ONLINE REPOSITORY

Immunologic Features of Infants with Milk or Egg Allergy Enrolled in an Observational Study (CoFAR) of Food Allergy

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Definition of Atopic Diseases

Atopic disease history in parents and siblings of enrolled infants was based upon previously accepted definitions.[1] A diagnosis of asthma was defined by a minimum of 3 reported episodes (history) of wheezing in addition to respiratory symptoms with response to beta agonists or signs of airway hyperreactivity (wheezing or severe coughing while exercising, cold weather, or disturbed coughing at night) without ongoing upper respiratory infection. Atopic dermatitis required[2;3] pruritus and an eczematous rash (acute, subacute, chronic) with typical morphology and age specific patterns and a chronic (3 or more weeks) or relapsing history (excluding scabies, seborrheic dermatitis, allergic contact dermatitis, ichthyoses, cutaneous lymphoma, psoriasis, and immune deficiency disease) and atopy (personal and/or family history or IgE reactivity) and xerosis. A family history of food allergy required typical symptoms such as urticaria, angioedema, or asthma, directly following consumption of the food.

For enrolled infants, asthma is graded by severity according to NHLBI guidelines and atopic dermatitis severity is graded by criteria previously described and published by Rajka and Langeland.[3] Briefly, the AD severity is graded as mild, moderate, or severe using the following parameters to compute a score summation: 1) extent of disease (by “rule of nine”), 2) course of disease (by history), and 3) intensity of disease (disturbance of night’s sleep by itching) each on a 3 point scale. Summation scores of 3–4 indicate mild disease, 5–7 moderate disease, and 8–9 severe atopic dermatitis. To avoid exclusion of milk or egg sensitized children who experienced improvement of atopic dermatitis by ongoing milk or egg restriction prior to consideration for enrollment, a historical grading of AD severity prior to dietary manipulation (change of formula or maternal exclusion of milk or egg) was allowed.

Categorization of food allergy

Because this is an observational study, repeated diagnostic oral food challenges could not be imposed upon infants at enrollment. Therefore, the following categorization scheme was developed and designed for longitudinal use.

At enrollment (and for the longitudinal course of the study) we define food allergy according to clinical history and test results, as well as by oral food challenges when clinically indicated. A

clinical history was considered ***convincing*** when there were symptoms within an hour of isolated ingestion that included at least: urticaria and/or angioedema, difficulty breathing, wheezing, throat tightness, and/or vomiting. Brief ingestion of cow's milk protein formula during the newborn period does not qualify as evidence of tolerance.

Based upon available studies for children under age 2 years, we considered food-specific IgE levels to have diagnostic accuracy of >95% when they were equal to or greater than 5 kU_A/L for milk[4], 2 kU_A/L for egg,[5] and 5 kU_A/L for peanut (see below).

We developed a novel classification scheme to categorize each study subject into one of the following food allergy diagnostic categories based upon the clinical history and standard IgE levels:

***Confirmed* IgE mediated reaction (~97% certainty):** A positive physician-supervised oral food challenge and sensitization to the food (food-specific IgE $\geq$ 0.35 kU_A/L and/or PST $\geq$ 3 mm) and/or a convincing reaction plus a >95% predictive food-specific IgE test result.

***Convincing*, but not confirmed IgE mediated reaction (~95% accurate):** a convincing history with sensitization demonstrated by a positive serologic test (milk or egg $\geq$ 0.35 but < 95% predictive levels) and/or a positive skin test; or history of flare of atopic dermatitis upon ingestion of the food AND food-specific plasma IgE > 95% predictive level.

***Serological* diagnosis:** a food-specific IgE test result that is >95% predictive of a clinical reaction but no ingestion of the food. Based upon available studies for children under age 2 years, we considered food-specific IgE levels to have diagnostic accuracy of >95% when they were equal to or greater than 5 kU_A/L for milk[4], 2 kU_A/L for egg,[5] and 5 kU_A/L for peanut. The predictive value for peanut is derived. Oral food challenges are not typically performed to peanut in this age group and therefore diagnostic properties of the test have not been determined in infants.[6;7] The predictive value of serum IgE for clinical reactions varies by age, with younger infants reacting at lower levels than school-age children. For example, previous studies of egg and milk allergy in infants[4;5;8] indicate that >95% react at IgE levels to egg and

milk that correspond to 50% reaction rates for 5-7 year olds (e.g., a level of 2 kU_A/L for egg and milk).[7] In studies of children at mean ages of 5-7 years,[6;7;9] a level of 5 kU_A/L to peanut is associated with a 70-90% clinical reaction rate. Based upon these studies of peanut, and studies on egg and milk showing that infants react at lower food-specific IgE levels than older children, we estimate that a peanut IgE level of $\geq$ 5 kU_A/L in the infants in this study would indicate a high (>95%) likelihood of current clinical peanut allergy. Based upon the above-referenced studies,[6;7;9] co-incident soy allergy affecting a small percent of peanut-allergic children, would not likely influence these predictive values.

***Potential* allergy:** no ingestion and an indeterminate positive test result, or a convincing history but no sensitization, or there was a flare of atopic dermatitis and sensitization to the food (but the food-specific IgE is not in diagnostic range).

***Not allergic-sensitized*:** detectable IgE antibody (sensitized, IgE $\geq$ 0.35 kU_A/L or PST $\geq$ 3 mm) but tolerates eating the food.

***Not allergic- not sensitized*:** No evidence of IgE antibody to the food and food tolerant.

***Not sensitized-never ingested*:** tested negative and has not ingested the food (does not include exposure to allergen in breast milk).

***Non-IgE food allergy*:** a positive oral food challenge but not sensitized (serum IgE < 0.35 kU_A/L and PST < 3 mm).

As described in the manuscript, we only evaluated mononuclear cell expression of key cytokine and regulatory genes for the clinical endpoints associated with allergy (confirmed/convincing) or no allergy categories.

Skin tests

A positive PST is defined by a mean wheal diameter of 3 mm or greater, after subtraction of the saline control. Tests were considered reliable if the wheal size of the histamine control was

at least 3 mm larger than the wheal size of the negative control. All sites used the same lot of reagents and training was performed to ensure consistency. The following extracts were used (Greer catalog number in parentheses, Lenoir, NC): cow milk (F293), chicken egg white (F272), and peanut (F171). For additional allergic characterization, PSTs were performed with environmental allergens: standardized cat (TE3), dog epithelia (Canis familiaris; mixed breed) (E7), Dermatophagoides pteronyssinus (B70), Dermatophagoides farinae (B64), mold mix #1 (alternaria, aspergillus, helmithosporium, cladosporium, penicillium) (MO1), and cockroach mix (American German) (B012). Skin tests to a specific agent could be deferred if the infant experienced an unequivocal recent episode of anaphylaxis to the substance.

ADDITIONAL RESULTS

Additional Demographic Features

The number of children enrolled at each site were: Denver, CO (99), Durham, NC (103), Baltimore, MD (109), New York, NY (107) and Little Rock, AR (94). Of the mothers, 79.1% had a college degree or higher and among fathers this was 73.0%. Parental atopic disease was reported among 67.4% of mothers, 59.8% of fathers and 43.6% were families with biparental atopy. The mean birth weight of participants was 3.42 kg (range 1.16-4.77). Full term pregnancy ($\geq$37 weeks) was reported for 92.4% of the participants, and 13 infants were born prematurely at $\leq$34 weeks. Pets in the household included dogs (24.8% of households) or cats (13.3%) and 3.1% had both types. Asthma was diagnosed in 28 (5.5%) of the entrants and when present, was mild-intermittent in 19, mild-persistent in 8 and moderate-persistent in 1. By parental report, 15.2% of the infants had experienced bronchiolitis. The sites were geographically diverse and distributions of race, ethnicity, household income, parental education, atopic dermatitis severity and pets in the home, but not gender, differed significantly ($p<.001$) by site. For some variables, the range of site-specific characteristics was substantial; for example, the Caucasian race rate ranged from 57.5% to 86.0%, incomes over $100,000 from 13.8% to 60.8% and pets in the home from 21.5% to 50.5%. **Table E1** shows the categories of allergy as described above. **Table E2** shows sensitization characteristics.

Additional Results and Quality Control Standards for PCR

Table E3 shows the mean delta Ct and percent of undetectable stimulations. Overall 96.8% of the PCR passed laboratory quality standards while 12.5% of the assays failed to detect the targeted gene (5% for anti-CD3/-CD28, 22% for medium). The 5 clinic sites demonstrated the ability to consistently prepare samples with quality success rates ranging from 95.3 to 97.4 % and non-detection rates ranging from 10.7- to 16.1%. As expected, CD25 up-regulation following positive control (anti-CD3/-CD28) and tetanus toxoid was readily detectable by PCR. Transcriptional changes were also evident upon allergen stimulation for several target genes in comparison to medium alone. To show quality control, **Figure E2** displays a high correlation of housekeeping genes and **Figure E3** shows typical consistent results across study sites for a representative stimulation (peanut stimulated IL4 gene).

References

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(2) Sampson HA, Scanlon SM. Natural history of food hypersensitivity in children with atopic dermatitis. J Pediatr 1989; 115:23-7.

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(9) Maloney JM, Rudengren M, Ahlstedt S, Bock SA, Sampson HA. The use of serum-specific IgE measurements for the diagnosis of peanut, tree nut, and seed allergy. J Allergy Clin Immunol 2008; 122(1):145-51.

Table E1. Demographic characteristics of the study participants (n=512 unless otherwise noted).

	N	%
Male	345	67.4
Age (months)at enrollment		
3-5	65	12.7
6-8	128	25.0
9-11	182	35.5
12-14	137	26.8
Race		
Caucasian	378	73.8
Black/African-American	79	15.4
Asian	40	7.8
Other	15	2.9
Ethnicity		
Hispanic or Latino	36	7.0
Household income		
$0-$49,999	86	16.8
$50,000-$99,999	135	26.4
>$100,000	215	42.0
Caeserian section Delivery	178	34.8
Breastfed		
Never	73	14.3
Yes	439	85.7
Atopic Dermatitis severity		
None	40	7.8
Mild	51	10.0
Moderate	258	50.4
Severe	163	31.8

Table E2. Sensitization rates (n = 503).

Sensitization parameter	**Milk**	**Egg**	**Peanut**
Positive PST (%)	68.7	87.1	53.7
IgE $\geq$ 0.35 to 2 kU_A/L (%)	22.1	23.7	20.9
IgE > 2 to 5 kU_A/L (%)	12.7	16.1	11.9
IgE > 5 kU_A/L (%)	26.6	34.6	27.8
Positive sensitization by positive PST and/or IgE $\geq$ 0.35 kU_A/L (%)	77.7	88.7	68.8
Mean IgE (kU_A/L)	9.1	10.4	9.8
25^{th} Percentile IgE (kU_A/L)	.1	.3	.05
Median IgE (kU_A/L)	0.9	2.1	0.9
75^{th} Percentile IgE (kU_A/L)	5.5	9.5	5.9

Table E3. Raw data showing Mean Delta Ct by Stimulation and % Undetectable

	Stimulant							
	Peanut		Medium		aCD3/28		Tetanus	
	% Undetectable	Delta CT	% Undetectable	Delta CT	% Undetectable	Delta CT	% Undetectable	Delta CT
Gene								
CD25	5.22	5.91	6.81	7.81	4.02	4.93	2.41	5.41
CISH	11.16	7.58	39.23	11.60	6.29	7.29	9.05	7.12
FOXP3	9.62	7.66	12.66	8.46	6.38	7.52	11.01	7.72
GATA3	2.46	4.66	4.97	5.07	2.33	4.94	2.18	4.37
IL10	35.66	12.09	60.57	14.49	14.03	11.84	39.37	12.40
IL4	36.50	12.48	59.45	14.36	11.48	11.37	20.32	9.99
INFG	21.66	9.17	38.63	12.60	5.93	5.47	4.80	6.10
RPL41	0.00	-1.72	0.00	-1.67	0.00	-1.80	0.00	-1.77
RPS9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TBET	9.43	8.57	12.87	10.32	2.34	6.48	7.41	8.03

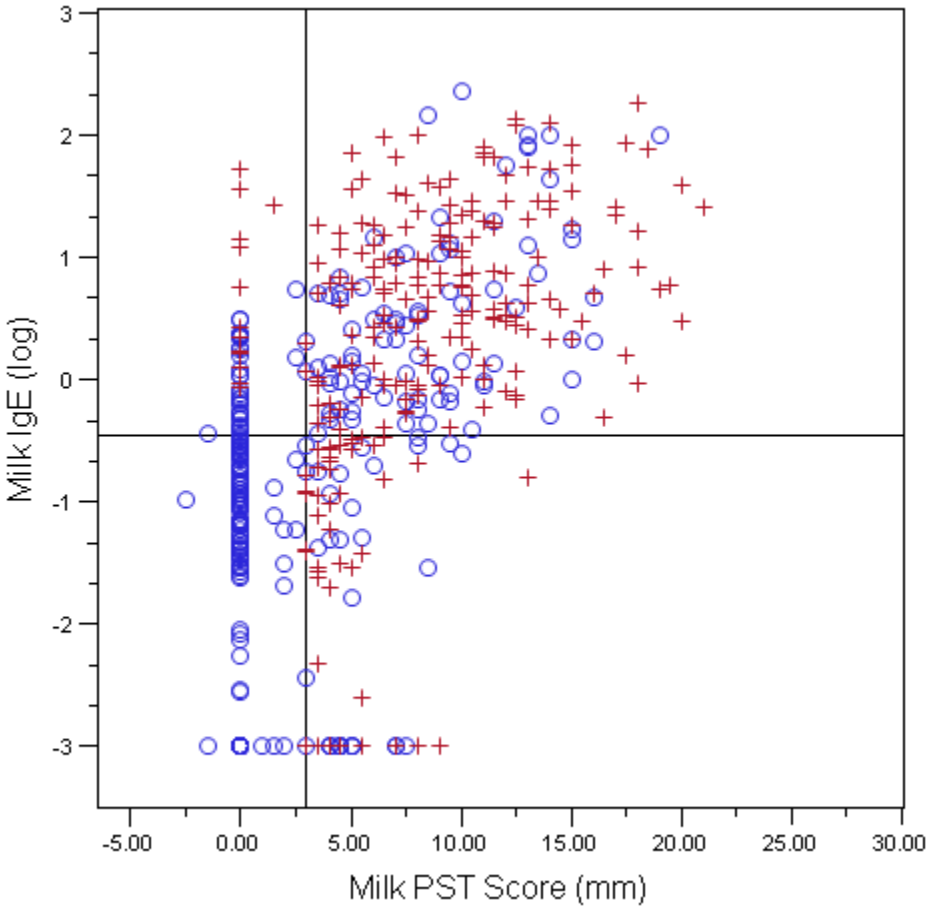
	Stimulant				All	
	Caseins		Egg White			
	% Undetectable	Delta CT	% Undetectable	Delta CT	% Undetectable	Delta CT
Gene						
CD25	6.57	5.57	4.77	5.89	4.96	5.93
CISH	10.60	7.11	15.44	7.78	15.23	8.08
FOXP3	11.94	7.03	12.29	7.59	10.60	7.68
GATA3	2.33	4.07	1.65	4.27	2.69	4.58
IL10	29.19	10.53	53.14	13.33	38.25	12.45
IL4	35.59	11.60	44.67	12.61	34.37	12.06
INFG	17.65	8.04	11.99	8.08	16.87	8.25
RPL41	0.00	-1.85	0.00	-1.80	0.00	-1.77
RPS9	0.00	0.00	0.00	0.00	0.00	0.00
TBET	18.81	10.14	9.57	9.00	9.93	8.73

Figure Legends.

Figure E1a-c. Relationship of skin test wheal sizes (mm) to serum IgE antibody levels (kU_A/L) for milk (1a), egg (1b) and peanut (1c) shown on a logarithmic scale. Spearman correlation coefficients are 0.64 for milk, 0.65 for egg and peanut. "+" symbols refer to those with confirmed/convincing milk/egg allergy; Spearman correlation coefficients are 0.47 for milk and 0.48 for egg, $p < 0.001$ for this subgroup. PSTs were recorded as negative if the saline control wheal was larger than the food test wheal.

Figure E2. Reproducibility of replicate PCR: Correlation of two housekeeping genes threshold cycle number from 2,708 PCR assays, using 5 stimulation conditions. Spearman correlation coefficient = 0.84.

Figure E3. Reproducibility across study sites: The 5 participating sites (DC-National Jewish Health, Denver, CO, DU-Duke, Durham, NC, JH-Johns Hopkins, Baltimore, MD, MS-Mount Sinai, NY, NY and UA- University of Arkansas, Little Rock, AR) prepared stimulated lymphocytes that were shipped to a central laboratory for qPCR analysis. The site specific results for the peanut stimulated IL4 gene Delta-Delta Ct are shown (N ranges from 77-91 per site; there was no significant difference between sites for the detection of IL4, p=0.32).


Milk IgE (log)
3
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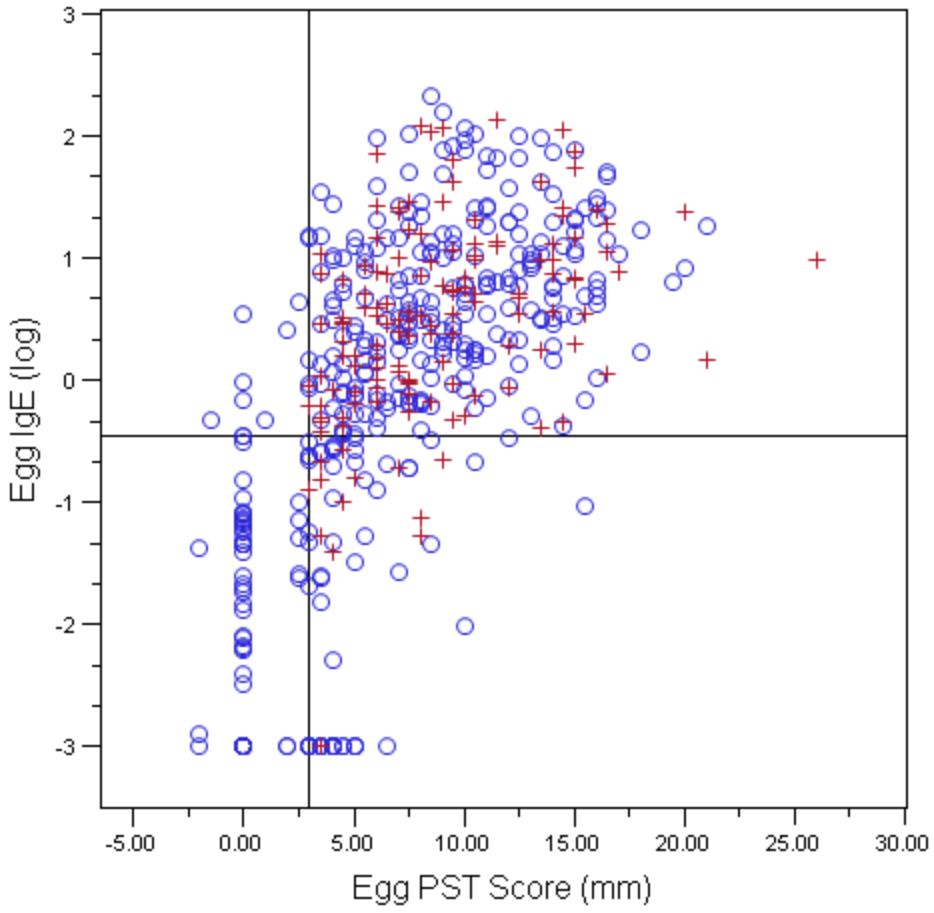
Milk PST Score (mm)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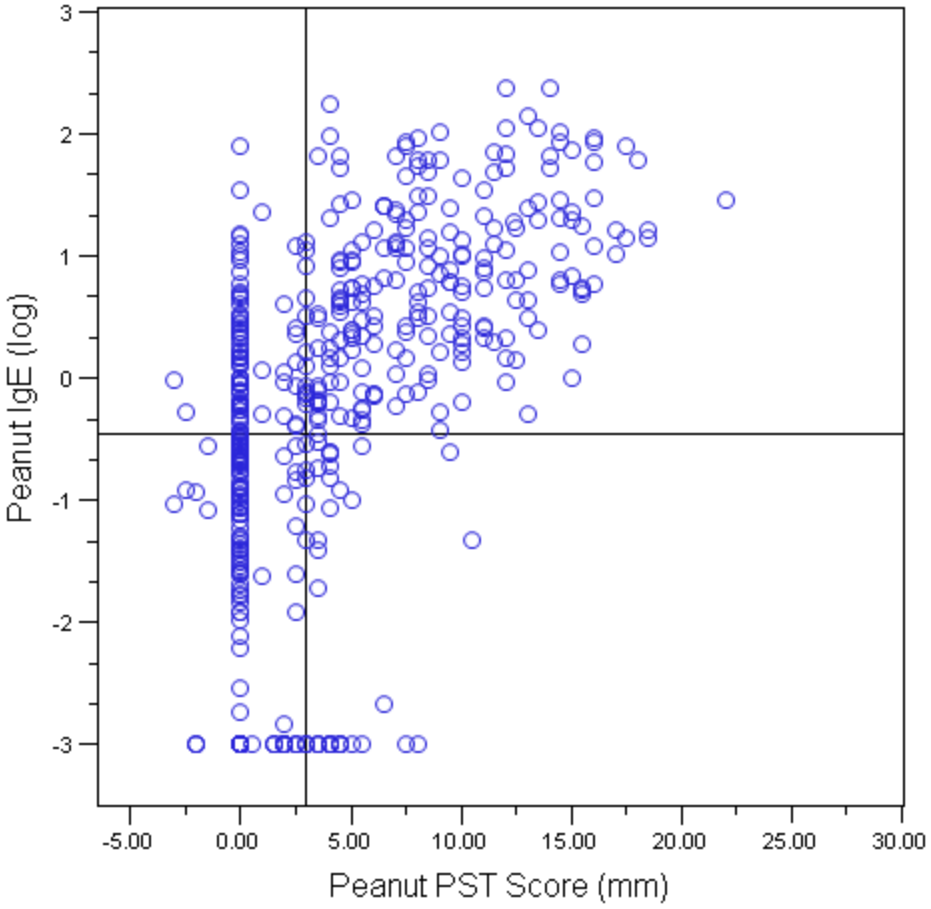
Egg PST Score (mm)


Peanut IgE (log)
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Peanut PST Score (mm)

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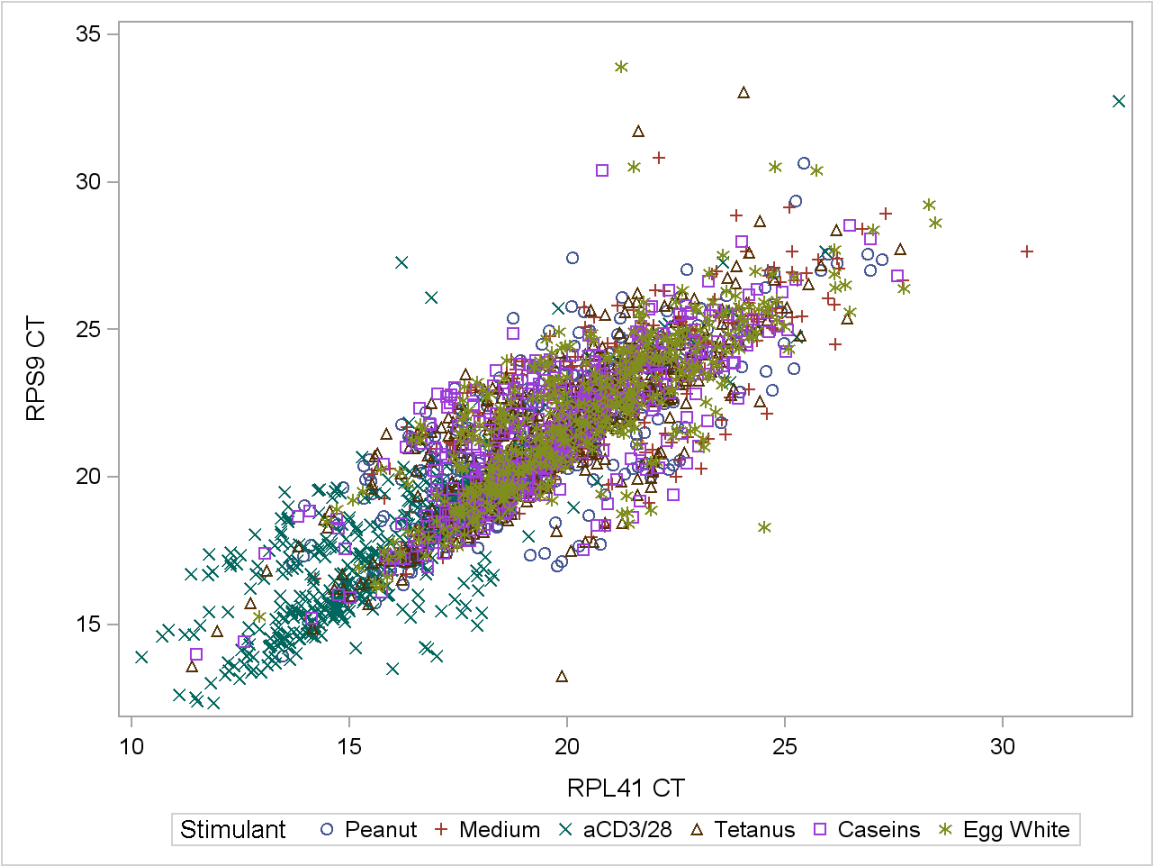

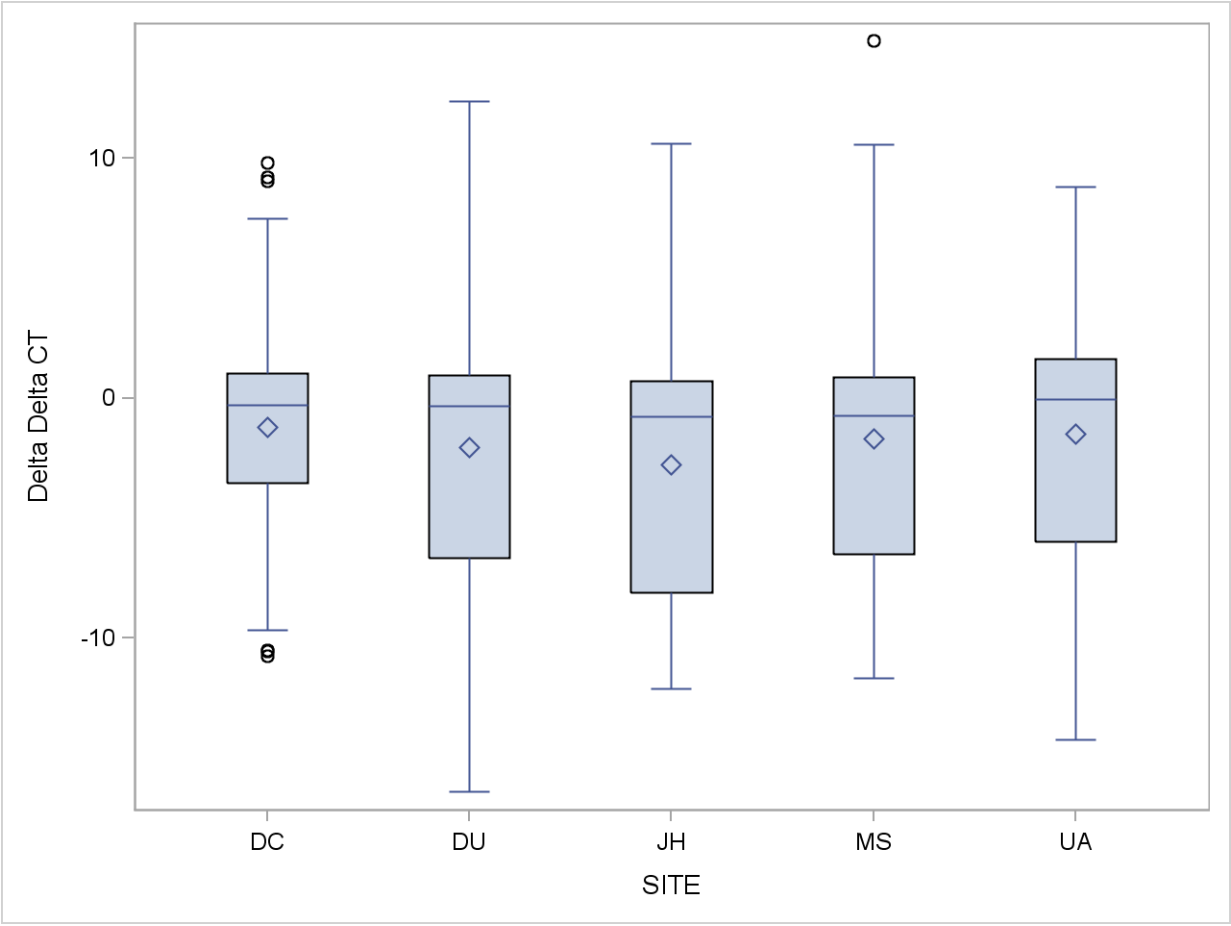
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