. The AE profile was consistent with that reported in large clinical trials of 12 SQ-HDM.³⁻⁶ Two events of hypersensitivity (mild local reactions) occurred.

Induction of HDM-specific IgG₄ and IgE-BF by 12 SQ-HDM, along with significant improvement in early phase NAC-induced nasal symptoms, suggests that IgE-BF generation may contribute to the mechanism of action of 12 SQ-HDM during the first 12 weeks of treatment. There was no significant effect on mucosal IL-5 and IL-13, or eosinophil-associated gene expression, indicating the early mechanism of action of 12

SQ-HDM is likely to be based on inhibition of mast cell activity and is unrelated to inhibition of eosinophil activity.

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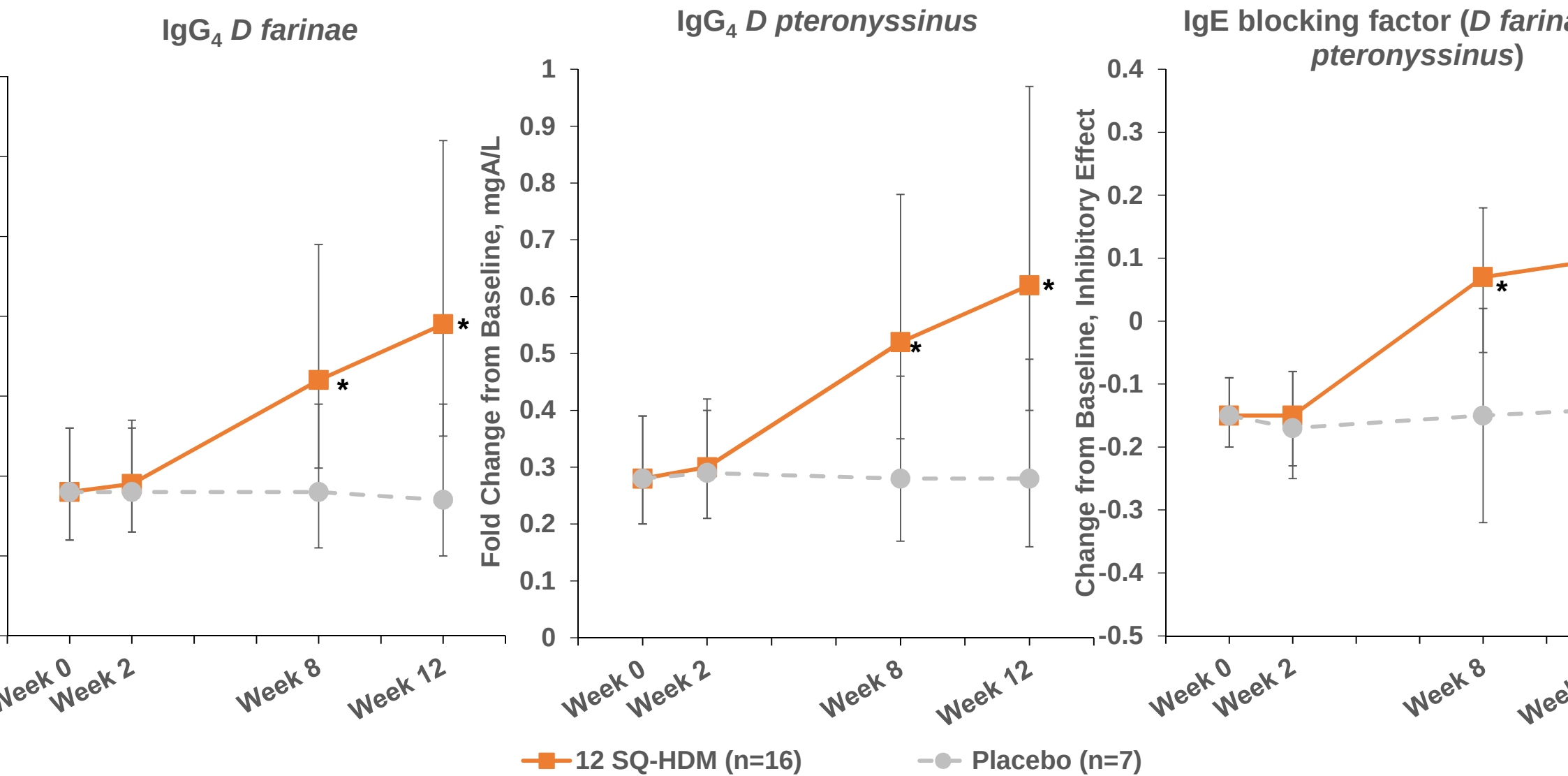
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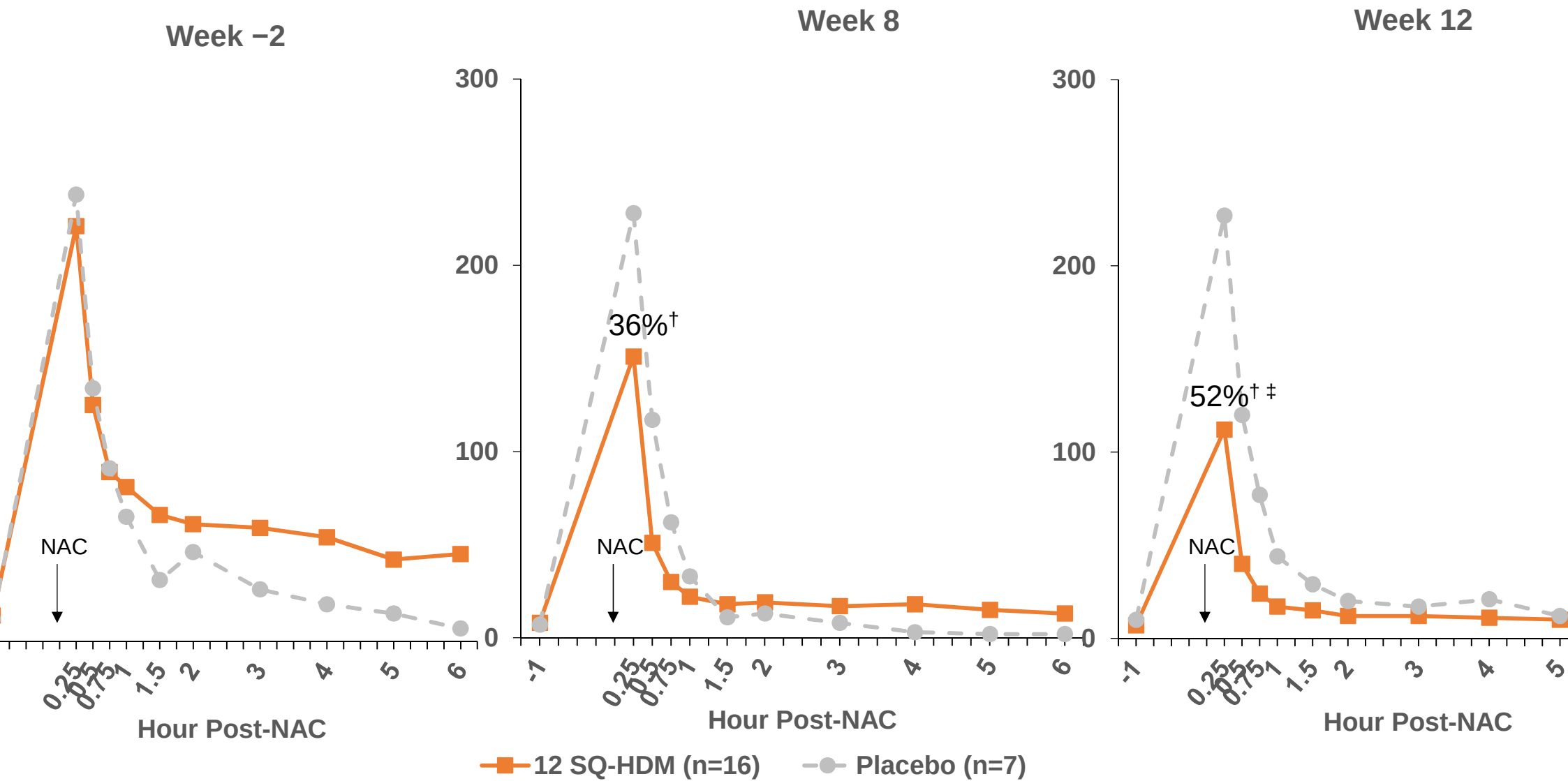
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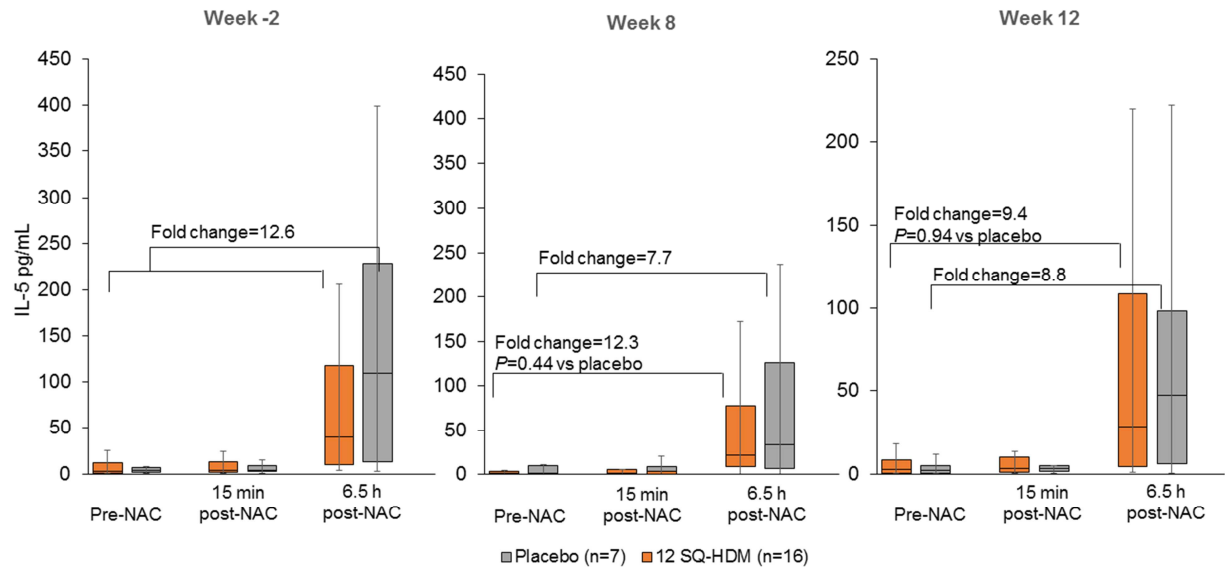
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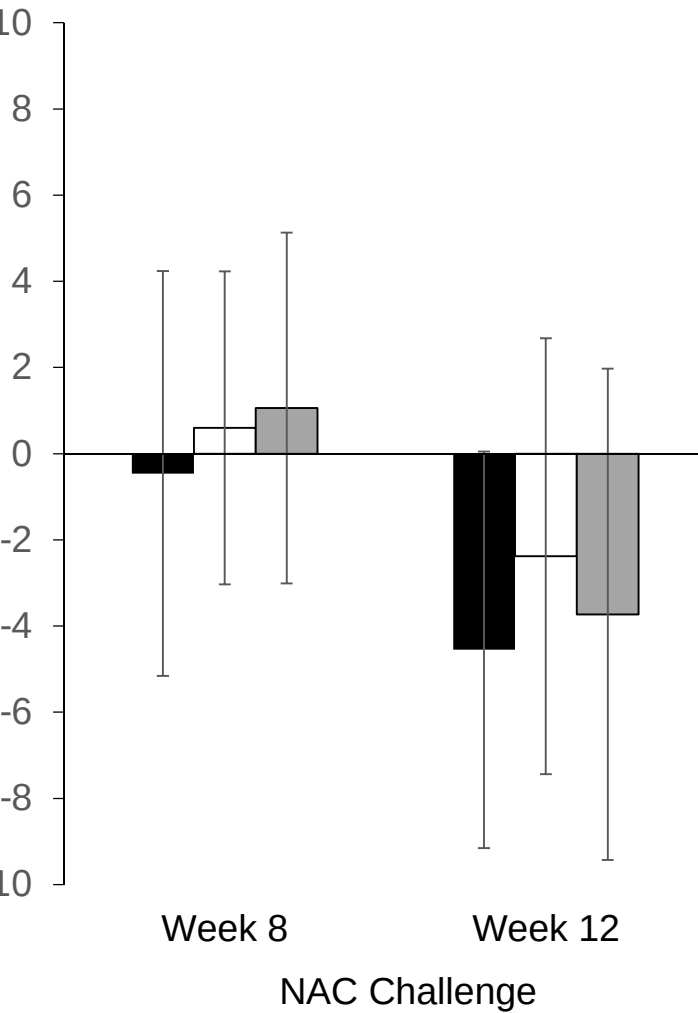
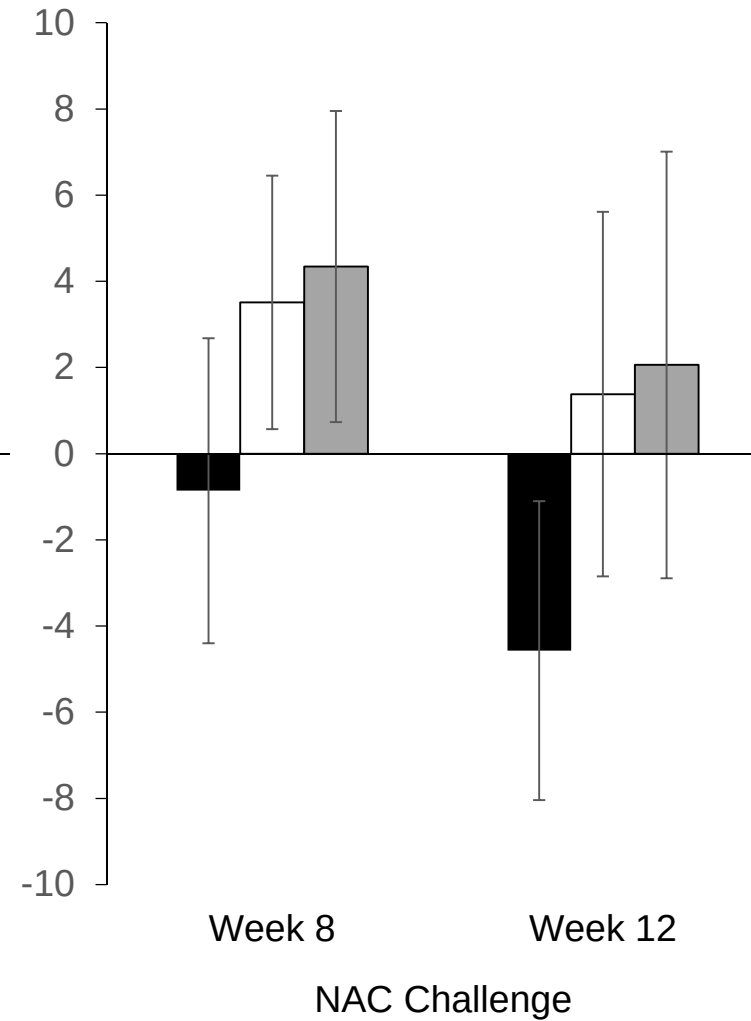
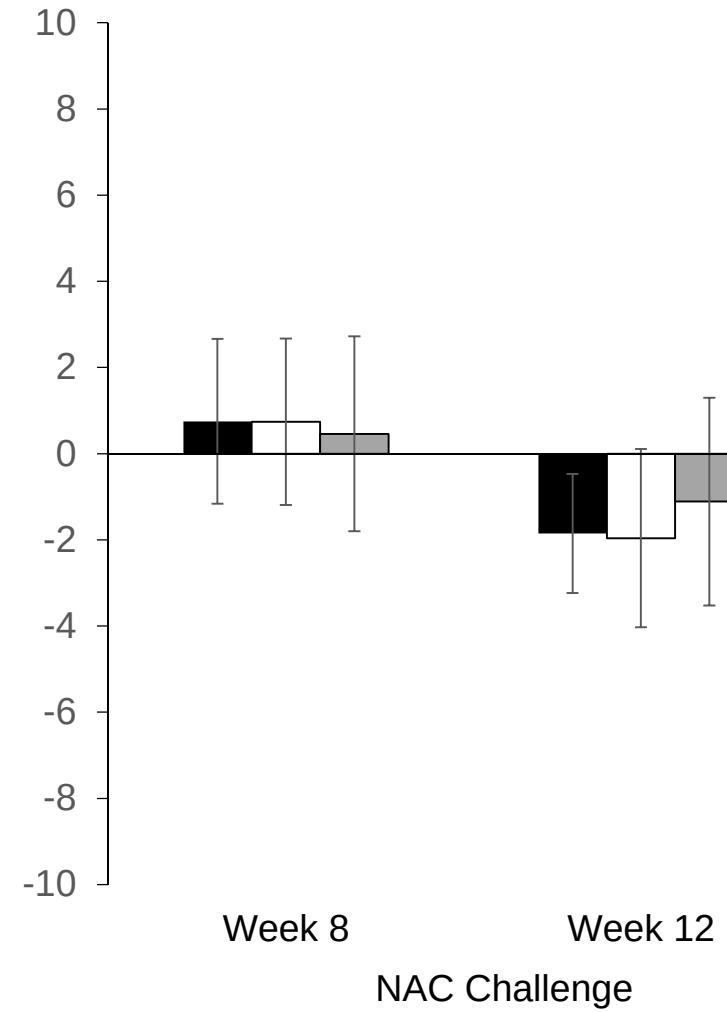
Figure 1. Effect of 12 SQ-HDM treatment on A) serum antibody-related parameters and B) NAC-induced symptoms. Values are least squares geometric mean $\pm$ 95% CI for IgG₄, and least squares mean $\pm$ 95% CI for IgE blocking factor. * P <0.05 for difference in ratio of geometric mean (IgG₄) or change from baseline (IgE blocking factor) vs placebo. [†]Percent change from baseline NAC at 15 minutes. [‡] P =0.02 vs placebo. HDM, house dust mite; mgA/L=mg antibody per liter; NAC, nasal allergen challenge; TNSS=total nasal symptom score.

Figure 2. Effect of 12 SQ-HDM treatment on NAC-induced A) IL-5 concentrations in mucosal lining fluid and B) nasal cell mRNA markers. Error bars for IL-5 concentrations indicate minimum and maximum values. Fold changes are estimated and the fold change at baseline is assumed to be the same for both treatment groups. HDM, house dust mite; NAC, nasal allergen challenge.







Eosinophil Markers**Mast Cell Markers****Th2 Markers**

■ Pre NAC

□ Post NAC

■ Fold Change

ONLINE REPOSITORY

METHODS

Randomization

Randomization was conducted by a computer-generated allocation schedule provided by the sponsor. Each site was given a range of randomization numbers from the allocation schedule and subjects were assigned a number sequentially. Blinding of investigators and subjects was maintained through the use of a placebo identical in appearance to active treatment. The protocol was approved and the study overseen by the Research Ethics Committee of NRES Committee South Central-Oxford A (Bristol, UK). All subjects provided written informed consent.

Key selection criteria

Subjects aged 18–55 years with a clinical history of AR with or without conjunctivitis to HDM and who used medications for its symptomatic relief within the last year were recruited. Intermittent mild asthma was allowed if it was controlled without regular use of corticosteroids (including inhaled corticosteroids) over the last 12 months. Subjects had a positive skin prick test response (average wheal diameter of duplicate tests ≥ 3 mm larger than the saline control) to *D pteronyssinus* (with or without a concomitant reaction to *D farinae*), and a serum-specific IgE to *D pteronyssinus* of ≥ 0.7 kU/L (with or without specific IgE to *D farinae*). Subjects experiencing symptoms from a respiratory tract infection or an allergic rhinoconjunctivitis exacerbation within 4 weeks prior to the screening NAC were excluded from the study.

Total nasal symptom score (TNSS) was assessed by visual analog scale (VAS) on a scale of 0 (absent) to 100 (severe) for each of the symptoms of nasal congestion, rhinorrhea, itching, and sneezing with a total maximum score of 400.¹ At screening (prior to baseline NAC) the VAS score was required to be <150/400 and participants had to demonstrate VAS score increases of ≥ 25 for at least 2 symptoms within two hours of the nasal challenge.

IgE blocking factor and IgG₄ measurement

Serum for measurement of IgE-BF and IgG₄ was collected at randomization, and weeks 2, 8, and 12. IgE-BF was measured using a validated immunoassay (ADVIA Centaur Specific IgE 2-step assay with ADVIA Centaur Specific IgE, Siemens Healthcare Diagnostics, Deerfield, Ill)² in a recently adapted version that used a 1:1 mixture of *D pteronyssinus* and *D farinae* extracts.³ HDM species-specific IgG₄ was measured by ImmunoCap (Phadia AB, Portage, MI) as previously described.⁴

Nasal allergen challenge (NAC)

Antihistamines and decongestants had to be discontinued for more than 3 days and nasal or ocular corticosteroids for 21 days before NAC.

Nasal lavage was performed as previously described using 5 mL of sterile normal saline flushed into the nasal cavity 20 times over approximately 1 minute prior to any sampling.⁵

House dust mite allergen extract (Aquagen, *D pteronyssinus*, 100,000 SQ-U/mL; ALK, Hørsholm, Denmark) was diluted in normal saline to 9,000 SQ-U/mL according to the

manufacturer's instructions and administered using a nasal Pfeiffer Bidose applicator (Aptar Pharma, Milton Keynes, UK). A single nasal spray (100 µL) was given to each nostril in each challenge (total bilateral challenge of approximately 1800 SQ-U). NAC was performed at week -2 (baseline), and weeks 8 and 12 of the study (**Figure E1**). Nasosorption was undertaken 1 hour pre-NAC (baseline), 15 minutes and 6.5 hours post-NAC (**Figure E1**). Nasal curettage was performed at 1 hour pre-NAC (baseline) and 6.5 hours post-NAC.

Total nasal symptom scores

TNSS using VAS out of a total score of 400 was recorded before NAC, every 15 minutes in the first hour after NAC, at 90 minutes, and hourly for six hours.

Peak nasal inspiratory flow (PNIF)

PNIF was measured using an inspiratory flow meter connected to a facemask according to published guidelines.⁶ Subjects were coached on the method and allowed to become familiar with the equipment. At each time point in the NAC (same timepoints as symptom scores), three measurements were collected of which the highest was recorded.

Nasosorption: collection of mucosal lining fluid (MLF)

Synthetic absorptive membranes (SAM) in 7 mm x 30 mm strips with rounded ends (Hunt Developments, Midhurst, West Sussex) were inserted into both nostrils using forceps under direct vision with a head-mounted lamp. This process was timed for 2 minutes with application of nasal clips to ensure good apposition on the mucosal surface of the inferior turbinate. After withdrawal, SAM strips were processed as

previously described and the collected eluate immediately stored at -80°C for subsequent immunoassay.⁷

Measurement of chemokines and cytokines in MLF

MLF spun through cellulose acetate centrifuge filters was stored frozen at -80°C at the sites. Concentrations of IL-5, IL-13, and TARC was determined using Mesoscale V-plex singleplex immunoassays (Mesoscale Diagnostics, Rockland, Maryland, USA). Fit for purpose validations were performed internally where inter- and intra-assay variability ($<20\%\text{CV}$), spike recovery ($>80\%$), linearity, sensitivity, and freeze/thaw stability ($<20\%$ change) were assessed and passed internal pre-specified cutoffs in each category.

Nasal mRNA collection and measurement

Nasal scrapes were collected using Rhino-Probe® plastic nasal curettes (Arlington Scientific, Springville, UT) from the inferior side of the inferior turbinate under direct vision. The nasal mRNA was assessed using NanoString's nCounter™ Analysis System (NanoString Technologies, Inc., Seattle, WA). Libraries for RNA sequencing were prepared by Beijing Genomics Institute (BGI: Philadelphia, PA or Hong Kong, China) using 100 ng of purified total RNA and the Truseq stranded total RNA RiboZero library preparation kit (Illumina Catalog #: RS-122-2201) strictly following the Illumina guide (15031048 E). Briefly, rRNA was depleted with rRNA Removal Mix and then the remaining RNA fragmented into $\sim 160\text{bp}$ fragments. These fragments served as templates for first-strand cDNA synthesis using random hexamer-primers, followed by second stand synthesis with the addition of buffer, dNTPs, RNase H and DNA polymerase I. Double-stranded cDNA was purified using the QiaQuick PCR extraction

kit (Qiagen) followed by end repair, base A addition and ligation of sequencing adapters. Ligated fragments were purified by magnetic beads and amplified via PCR. The resulting library products were quantified with the Agilent2100 bioanalyzer and were sequenced using an Illumina HiSeqTM 4000 for a total of 3GB of 50bp paired-end read data per sample.

Endpoints

The co-primary endpoints were the change induced by 12 SQ-HDM from baseline in HDM-specific (against *D farinae* and *D pteronyssinus*) IgG₄ and IgE-BF antibodies (against both species together) at week 12. Secondary endpoints were changes in nasal MLF IL-5 concentration after treatment and in response to NAC, and changes in time-weighted average TNSS during NAC for early (baseline to 1 hour post NAC) and peak (15 min post NAC) responses. Changes in TNSS during NAC for late (2 hours to 6 hours post NAC) responses were also evaluated. Safety was assessed through adverse event (AE) reporting. Exploratory endpoints included nasal MLF IL-13 and TARC concentrations, PNIF, and nasal mRNA signature.

Statistical methods

Endpoint analysis was performed for the per-protocol population, which in this study was also the all subjects as treated population. Descriptive statistics were calculated for the co-primary endpoints of IgG₄ and IgE-BF. Within-group change from baseline and between-group differences in serum HDM-specific IgG₄ antibody were evaluated based on a constrained longitudinal data analysis (cLDA) method with log transformed data.⁸ The model included time (categorical variable), treatment, and time by treatment interaction as fixed effects and subjects as random effect. The 90% two-sided

confidence interval (corresponding to a 95% one-sided CI) for the geometric mean ratio of the endpoints in 12 SQ-HDM-treated patients to those in placebo-treated patients at week 12 was reported. Change from baseline in IgE-BF was analyzed in a similar manner to IgG₄, but on the original scale (not log-scale).

To preserve the overall Type I error at a one-sided $\alpha = 0.05$ level, analysis of the co-primary endpoints was tested in a step-down procedure. Specifically, the evaluation of the HDM-specific IgE-BF response was only tested if the HDM-specific IgG₄ response was statistically significant. Furthermore, as the IgG₄ response was evaluated for the two HDM species (*D farinae* and *D pteronyssinus*), a Hochberg step-up procedure was used and the hypothesis was considered successful if statistical significance was found for at least one of the 2 HDM species.

Gene expression alignment and transcript quantitation was performed in Omicsoft Array Studio version 7.2.2.29. Briefly, cleaned reads were aligned to the human B37.3 genome reference using the Omicsoft Aligner with a maximum of 4 allowed mismatches. Gene level counts were determined by the RSEM algorithm as implemented in Omicsoft Array Studio and using RefGene transcript annotation prepared March 21st, 2014.

TNSS and PNIF were measured as average time-weighted scores from baseline (1 hour pre NAC) through 1 hour post NAC (early phase NAC). Least square means and 95% two-sided confidence intervals were calculated for each treatment group at each NAC. Between-group differences in change from pre-treatment NAC and 90% two-sided confidence intervals were calculated for TNSS, PNIF, and cytokine levels using the same cLDA model used for the co-primary endpoints.

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Table E1. Subject demographics and baseline characteristics.

	12 SQ-HDM (n=16)	Placebo (n=7)
Women, n (%)	8 (50)	3 (43)
Mean age (range), y	31 (19–52)	36 (23–53)
White, n (%)	14 (88)	6 (86)
Blood eosinophil levels (min, max)	0.2 (0.1, 0.7)	0.2 (0.1, 0.4)
<i>D farinae</i> SPT wheal size, mean mm (min, max)	11.3 (6.6, 16.0)	12.4 (4.3, 18.5)
<i>D farinae</i> serum specific IgE, mean kU/L (min, max)	19.9 (1.6, 58.8)	16.8 (1.6, 36.0)
<i>D pteronyssinus</i> SPT wheal size, mean mm (min, max)	12.5 (5.9, 19.0)	10.6 (6.0, 14.8)
<i>D pteronyssinus</i> serum specific IgE, mean kU/L (min, max)	33.4 (1.6, 98.2)	27.7 (3.7, 60.0)
HDM, house dust mite; SPT, skin prick test.		

175 **Table EII.** Fold change from baseline for IgG₄ and change from baseline for IgE
 176 blocking factor.

Treatment	Week	Min	Median	Max	Least Square GM/Mean (95% CI) ^a	GMR/CFB vs Placebo (90% CI) ^b	P Value
IgG₄ <i>D. farinae</i>							
12 SQ-HDM	0	0.01	0.18	1.00	0.18 (0.12, 0.26)	-	-
	8	0.04	0.32	1.68	0.32 (0.21, 0.49)	1.83 (1.20, 2.81)	0.024
	12	0.04	0.46	2.36	0.39 (0.25, 0.62)	2.36 (1.52, 3.66)	0.003
Placebo	0	0.13	0.17	0.47	0.18 (0.12, 0.26)	-	-
	8	0.11	0.16	0.43	0.18 (0.11, 0.29)	-	-
	12	0.11	0.15	0.45	0.17 (0.10, 0.29)	-	-
IgG₄ <i>D. pteronyssinus</i>							
12 SQ-HDM	0	0.03	0.29	1.52	0.28 (0.20, 0.39)	-	-
	8	0.08	0.60	2.60	0.52 (0.35, 0.78)	1.86 (1.24, 2.78)	0.015
	12	0.07	0.63	2.94	0.62 (0.40, 0.97)	2.18 (1.36, 3.49)	0.010
Placebo	0	0.17	0.27	0.75	0.28 (0.20, 0.39)	-	-
	8	0.14	0.30	0.78	0.28 (0.17, 0.46)	-	-
	12	0.17	0.26	0.79	0.28 (0.16, 0.49)	-	-
IgE blocking factor							
12 SQ-HDM	0	-0.42	-0.17	0.14	-0.15 (-0.20, -0.09)	-	-
	8	-0.28	0.06	0.57	0.07 (-0.05, 0.18)	0.22 (0.05, 0.38)	0.038
	12	-0.25	0.07	0.62	0.10 (-0.03, 0.23)	0.24 (0.07, 0.41)	0.027
Placebo	0	-0.27	-0.15	0.00	-0.15 (-0.20, -0.09)	-	-
	8	-0.38	-0.13	0.03	-0.15 (-0.32, 0.02)	-	-
	12	-0.41	-0.10	0.03	-0.14 (-0.32, 0.02)	-	-

0.04)

CFB, change from baseline; GM, geometric mean; GMR, ratio of geometric mean;
HDM, house dust mite.

^aGM for IgG₄ and mean for IgE blocking factor.

^bGMR for IgG₄ and CFB for IgE blocking factor.

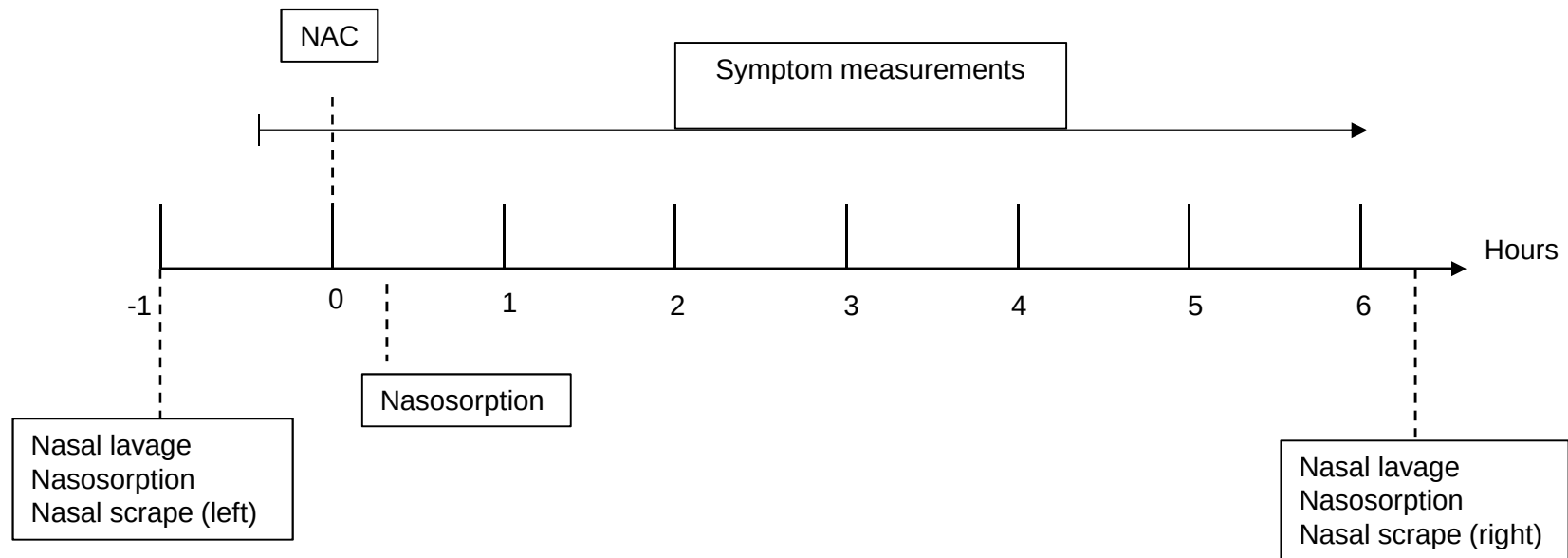
Figure Legends

Figure E1. A) Study design; B) sampling schedule at weeks -2, 8 and 12. NAC, nasal allergen challenge.

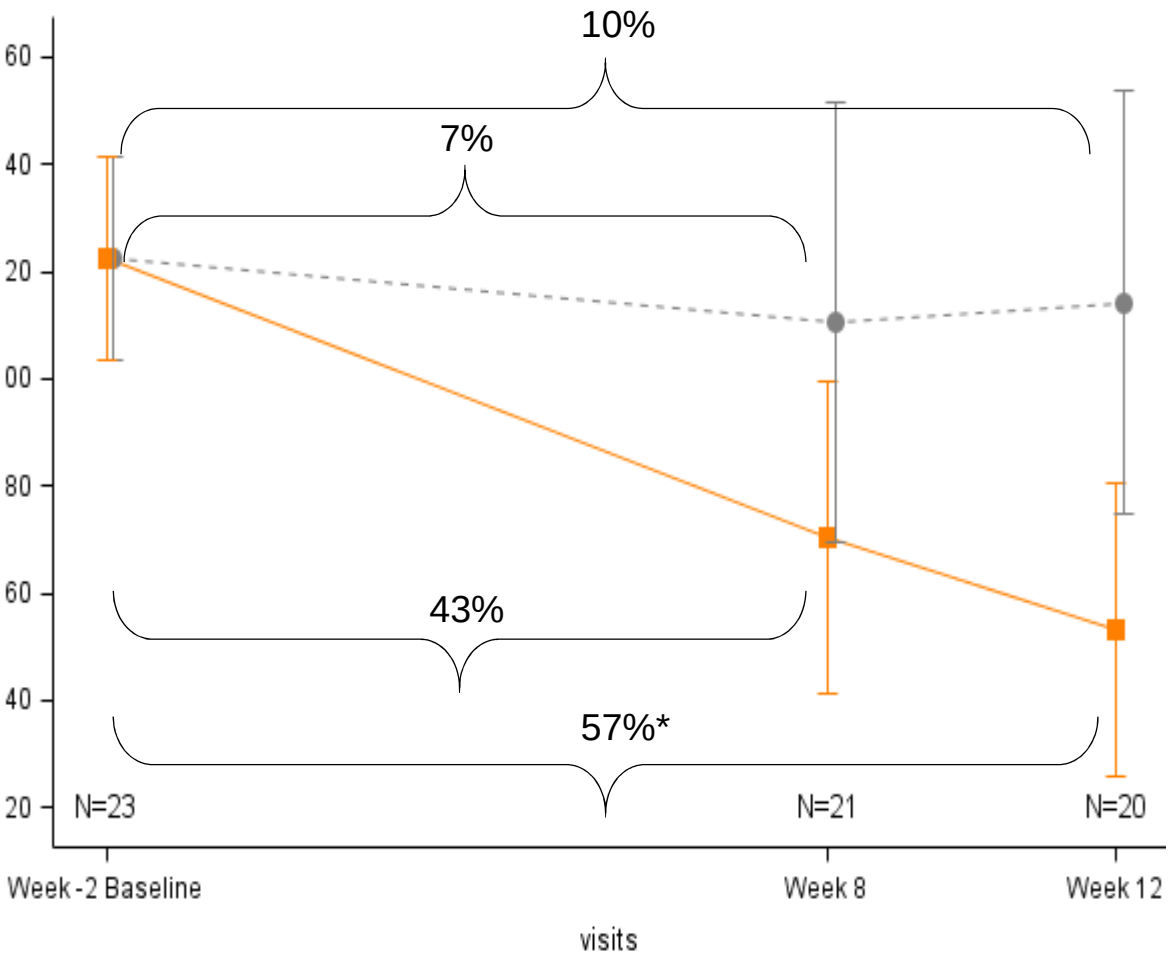
Figure E2. Total nasal symptom score (TNSS) during early and late phases of nasal allergen challenges at weeks -2, 8, and 12. Percentages indicate change from baseline. * $P \leq 0.05$ for change from baseline vs placebo. HDM, house dust mite; NAC, nasal allergen challenge.

Figure E3. Subject-level protein concentrations in nasal mucosal lining fluid at 1 hour pre-NAC, 15 minutes post NAC (except TARC), and 6.5 hours post NAC at weeks -2, 8 and 12 for A) IL-5, B) IL-13, and C) TARC. BLOQ, below limit of quantitation; HDM, house dust mite; NAC, nasal allergen challenge.

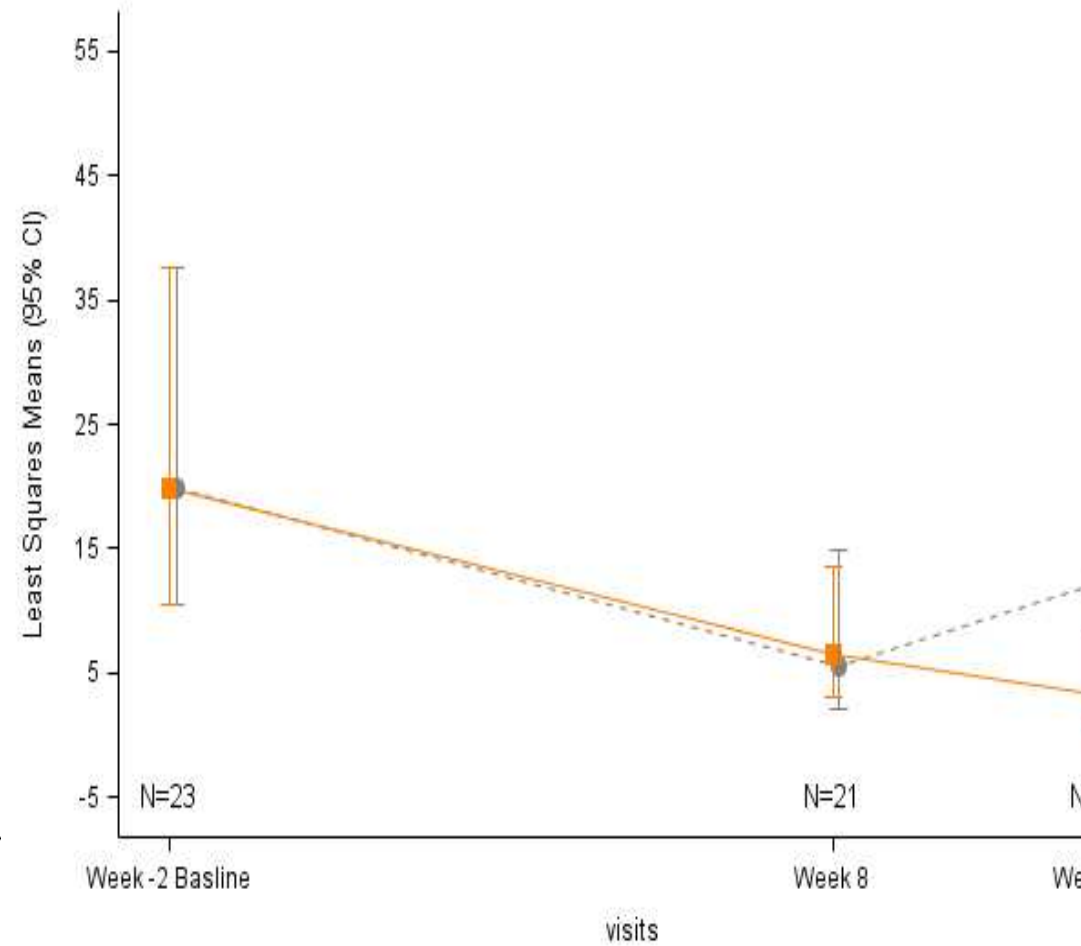
Figure E4. Correlations between mRNA markers.

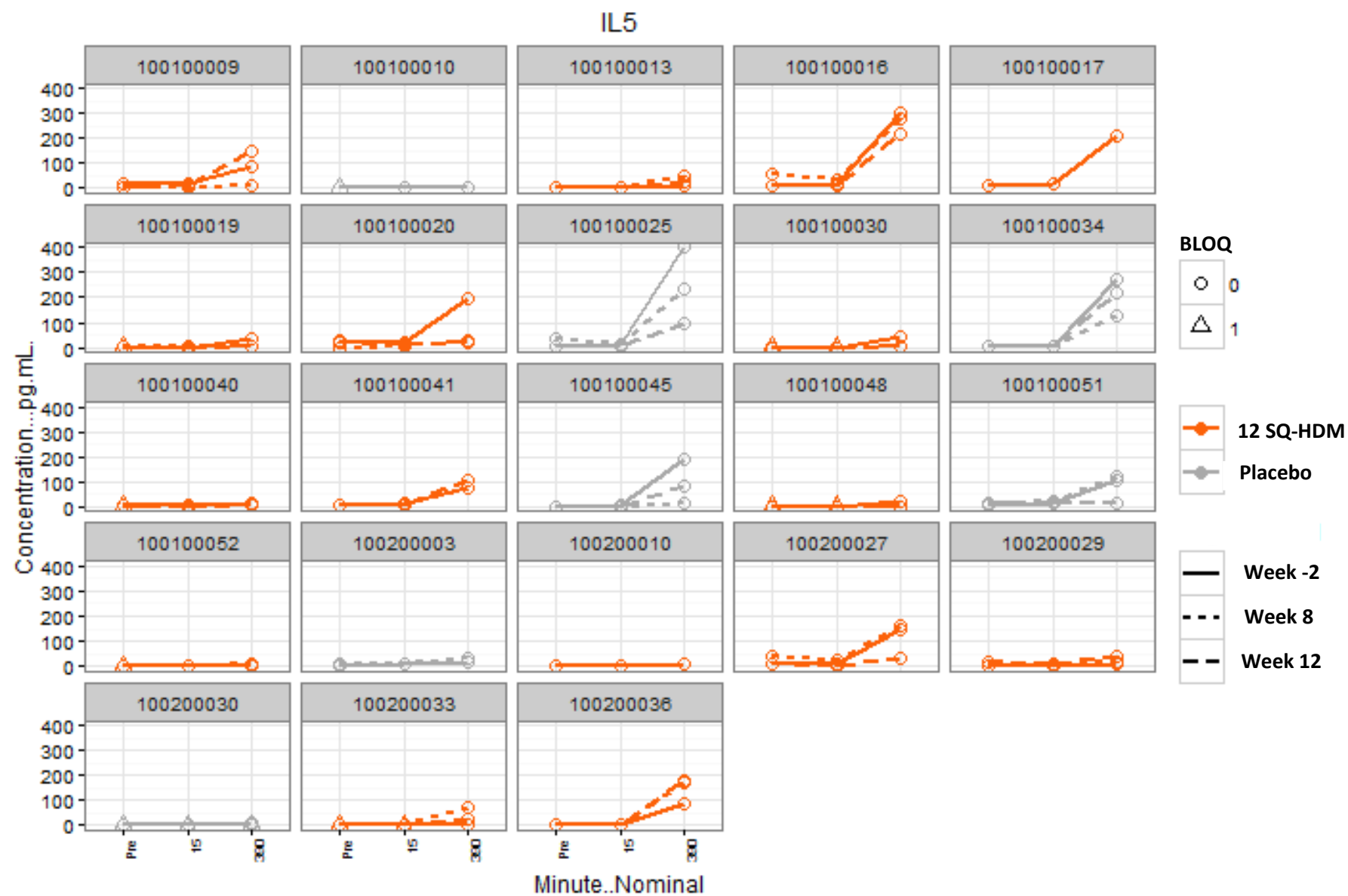


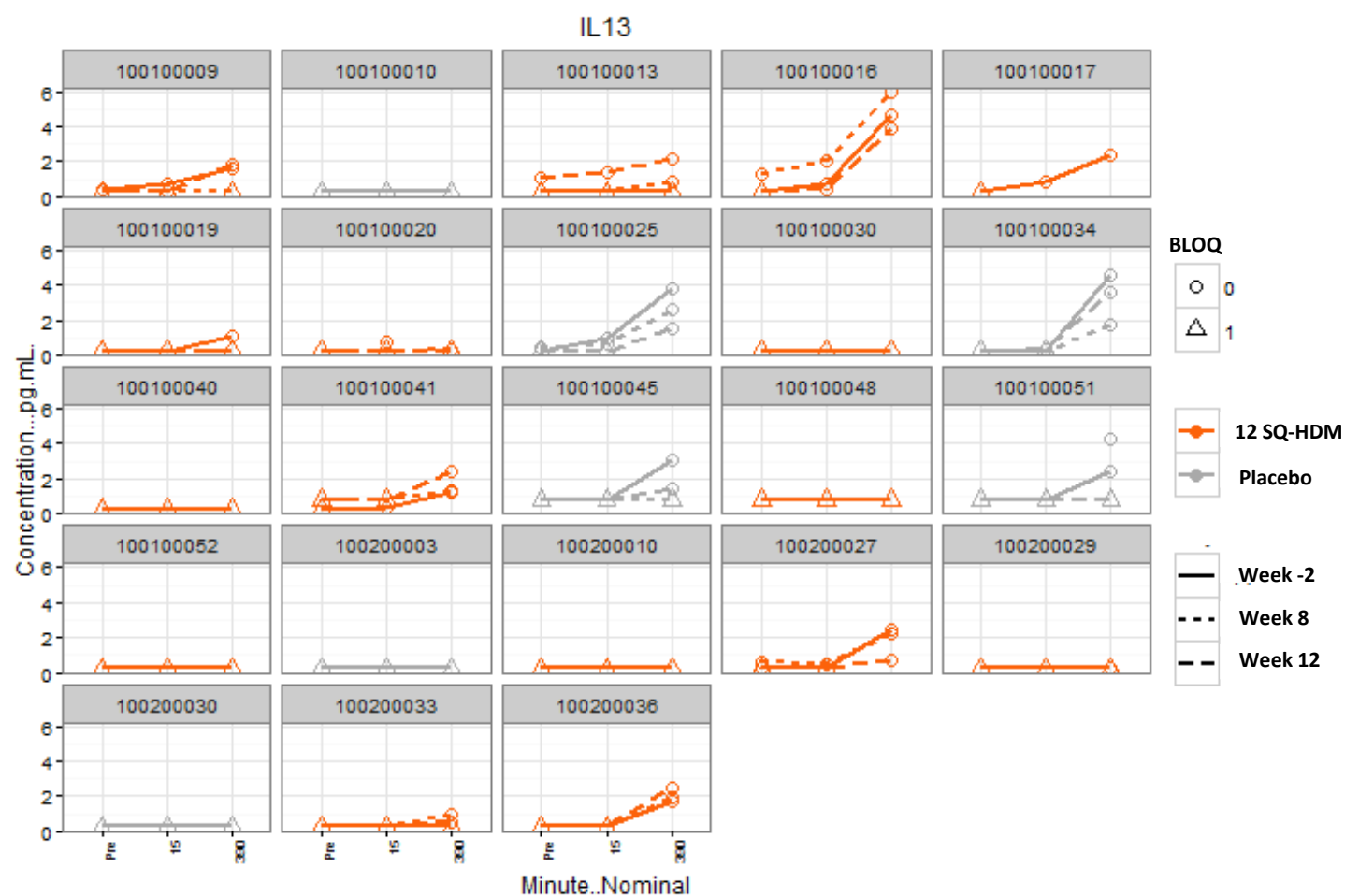
Early Phases of NAC (Hours -1 to 1)

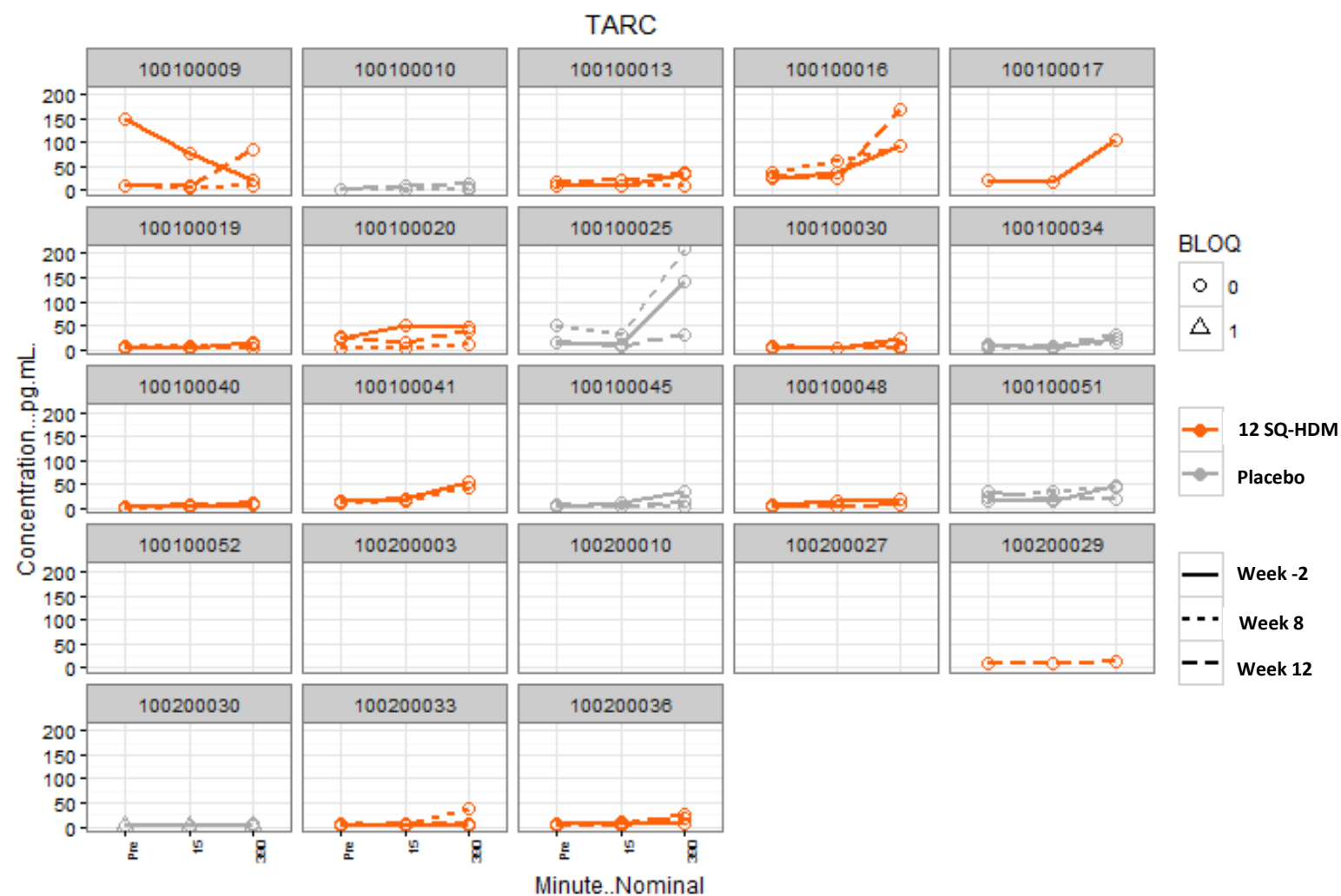


Late Phases of NAC (Hours 2-6)









Eosinophil Markers

LGAL S12	DACH 1	CEBPE	HRH 4	EMR 1	EMR 4P	CLC	SIGLE C8	VSTM 1
1	0.94	0.92	0.96	0.97	0.94	0.98	0.89	0.89
	1	0.94	0.93	0.95	0.92	0.95	0.87	0.94
		1	0.96	0.96	0.96	0.95	0.93	0.99
			1	0.96	0.96	0.98	0.92	0.95
				1	0.97	0.97	0.90	0.94
					1	0.95	0.84	0.93
						1	0.92	0.92
							1	0.92
								1

Mast Cell Markers

	RGS1 3	C1orf1 8	MS4A2	TPS B2	TPS AB1	CPA 3
RGS13	1	0.69	0.89	0.91	0.85	0.86
C1orf18		1	0.84	0.84	0.86	0.85
MS4A2			1	0.99	0.97	0.98
TPSB2				1	0.98	0.98
TPSAB 1					1	0.99
CPA3						1

Th2 Markers

	IL4	IL5	IL9
IL4	1	0.98	0.90
IL5		1	0.93
IL9			1
IL13			