RISK FACTORS FOR PEANUT SENSITIZATION AND ALLERGY
NOVEL DISEASE AND GENE-ENVIRONMENT INTERACTIONS AND BIOMARKERS OF DISEASE

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RISK FACTORS FOR PEANUT SENSITIZATION AND ALLERGY: NOVEL DISEASE AND GENE-ENVIRONMENT INTERACTIONS AND BIOMARKERS OF DISEASE

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Background: Peanut allergy (PA) is responsible for life-threatening allergic reactions. Household peanut consumption (HPC), used as an indirect measure of environmental peanut exposure (EPE), is associated with PA especially when compared against atopic controls.

Aims: To determine the association between EPE, peanut sensitization (PS), PA and explore the modifying effect of an impaired skin barrier. To assess the route of PS using peanut specific immune responses in skin versus gut derived T-helper (Th) cells.

Methods: Peanut antigen in dust was assessed using ELISA, Mass Spectrometry (MS) and basophil activation test (BAT). HPC was compared to peanut-dust levels and airborne peanut. The impact of EPE on PS was determined in three cohorts with genotypic and phenotypic skin barrier function measures. Recall responses to peanut in skin versus gut-homing memory Th-cells were assessed using gene expression profiles and intracellular cytokine staining.

Results: HPC was the most important factor for peanut-dust in the infants' environment. BAT confirmed biological activity of peanut in dust; MS confirmed whole sequences of major peanut allergens. Airborne peanut was only transiently above peanuts being shelled. Early EPE increased the risk of PS; this was augmented by atopy and markers of skin barrier impairment. Th2 gene expression was not increased in skin versus gut-homing memory CD4+Th cells from peanut allergic children. *IL9* was the most accurate classifier for PA versus PS and atopic non-peanut allergic (NA) children.

Conclusions: EPE is a risk factor for PS in atopic children, especially when skin barrier is impaired; this supports the concept of epicutaneous peanut sensitization. Peanut is unlikely to be sufficiently airborne to induce inhalational sensitization. Although, there was no differential expression of Th2 cytokines in skin versus gut-homing Th cells, longitudinal assessment as children progress from PS to PA may show Th2 cytokines initiate in skin-homing Th cells then spread to gut-homing Th cells. IL9 may be a useful biomarker for peanut allergy. The role of IL9 in mast cell activation, trafficking and proliferation also

provides a compelling explanation for the immunobiology underlying epicutaneous sensitization and elicitation of allergic reactions.

Peer reviewed publications arising from this PhD

1. Brough H.A., Makinson K, Penagos M, Maleki S, Cheng H, Stephens AC, Turcanu V, Lack G. Distribution of peanut protein in the home environment. J Allergy Clin Immunol 2013;132(3):623-9

2. Brough H.A., Santos A, Makinson K, Penagos M, Stephens AC, Turcanu V, Lack G. Peanut protein in household dust is related to household consumption and is biologically active. J Allergy Clin Immunol 2013; 132(3):630-8

3. Brough H.A., Simpson A., Makinson K., Hankinson J., Brown S., Douiri A., Belgrave D.C.M., Penagos M., Srephens A.C. Mclean W.H.I., Turcanu V. Nicolaou N., Custovic A., Lack G. Peanut allergy: Impact of environmental peanut exposure in children with a filaggrin loss-of-function mutation. J Allergy Clin Immunol. 2014;134:867-75

4. Brough H.A., Liu A., Sicherer S., Makinson K., Douiri A., Brown S.J., Stephens A.C., McLean W.H.I. Turcanu V., Wood R., Jones S.M., Burks W., Dawson P., Stablein D., Sampson H., Lack G. Atopic dermatitis increases the impact of exposure to peanut antigen in dust on peanut sensitization and allergy. J Allergy Clin Immunol. 2015;135(1):164-170.

5. Brough H.A., Cousins D.J., Muntaenu A., Wong Y.F., Sudra A, Makinson K., Stephens A.C., Arno M., Ciortuz L., Lack G., Turacnu V. IL9 is a key component of memory Th cell peanut-specific responses from peanut allergic children. J Allergy Clin Immunol. 2014;134(6):1329-1338

Abstracts arising from this PhD

1. Brough H.A., Penagos M, Stephens AC, Turcanu VT, Lack G. Household consumption of peanut over 6 months is positively correlated with measurable peanut protein in an infant's home environment. Allergy 2010; 65(s92): 31-32

2. Brough H.A., Makinson K, Penagos M, Stephens AC, Turcanu V, Lack G. The aerosolizeability of peanut protein. Allergy 2012; 67 (s96): 608

3. Brough H.A., Simpson A., Makinson K., Jenny H. Sara B., Douiri A., Belgrave D., Penagos M., Stephens AC, Mclean I, Turcanu V, Nicolaou N, Custovic A, Lack G. Filaggrin loss-of-function mutations increase the impact of early environmental peanut exposure on peanut sensitization. Allergy 2013; 68 (s97):108

4. Brough H.A., Simpson A., Makinson K., Jenny H., Sara B., Douiri A., Belgrave D., Penagos M., Stephens A.C., Mclean I., Turcanu V., Nicolaou N., Custovic A., Lack G. Early exposure to peanut protein in household dust increases the risk of peanut sensitization in children carrying a filaggrin loss-of-function mutation. Clin Exp Allergy 2013; 43 (12): 1464-1465

5. Brough HA, Liu A, Sicherer S, Makinson K, Douiri A, Brown S, Stephens AC, McLean WHI, Turcanu V, Wood R, Jones, SM, Burks W, Dawson P, Stablein D, Sampson H, Lack G. The impact of atopic dermatitis on exposure to peanut antigen in dust on peanut sensitization and likely peanut allergy. Allergy 2014; 69(s99):579

Awards arising from this PhD

1. **Barry Kay Award** 07/2013

British Society of Allergy and Clinical Immunology (BSACI) Annual Meeting

2. **Prize for Outstanding Abstract Presentation** 06/2013

European Academy of Allergology and Clinical Immunology (EAACI) and World Allergy Organization (WAO) Annual Congress

3. **Postgraduate show-case award, runner up** 03/2012

King's College London

4. **Travel Fellowship** 07/2012

British Society of Allergy and Clinical Immunology Annual Meeting

5. **International Fellow in Training Travel Grant** 03/2011

American Academy of Allergy, Asthma, and Immunology (AAAAI) Annual Meeting

6. **Junior Member Association (JMA) poster award** 06/2010

BSACI and European Academy of Allergology and Clinical Immunology (EAACI) Annual Congress

PhD hypothesis, aims and structure

The overriding hypothesis of this PhD is that early EPE, measured by peanut protein levels in household dust, is a risk factor for the development of PS and PA in children with an impaired skin barrier. The aims of this PhD were to describe characteristics of environmental peanut exposure, the association between this and PS and PA in children with an impaired skin barrier and finally in-vitro work to support the concept that PS occurs through the skin using recall responses to peanut from memory Th2 cells. This PhD thesis is a thesis incorporating publications, therefore the results section comprises accepted publications instead of conventional results chapters (where the results of this PhD have been published).

The structure of this PhD thesis comprises an Introduction section separated into several chapters (Chapters 1-5) to evaluate the literature surrounding the hypothesis of this PhD. The Methods section (Chapter 6) summarizes the methods incorporated into the publications and describes in full the methods for the work that has not yet been published. The Results sections (Chapter 7) contains articles accepted for publication (Publication 1-5 described in the section on peer reviewed publications arising from this PhD); where the work has not yet been published, these results are described in the conventional way. This is followed by a Discussion section (Chapter 8), References (Chapter 9) and Appendices (Chapter 10) which comprise further details required for background, optimisation and justification of methods and statistics employed as well as standard operating procedures for certain methods, participant questionnaires and information sheets used.

In keeping with the aims of this PhD there are three themes to this thesis as described below:

Characterization of environmental peanut exposure (EPE):

a. Validation of environmental sampling methods and peanut protein quantification
b. Household peanut consumption and its relationship with peanut levels in dust
c. Household characteristic and participant factors related to peanut levels in household dust

d. Persistence of peanut protein on human hands and saliva after peanut consumption
e. Persistence of peanut protein in household bed and furnishings
f. Analysis of airborne peanut
g. Basophil activation test to confirm biological activity of peanut in dust
h. Mass spectrometry to determine major peanut protein allergens in dust

2) Influence of EPE on PS and PA in children with an impaired skin barrier

a. Comparison of cohort studies employed (MAAS, CoFAR and BAMSE)
b. Review of potential confounding factors for influence of EPE on PS and PA
c. Overview of *FLG* genotyping in three cohort studies
d. Statistical powering and analysis for influence of EPE on PS and PA in children with an impaired skin barrier
e. Evaluation of threshold level of EPE for PS and PA

3) In-vitro evaluation of the route of PA using memory Th cell responses to peanut in skin versus gut-homing Th subsets

a. PBMC isolation, culture, staining and sorting into peanut specific memory Th cell in skin versus gut-homing Th subsets in children with PA and NA children
b. Gene expression of sorted cells following RNA extraction, cDNA synthesis, amplification, biotin labelling and fragmentation for Affymetrix gene microarray
c. Analysis of gene microarray results using Partek Suite ANOVA analysis and Random Forest automated classification approach
d. Confirmation of gene microarray findings by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) in PBMCs from children with PA, PS and NA children
e. Confirmation of gene microarray findings on a protein level by intracellular cytokine staining in PBMCs of children with PA versus PS

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Glossary of terms and abbreviations

AAAAI	American Academy of Allergy, Asthma, and Immunology
Allergy	Immune response to a usually innocuous protein in individuals sensitized to that protein which results in an adverse reaction
Allele	One member of a pair or series of alternative genes occupying the same position on matching chromosomes.
Allergen	Substance capable of inducing an allergic immune response
α4β7+ / β7+	Alpha-4, beta-7 Integrin surface gut-homing marker on T cells
Angioedema	Swelling due to influx of fluid subcutaneously or in the submucosa
Anaphylaxis	Severe form of an allergic reaction which may cause respiratory or cardiovascular compromise
Antibody	An immunoglobulin protein produced by B-cells that binds specifically to an antigen
APC	Antigen presenting cell
Ara h 1	Arachis hypogaea 1 is a major peanut allergen, which belongs to the vicilin family of seed storage proteins
Ara h 2	Arachis hypogaea 2 is a major peanut allergen, which belongs to the conglutin family of seed storage proteins
Basophil	Lymphocyte usually circulating in the blood, which has granules containing inflammatory mediators such as histamine
BAMSE	'Barn' (children) Allergy Milieu (environment) Stockholm Epidemiology study recruited 4089 unselected newborn children from 1994-1996

mMCP-1	Mouse mast cell protease is indicative of mucosal mast cell degranulation and increases in mice undergoing systemic anaphylaxis
β-lactoglobulin	Beta-lactoglobulin: A major cow's milk allergen
Biorad Experion chip	Automated quantitation of protein using gel electrophoresis
Biorad Protein assay	A dye-binding protein quantitation assay; a differential colour change of a dye occurs in response to various concentrations of soluble protein
Bla g 1	Blattella germanica Allergen 1: A major cockroach allergen
bp	base pair is a pair of complementary nitrogenous molecules that are the building blocks of the DNA double helix.
Brefeldin A	Redistributes intracellularly produced proteins from the cis/medial Golgi complex to the endoplasmic reticulum
Bronchoalveolar lavage	Bronchoscope washes lungs with fluid in order to take samples for microscopy, culture or histology
BSACI	British Society of Allergy and Clinical Immunology
CD	Cluster of differentiation molecules are cell surface markers useful for the identification and characterization of white blood cells
CD4	CD4 is a glycoprotein present on the surface of various immune cells such as T helper cells, and antigen presenting cells such as dendritic cells and macrophages. T cells that express CD4 are called T helper cells.
CD8	CD8 is a transmembrane glycoprotein present on the surface of circulating T cells and serves as a co-receptor for the T cell receptor. T cells that express CD8 are called cytotoxic T cells.

CD14	CD14 is a glycoprotein expressed in the cell membrane of monocytes and macrophages. It is an important component of the innate immune system as it is a co-receptor for the detection of bacterial lipopolysaccharide protein
CD63	CD63 is a cell surface marker which is expressed after cross-linking of the high affinity IgE receptor on basophils and mast cells
CD203c	CD203c is a transmembrane glycoprotein in basophils and mast cells
CLA	Cutaneous Lymphocyte Antigen is an adhesion molecule that binds to E-selectin in skin capillaries, ensuring the preferential homing of CLA+ T cells to the skin
CoFAR study	Part of the NIH-sponsored Consortium of Food Allergy Research; 512 children aged 3-15 months with a history of eczema and milk or egg allergy, but without known PA were recruited from 2006
COT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
CRD	Component resolved diagnostics: component allergen analysis of major allergenic proteins
CT	Cholera toxin is a common adjuvant used to sensitize mice to an allergen
Ct	Cycle threshold is the number of cycles required for the fluorescent signal to cross the threshold in polymerase chain reactions
CV	Coefficient of variation is used to define variability: It is the standard deviation of a group of values divided by their mean
Da	Dalton: standard unit used to indicate the atomic mass
DACT1	Dapper antagonist of β catenin homolog 1: Inhibits dishevelled activation of JNK (c-Jun N-terminal kinase) and β catenin signalling pathways
DBPCFC	Double-blind placebo-controlled food challenge

Deionised	Water free from ions (molecule with net positive or negative charge)
Densitometry	Assay which uses optical density to measure substances
Der p 1	Dermatophagoides pteronyssinus Type 1: A major HDM allergen
Der p 2	Dermatophagoides pteronyssinus Type 2: A major HDM allergen
DNA	Deoxyribonucleic acid is a molecule made of repeating units called nucleotides that encode the genetic instructions for the functioning of cells and organisms
DNase	Deoxyribonuclease is an enzyme that degrades DNA by catalysing the cleavage of phosphodiester linkages in the DNA backbone
EAT	Enquiring About Tolerance study is a large population based randomised controlled study assessing whether early introduction of solids with concomitant breastfeeding protects against food allergy at 3 years of age
EAACI	European Academy of Allergy and Immunology
ELISA	Enzyme linked immunosorbent assay: antibody based assay used in the detection of proteins
Eppendorf	Generalised trademark of laboratory tubes which may be centrifuged
Epicutaneous	On the skin
EDC	Epidermal differentiation complex: a cluster of genes involved in the terminal differentiation of keratinocytes.
Eosinophilia	Increase in the number of eosinophils; a lymphocyte which predominates in allergy, parasitaemia and some forms of leukaemia
EPE	Environmental peanut exposure

EPIT	Epicutaneous immunotherapy
Epitope	Part of a protein recognised by the immune system
FACS	Flow-cytometry
FAM	6-carboxyfluorescein reporter fluorescent marker in Taqman PCR probes
FcεRI	High-affinity receptor for the Fc region of Immunoglobulin E which is found on mast cells and basophils
Fel d 1	Felis domesticus allergen 1: A major cat allergen
Fluff	Unsieved larger particles in dust such as hair and cloth fibres.
FFQ	Food frequency questionnaire
Filaggrin	A protein which forms a dense protein lipid matrix in the skin. It is important for maintaining the skin barrier and mutations of the gene that codes for profilaggrin (the precursor of filaggrin) are associated with eczema.
FLG	Gene that codes for the protein profilaggrin (the precursor of filaggrin) within the epidermal differentiation complex
FTMS	Fourier transform mass spectrometry
g	gravitational force which refers to the acceleration applied to the centrifuge
GALT	Gut-associated-lymphoid tissue
Glycan	A polysaccharide or oligosaccharide
GNG8	Guanine nucleotide-binding protein (G protein): Modulator /transducer in various transmembrane signalling systems
Haplotype	Groups of closely linked alleles that tend to be inherited together

HDM	HDM – small arachnids that feed off shed human skin cells and are usually found in soft furnishing and bedding of homes
HLA	Human leucocyte antigen is part of the human Major Histocompatibility Complex (MHC) which helps the body differentiate between self and non-self.
HUWE1	HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase
Horse-radish peroxidase	Enzyme which is used to amplify signals in many immunoassays
HPLC	High performance liquid chromatography: Separation of components in a mixture by passing a pressurized liquid and a sample mixture through a column packed with a solid adsorbent material. Each component in the sample mixture interacts differently with the adsorbent material thus leading to differential flow and elution off the column
HSPA5	Heat shock 70kDa protein 5 encodes a protein that is localized in the lumen of the endoplasmic reticulum and is involved in the folding and assembly of proteins
IgG	Immunoglobulin G: One of the five major classes of immunoglobulin and part of the adaptive immune system
IgE	Immunoglobulin E: One of the five major classes of immunoglobulin responsible for allergy and in protecting against parasites
IIS	Infant Immune Study: Birth cohort of 400 children from Tucson, Arizona, US in whom 50% are high-risk for atopy as their mothers have allergic rhinitis
IL	Interleukin: Cytokines (signalling molecules) which are produced predominantly by activated CD4+ Th cells, but also B cells, mast cells, macrophages and endothelial cells
IL	Gene encoding interleukin (e.g. *IL9* is the gene encoding IL9)
Immunoblotting	Western blotting for detection of proteins

Incidence	Number of new cases of a disease that occur over a particular time period in a defined population
IOM	Inhalable occupational medicine: type of sampling head used for the Casella Tuff Personal Air Monitor
ISAAC	International Study of Asthma and Allergies in Childhood
iTregs	Inducible T regulatory cells are cells that acquire a regulatory function after coming out of the thymus. These include: T regulatory 1 cells, which secrete IL-10; T helper 3 cells, which secrete TGF-β, and Foxp3+ Treg cells.
JACI	Journal of Allergy and Clinical Immunology
kDa	kilo Dalton: the molecular mass of 1000 Dalton which is the standard unit used to indicate the atomic mass
L	Litres
LC	Liquid chromatography
LEAP	Learning Early About Peanut Study is a high-risk randomised controlled study assessing whether early introduction of peanuts in the diet protects against peanut allergy at 5 years of age
LLQ	Lower limit of quantitation
LN	Lymph node
LR	Logistic regression: statistical analysis to determine associations between exposure and outcome variables
LTA	Lymphotoxin A
MAAS	Manchester Asthma and Allergy Study: Birth cohort study of 1184 children from the UK from 1995-1997
Mast cells	Cells which reside in tissues and are characterized by granules which contain inflammatory mediators such as histamine

MS	Mass spectrometry: analytical tool involving the ionisation of molecules and their subsequent separation and detection based on their mass (m)- to-charge (z) ratios (m/z).
Matrix effect	Substances inside a sample other than the substance one is measuring which may interfere with the analysis
MAF	Minor allele frequency is the frequency of the least common allele in a population
Methacholine	Parasympathomimetic drug that induces increased secretions and smooth muscle activity in the lung; it is used as a test for asthma
µl or mcl	Microlitre (1000^{th} of a millilitre)
µg or mcg	Microgram (1000^{th} of a milligram)
µm	Micrometres (1000^{th} of a millimetre)
MGB	Minor Groove Binder: Taqman MGB probes contains a non-fluorescent quencher (NFQ) to absorb energy (light) emitted from the reporter dye during real-time quantitative polymerase chain reactions
MHC	Major Histocompatibility Complex allows differentiation of self versus non-self and is called the Human Leucocyte Antigen (HLA) in humans.
MMCP-1	Mouse mast cell protease 1 is a member of the chymase family of serine proteases and is a cardinal feature of IgE mediated anaphylaxis in mice
Monensin	Prevents protein secretion by blocking transport within the Golgi stack; the Golgi stack modifies proteins delivered from the endoplasmic reticulum then packages and sends them to different parts of the cells
Monoclonal antibody	Antibodies produced by the same parent cell (cloned) which are directed against one epitope

m/z	m stands for mass and z stands for number of charged ions; this provides a mass to charge ratio for ionized peptides which can be used to identify proteins of interest
N	Normality means the number of equivalent weights of a solute per litre of a solution
NA	Non-peanut allergic
n/a	Not applicable
NDFIP2	Nedd4 family interacting protein 2: Promotes NF-κB signalling
ng	Nanogram (1000^{th} of a μg)
NICE	National Institute for Health and Care Excellence
NMF	natural moisturizing factor is a collection of water soluble molecules in the cornified layer formed predominantly from breakdown products of filaggrin
NPV	Negative predictive value
OD	Optical density is the absorbance of light at a particular wavelength; in this case through the ELISA well sample
OFC	Oral food challenge
OVA	Ovalbumin: A major hen's egg allergen
P	P-value
PA	Peanut allergy
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline (without calcium or magnesium)
PCA	Principal Component Analysis shows the variation in all the genes expressed in a gene microarray analysis in one 3-dimensional image

ppm	Parts per million. 1 ppm = 1mg/litre or 1µg/ml or 1ng/µl
Polyclonal antibodies	Mix of different antibodies produced by different B-cells which are directed towards different epitopes of a protein
PPV	Positive predictive value
Prevalence	Total number of cases of a disease at a given time-point in a defined population
PS	Peanut sensitization
Random Forest	Automatic classification approach
RAST	Radio-allergosorbent test is a radioimmunoassay to test for specific IgE
RF	Random Forest is a supervised classification algorithm
ROC	Receiver Operating Characteristic curve is a graphical representation of the predictive accuracy of a model or diagnostic tool, combining sensitivity and 1-specificity to determine the area under the curve at all possible cut-offs
ROX	ROX is a dye within the PCR Mastermix which is used to normalise the experiment
RNA	Ribonucleic acid: a family of biological molecules essential for coding and expression of genes
RNase	Ribonuclease is an enzyme which catalyses the degradation of RNA and is ubiquitously present in the environment and is secreted by human skin cells
RP-HPLC-ESI-MS	Reverse phase high performance liquid chromatography electrospray ionisation mass spectrometry
RPMI	Roswell Park Memorial Institute medium
RQ	Relative quantitation in real-time quantitative polymerase chain reaction relates the PCR signal for a target gene against another sample such as a negative control or comparator group

r_s	Spearman Rank: non-parametric correlation coefficient for non-normally distributed data
RT-qPCR	Real time quantitative polymerase chain reaction
SCIT	Subcutaneous immunotherapy
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis is used to separate out proteins according to their molecular size and weight. SDS denatures proteins into polypeptides
Sensitization	Immune system produces IgE antibodies against a usually innocuous antigen such as a pollen or food
Sensitivity	Number of true positive results divided by the combination of true positive and false negative results
siRNA	Small interfering RNAs are 20-25 nucleotide double stranded RNA molecules that interfere with the expression of a specific gene
SPT	Skin prick test: Allergy tests where purified standardized allergen is applied to the skin and pierced with a lancet; wheal and flare are measured at 15 minutes
SNP	Single nucleotide polymorphisms occur when a single base differs between members of paired chromosomes in an individual
SOP	Standard operating procedure: a consistent set of instructions for research work
Specificity	Number of true negative results divided by the combination of true negative and false positive results
Spectrophotometry	Assay which measures the colour of a solution by determining the amount of light absorbed in the ultraviolet, infrared, or visible spectrum
SPRRs	Small proline-rich proteins assist in the formation of the cornified envelope of the epidermis
STAT6	Signal transducer and activator of transcription 6

Stratum corneum	Outermost layer of the skin (epidermis)
ST6GALNAC1	ST6 (alpha-N-acetyl-neuraminyl-2, 3-beta-galactosyl-1, 3)-N-acetylgalactosaminide alpha-2, 6-sialyltransferase 1
TCR	T cell receptor
TGF-β	Transforming growth factor beta family of cytokines are peptides which regulate cellular differentiation, proliferation, adhesion and migration
Tgif1	TGFβ-induced factor homeobox 1
Th	T-helper (Th) cells are a sub-group of lymphocytes which express CD4 on their cell surface and help B-cells to produce antibodies.
Th1	T-helper cell Type 1: Produce cytokines which are important in defence against pathogens such as IFN-γ and IL-2
Th2	T-helper cell Type 2: Produce cytokines such as IL4, IL5 and have an important role in allergy and defence against parasites
TLR	Toll-like receptor: Class of proteins which reside on APCs in order to recognise microbial pathogen associated molecular patterns (PAMPs) and activate the innate immune system
TMB	Tetramethylbenzene; a substrate broken down by enzyme horse-radish peroxidase which gives rise to colour change measured by spectrophotometer
Tris	Tris (hydroxymethyl) aminomethane is a primary amine (one of three hydrogen atoms of ammonia is replaced by a hydrocarbon group). It is commonly used as a buffer
Trypsin	A serine protease produced by the pancreas which hydrolyses (breaks down) proteins
Tween	Common detergent used in biochemistry
UK	United Kingdom
Urticaria	Itchy red rash resulting from degranulation of mast cell inflammatory mediators such as histamine; also called ‘nettle rash’ or ‘hives’

US	United States of America
VIC	Reporter fluorescent marker in Taqman PCR probes. Proprietary marker thus no further details available through Applied Biosystems
w/v	weight /volume; to prepare solid in liquid solvent; 1% w/v is 1gram per 100 ml, 100% is 100grams in 100 ml

Chapter 1 Introduction

1.1 Statement of the problem

Food allergy is an increasingly important public health concern. [1] In prospective studies challenge proven food allergy has been shown to be between 5% and 6% (depending on whether defined by double blind placebo controlled food challenges or open challenge and a good clinical history respectively) in the United Kingdom (UK) [2] and 8% [3] in the United States of America (US) in the first three years of life. PA is characterised by severe reactions [4;5] and significantly impairs quality of life for both the child and their family. [6-8] Although there are exciting research developments in the prevention (www.leapstudy.co.uk) and treatment of established PA,[9-11] at present dietary avoidance and appropriate rescue treatment of allergic reactions secondary to accidental exposure remains the mainstay of management; thus prevention is key.

In this PhD an epidemiological approach was employed to determine a novel, previously unexplored, route of PS via EPE and the role of the skin barrier in mediating this effect. Subsequently genetic biomarkers of PA versus tolerance were evaluated in recall responses of skin versus gut-homing memory T helper (Th) cells in peanut allergic, peanut sensitized and non-peanut allergic (NA) children using gene and protein expression analyses. To highlight the clinical relevance of this PhD the introduction first provides an overview of PA followed by a review of known genetic and environmental risk factors for PS and PA. The evidence surrounding potential routes of sensitization to peanut, in particular epicutaneous sensitization is described as well as the role of environmental allergen exposure in the development of other allergic diseases. Validation of the method to quantify peanut protein in dust and surface wipes was carried as part of my MSc thus I will discuss data from my MSc as part of the introduction to this PhD.

1.2 Increasing prevalence of PA

Sequential cohort studies have demonstrated an increase in the prevalence of PA from 0.5% (1989) to 1% (1996) in the UK [12] and of self-reported PA from 0.4% (1997) to 0.8% (2002) then 1.4% (2008) in the US.

[13;14] Subsequently the Isle of Wight study assessed three cohorts of children (aged 3-4 years old) either born in 1989 (Cohort A), from 1994-1996 (Cohort B) or from 2001-2002 (Cohort C). They showed an initial increase in the prevalence of PA from 0.5% (Cohort A) to 1.4% (Cohort B) then levelling off at 1.2% (Cohort C). [15]However, two UK studies published after 2002 found a rate of PA of 1.8% [16] and 1.85% [17] in school aged children either confirmed by oral food challenge (OFC) or allergy testing above the 95% positive predictive value (PPV) for peanut with peanut skin prick test (SPT) ≥8mm or peanut specific IgE (sIgE) ≥15kU/L.[18] The Australian HealthNuts Study also confirmed a prevalence of 1.9% PA using diagnostic OFCs; however, infants were recruited to this study from baby clinics, thus may have had eczema for which the parents were seeking advice.[19]

The rise in PA prevalence coincides with a general increase in allergic disease. Using objective markers of allergic disease, hospital admission rates for anaphylaxis and food allergy are increasing. In the UK, out of a total of 49,300 admissions for systemic allergic reactions between 1990-2001, which were obtained from hospital discharge codes based on the international classification of diseases (ICD), anaphylaxis rates rose from 6 to 41 per million population and food allergy rates rose from 5 to 28 per million population. [20] No detail as to the responsible food allergen was provided. A similar study in Australia using data from ICD discharge codes found an increase in hospital admissions for food induced anaphylaxis of 350% between 1994-2005.[21] In children between 0-4 years old the increase was due mainly to peanut induced anaphylaxis. Thus one could argue that because PA has become more prevalent and is characterised by more severe allergic reactions that the rise in admissions for anaphylaxis is due, to a certain extent, to the rise in PA prevalence.

1.3 Severity of peanut and tree nut allergic reactions

PA is characterised by severe reactions. [4;5] In registries of food related anaphylactic deaths, there is often no differentiation between peanuts and tree nuts, therefore the following section will include reference to both peanuts and tree nuts. Peanuts and tree nuts are the commonest cause of food related anaphylactic

deaths in the UK and USA in children and adults; [4;22-24] this may be due to the increased prevalence of PA, [14;16;19] the increased allergenicity that may occur through peanut processing (predominantly roasting) [25] and a higher rate of food cross-contamination due to more widespread use of peanuts in processed and restaurant foods.[26]

Several studies have confirmed that peanut and tree nuts are the most common cause of food related anaphylactic reactions treated in hospital emergency departments. [21;27;28] At the Royal Children's Hospital in Australia, a review of all emergency department attendances between 1998-2003 (coding for 'anaphylactic shock caused by adverse food reaction'), found that peanut and tree nuts (predominantly cashew nut) combined accounted for 37/104 (36%) of food-related anaphylaxis. [29] Confirmatory findings were obtained from Australia's National Hospital Morbidity Database, where they found that 23% of food related anaphylactic admissions were due to peanut, and 39% were due to peanut and tree nuts combined.[21] In the US a database from the Health Maintenance Organization in Washington identified cases of anaphylaxis from 229,422 children using IDC codes between 1991 and 1997. [28] Peanut was the most common cause of anaphylaxis, with 10 peanut attributable cases out of 67 confirmed cases of anaphylaxis. A further 7 cases were due to tree nuts and 5 due to unspecified nuts, thus the total percentage contribution for anaphylaxis from peanut and tree nuts was up to a maximum of 22/67 (33%).

In the US PA is the leading cause of food allergy related fatalities, followed by tree nuts. Bock et al. (2001) reported on the cases of deaths as a result of food allergies from a registry kept by members of the American Academy of Allergy, Asthma & Immunology (AAAAI) and the Food Allergy and Anaphylaxis Network (FAAN). Over 6 years (1994 to 1999), there were 32 registered food allergy related deaths between ages 2 to 33 years. [4] In 10 cases there was only a probable identification of the likely food culprit. Peanut was responsible for 20/32 (62.5%) fatalities and peanut and tree nuts combined were responsible for 30/32 (94%) fatalities. Over a subsequent 6 years (2001-2006) Bock et al.(2007) reviewed a further 31 food allergy related deaths aged 5 to 50 years of which 17/31 (55%) were due to peanut and 25/31 (81%) were due to

peanuts or tree nuts.[22] In the second series peanut and tree nuts were accounting for fewer fatalities; with cow's milk being responsible for 4 fatalities and shrimp for 2 fatalities. As this was a voluntary register rather than data capturing all fatalities due to anaphylaxis, this decrease in nut-related fatalities may be due to increased awareness of the potential for other food allergens to cause allergic reactions.

In the UK food allergy related fatalities were sought from the Office of National Statistics which keeps a record of all UK death certificates; since 1993 these have been coded to allow searches for anaphylaxis as a cause of death. Over seven years (1992 to1998) there were 37 food allergy related fatalities due to peanut and 25/37 (68%) were due to peanut and/or tree nuts combined. [24] Age was not specified for each case but they specified that no nut allergic fatalities occurred before 13 years of age. Over the next 7 years (1999-2006), 48 fatalities ranging from 5 months to 85 years were related to food anaphylaxis, of which 9/48 (19%) reacted to peanuts and 18/48 (37.5%) reacted to peanuts and/or tree nuts combined. [23] Death occurred typically from respiratory arrest.[23] The rate of fatalities from food allergy related anaphylaxis has not increased (6-7 per year) in the UK even though the rate of hospital admissions for anaphylaxis has.[20] This may indicate better management of food allergic reactions when these reactions do occur.

In a recent article in press by Turner et al. 2015,[30] fatalities from the same Office of National Statistics were reviewed between 1992-2012, thus comprised the data obtained above and extended this by another 7 years. Of the 124 of fatalities highly likely to be secondary to a food allergen during this period, 95 (77%) were attributed to a known food allergen. Peanut was responsible for the fatality in 19/85 (22%) adults and 6/39 (16%) children. Peanut and /or tree nuts were responsible in 30/85 (35%) adults and 11/39 (28%) children; this calculation included unspecified nuts.

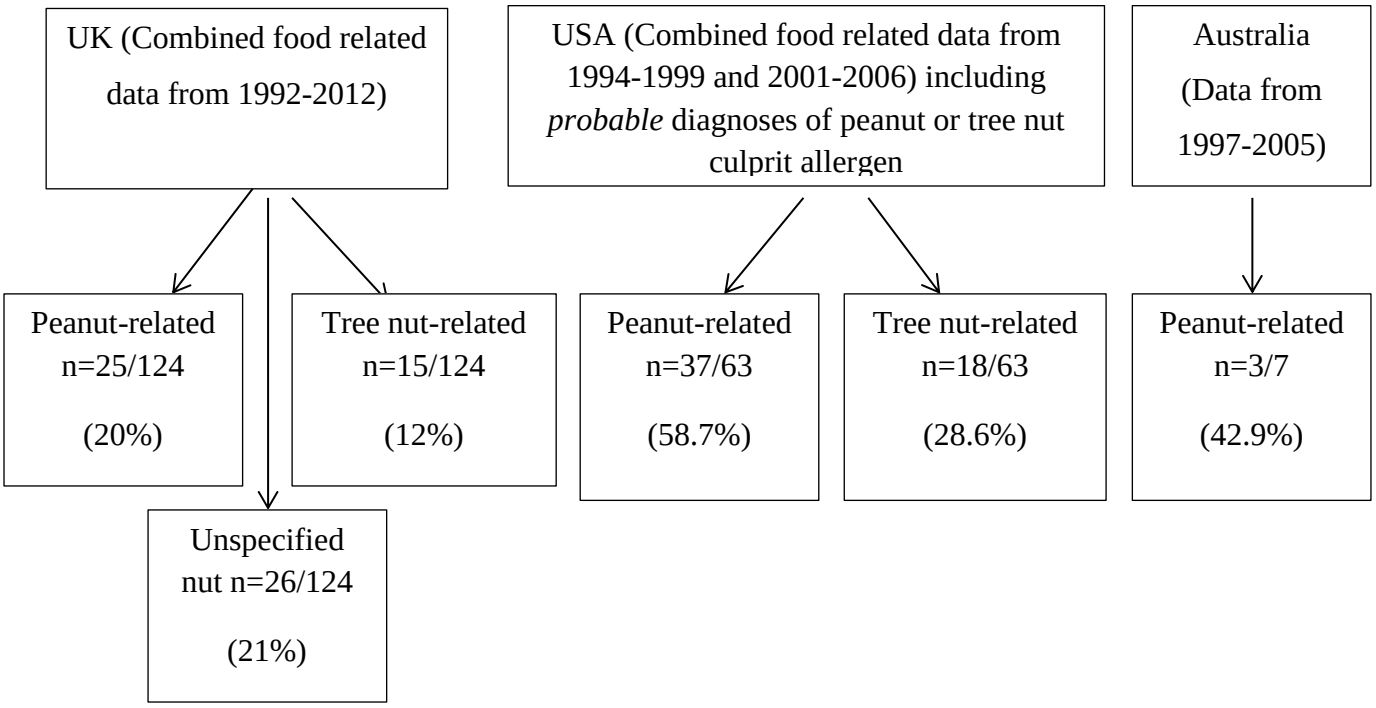
In Australia, over 9 years (1997-2005) there were 112 anaphylaxis fatalities based on death certificates entered into a National Mortality Database. Further details were sparse and a large proportion of

anaphylactic deaths were unspecified. However of the 7 food induced anaphylaxis fatalities (aged 5-35 years), peanut was the offending food in 3 of the 5 cases where data were available.[21]

The percentage of fatalities attributed to peanut or tree nuts was lower in the UK and Australia than the US, however, peanut and tree nuts were still the main culprit foods for food allergy related fatalities in all three countries. There are limitations in the available data that may account for this discrepancy. Firstly in the UK and Australia there was uncertainty regarding the food responsible for the fatality; for example, in the UK 18 unspecified food allergy related fatalities may have also been due to peanut or tree nuts bring the total up to a maximum of 36/48 (75%) cases. Conversely the US data is likely to have been subject to selection bias towards peanut and tree nut reported deaths as these cases came from a voluntary registry. [4;22] There are a reported 100 food related anaphylaxis deaths in the US each year; [31] this would equate to approximately 600 fatalities over 6 years, which is much higher than the registered approximately 30 fatalities reported by Bock et al.(2001, 2007) over each 6 year period. [4;22] To date no study has evaluated the culprit food in all US food related anaphylactic deaths; however, if this were performed it is likely that there would be a similar rate of peanut anaphylaxis related deaths to that reported in the UK and Australia, as they are similarly westernized countries where roasted peanuts are commonly consumed.

Two recent publications from US national public databases assessed the rate of food anaphylaxis fatalities. Ma et al. (2014) reviewed data from 3 public US databases from admissions, emergency and death reports; between 1999-2009, each year the fatalities due to food anaphylaxis ranged from 4-7%.[32] Jerschow et al. (2014) assessed records from the National Centre for Health Statistics' Multiple Cause of Death between 1999-2010.[33] The authors showed a similar rate of food related anaphylactic related deaths (6.7%) to the publication by Ma et al. (2014) of 164 (6.7% of all anaphylactic deaths, making it the least common cause of anaphylactic deaths). Both publications were limited by the use of ICD-10 which prevented the identification of the culprit food; however, Ma et al (2014) did show that peanut related anaphylaxis resulting in emergency attendances and admission had increased from n=256 (4%) to n=512 (7 %). A summary figure of the studies described above is depicted in Figure 1.

Figure 1: Peanut and tree nut related anaphylactic deaths in adults and children



In the UK, over half of deaths related to peanut anaphylaxis occurred in individuals whose previous peanut allergic reactions had been mild. [23] Thus a previous mild allergic reaction cannot reassure the patient that they are less likely to have a severe reaction in the future. Risk factors for fatal peanut allergic reactions include asthma (even well-controlled), location of exposure remote to the home environment and non-timely delivery of adrenaline (although mortality was not invariably prevented by early adrenaline alone).[22-24]

1.4 Impact on the quality of life in children and families with PA

Given the potential for severe allergic reactions it is not surprising that PA leads to heightened anxiety and reduced quality of life in the individual and family of the individual who has PA. When PA was compared with other chronic health conditions such as diabetes mellitus [7] and chronic rheumatoid arthritis,[6] children and families with PA fared worse. In particular they suffered from increased anxiety [7] and disruption in their daily activities and in their familial and social life.[6]

Avoiding peanut in the diet not only entails avoidance of peanut as an ingredient in food but patients may also be advised to avoid known cross-reactive legumes and seeds. This may compromise nutrition. [34] Pre-packed foods frequently have an advisory warning referring to possible nut contamination; these advisory labels are increasingly more prevalent and frequently contribute to anxiety in food allergic individuals. [35;36] The Anaphylaxis Campaign found that 56% (71/127) of foods randomly selected from a shopping basket which would not normally contain nuts as an ingredient indicated a risk of nut contamination. [37] In a more detailed evaluation they found that the peanut or tree nut allergic individual was unable to match a substitute for 18% of the items and in 9% of cases had to make do with a product of poorer quality. Additionally, nut allergic individuals took almost 40% longer to shop and paid on average 11% more than individuals without nut allergies.

1.5 Natural history of PA

PA usually persists into adulthood, and was previously thought to never be outgrown. However, several studies have now shown this is not the case; studies have shown that between 18-59% of children outgrow their PA (references in ascending order of PA resolution). [38-45] Although all of these studies confirmed resolution of PA by OFC, only two of these studies confirmed PA at baseline with a diagnostic OFC. [39;41] Most other studies based their definition of PA at baseline on a history of clinical reaction or positive SPT or sIgE with no history of ingestion; thus it is likely that some of these children were peanut sensitized rather than allergic at baseline which would have falsely elevated the rate of PA resolution described. Chances of outgrowing PA were highest if the child had a milder initial allergic reaction and low SPT/sIgE tests on first presentation, [38;40;42] and if they had fewer additional allergies (implying less complex atopic disease). [46] Unfortunately PA which has been confirmed as resolved on diagnostic OFC can reoccur in up to 8% of cases; [44] usually, however, after peanut consumption had been avoided following a negative peanut challenge.

1.6 Immunological process of food allergic sensitization

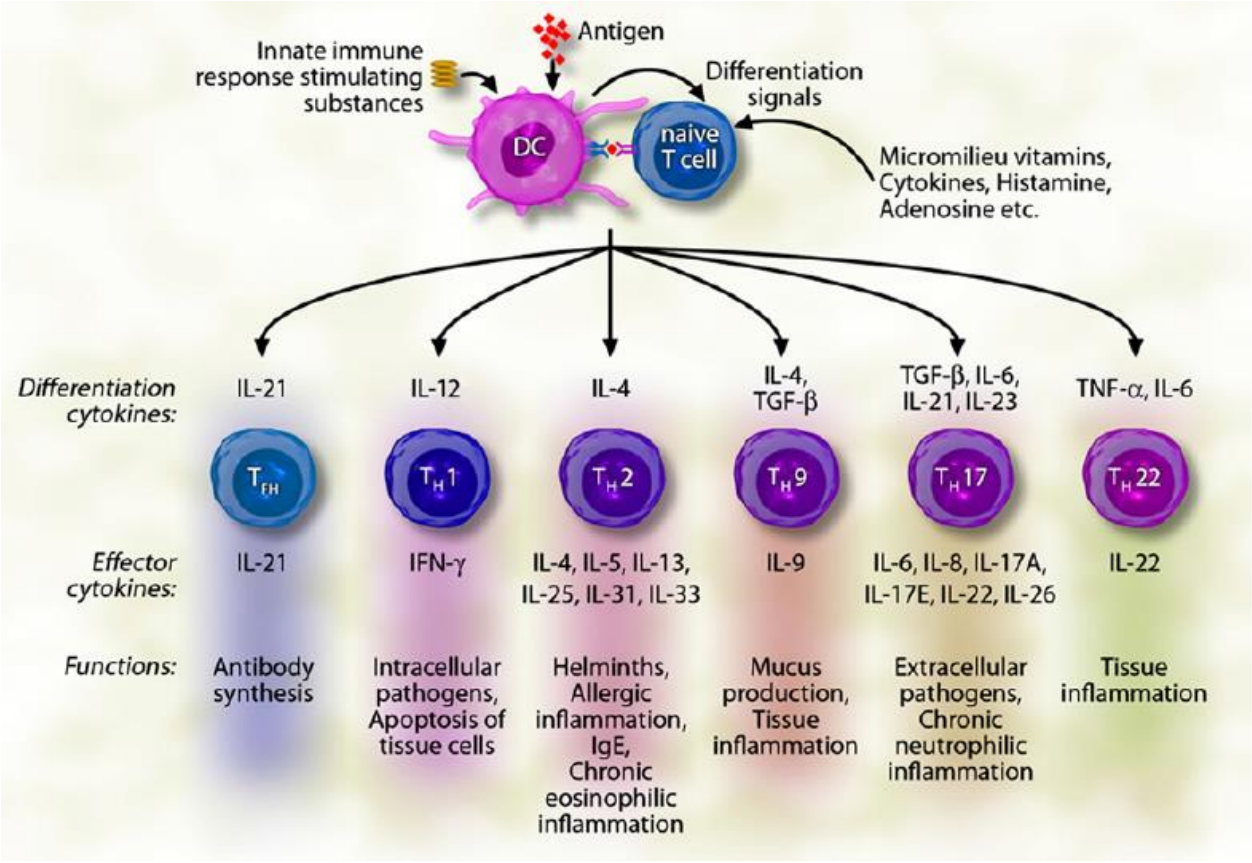
Many children have their first documented allergic reaction to peanut on first known oral exposure which implies a high frequency of prior occult sensitization. [47;48] In three observational studies 72-100% of children reacted to peanut on first known exposure.[5;47;49] To develop PA one must first be exposed to peanut protein or a cross-reactive protein in order for the immune system to be primed to produce the IgE specific for peanut which binds to the surface of mast cells. This process of 'sensitization' is often followed by clinical allergy. The process of sensitization starts with the interaction between an antigen presenting cell (APC), which has engulfed antigen/allergen and a naïve CD4+ T helper lymphocyte. T lymphocytes (or T cells as they will be called in this PhD) are the conductors of the immune system. T helper (Th) lymphocytes assist other lymphocytes in their immunological process such as activating and maturing B cells to produce antigen specific antibodies and activating cytotoxic CD8+T cells and macrophages. They are defined as T helper cells by the presence of the CD4 glycoprotein on their cell surface. An ever expanding array of T cell subsets is being discovered such as Th1, Th2, Th17 and Th9 cells; these T cell subsets may work together or oppose each other in their actions. [50]

'Naïve CD4+ Th cells leave the thymus after the process of thymic education through positive and negative selection has been completed in order for these cells to effectively identify pathogens without harming their host. This occurs via interaction of thymocytes through their T cell receptor with peptide-MHS complexes that are expressed by thymic epithelial cells and dendritic cells in the cortex of the thymus, thus also confers antigen specificity of the naïve Th cell.[51] They are considered to be immunologically naïve until they encounter MHC-peptide complexes for which their T cell receptors have a high affinity.[52] Professional APCs such as dendritic cells, Langerhans cells and macrophages express both Major Histocompatibility Complex (MHC) Class I and II on their cell surface (see Section 1.7.1) and are able to activate or prime naïve T cells. Naïve T cells are dependent on co-stimulatory molecules for activation and require higher concentrations of antigen than memory T cells; however, investigators have not found differences in antigen sensitivity between naïve and memory T cells.[52;53],

During the process of sensitization, antigens (food allergens) are engulfed and processed by APCs which then migrate to the regional LN. Within the cortical region of the regional LN the APC presents the antigen in a recognizable form (as a peptide on a MHC Class II receptor) to naïve CD4+ Th cells via a T cell receptor (TCR) specific for that peptide. Once the naïve CD4+ Th cell recognizes a peptide: MHC ligand, signaling through the TCR induces a conformational change in the cell adhesion molecules (e.g. lymphocyte function associated antigen -1 [LFA-1] on the T cell develops a greater affinity for ICAM-1 or ICAM-2 on the APC) which stabilizes the association between the two cells. During this time the naïve CD4+ Th cell differentiates into effector CD4+ Th2 cells and proliferates. This process is regulated by other concomitant signals (co-stimulatory molecules e.g. B7 on the APC binding to CD28 on the T cell which promotes the survival and expansion of T cells) and cytokines (e.g. IL-21; IL-12; IL4; IL4 + TGF-β; IL6 + TGF-β (+/- IL21 / IL23); IL6 + TNF-α or TGF-β) which direct Th cell differentiation into various Th subsets (e.g. follicular Th; Th1; Th2; Th9; Th17; Th22 or T regulatory in order of previously stated cytokines).[54] The cytokines responsible for Th cell differentiation and the function of different effector cytokines produced by different Th cells subsets are depicted in Figure 2.

Figure 2: Differentiation of naïve T cell into Th subsets following antigen presentation by dendritic cell (DC) to naïve T cell.

Reprinted from Akdis M et al. (2011) with permission from Elsevier.[54]



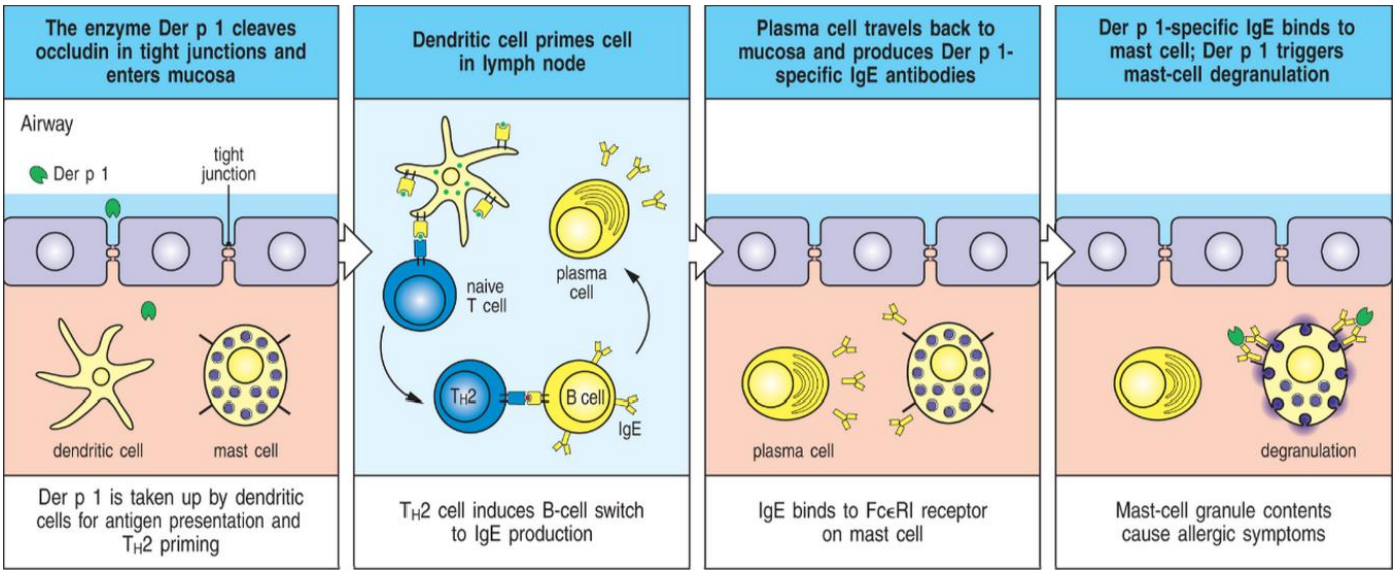
Th2 differentiation induced by IL4 operates through the transcription factor STAT6 (signal transducer and activator of transcription 6). [55] Effector CD4+ Th2 cells secrete IL4 and IL13 which promotes B lymphocyte immunoglobulin class-switching from IgM, IgG or IgA to IgE, and initiates B cell differentiation from naïve B cells to memory B cells and IgE-secreting plasma cells. [56;57] These plasma cells, which may be long-lived, [58] continue to produce and secrete sIgE antibodies directed against a specific peptide of the offending food protein. SIgE antibodies subsequently bind to the high-affinity receptor for the Fc region of IgE (FcεRI) on the surface of mast cells in target organs such as the skin, gastrointestinal and respiratory tract, and basophils in the blood circulation. [59] Upon subsequent re-exposure, sIgE on the surface of mast cells and basophils binds the specific peptide resulting in IgE cross-linking, degranulation and release of inflammatory mediators such as histamine, leukotrienes and

prostaglandins. [59] This results in the allergic manifestations of immediate hypersensitivity reactions in different target organs including the skin (hives, angioedema), gastrointestinal tract (vomiting and diarrhea), respiratory tract (stridor, wheeze and ensuing difficulty breathing) and cardiovascular system (pallor, low blood pressure, collapse). This process is displayed below for allergic sensitization to HDM allergen Dermatophagoides pteronyssinus Type 1 (Der p 1) as an example (Figure 3). Der p 1 has cysteine protease activity that breaks down the tight junctions by breaking down transmembrane protein occludin.[60] Peanut proteins are not known to have protease activity; however, this has not been actively studied.

Figure 3: Mechanism of allergic sensitization using HDM allergen (Der p 1) as an example.

Copyright from Janeway's Immunobiology, 8th Edition by Murphy et al. (2012)

Reproduced by permission of Garland Science/Taylor & Francis LLC. [61]



1.7 Genetic risk factors for PA

There is a significant genetic influence on the development of PA as shown by the 64% concordance rate for PA in monozygotic twins. [62] In a UK study which assessed 622 adults and children and their families with suspected PA, grandparents reported PA in 0.1% (3/2409), aunts and uncles in 0.6% (7/1213), parents in 1.6% (19/1218), and siblings in 6.9% (42/610). [63] On further evaluation by SPT and diagnostic OFC the researchers found a 7% prevalence of PA in siblings of peanut allergic individuals. [63] Identifying genes which predispose to the development of PA would facilitate the provision of personalized medicine.

1.7.1 Major Histocompatibility Complex (MHC)

Given that APCs present processed antigen to naïve CD4+ Th cells via the MHC Class II ligand (see Section 1.6) one could anticipate that variations in the genes that code for the MHC would be important predictors of allergic disease. MHC allows differentiation of self versus non-self and is called the Human Leucocyte Antigen (HLA) in humans. HLA Class 1 molecules regulate inflammatory responses and apoptosis (programmed cell death) and HLA Class II molecules, present on APCs, activate T cells and have been shown to control T cell recognition of peanut antigens.[64] Variation in the genes coding for HLA Class II DRB1*11, [65] DRB1*08, DRB1*08/12 tyr16 and DQB1*04 [66] have been associated with PA versus atopic controls. However, more recently, two studies compared HLA Class II genes in children with PA versus their siblings without PA.[67;68] The studies were designed to focus on variations in HLA Class II genes due to an association with PA rather than due to differences in the HLA Class II genes of non-related individuals. [67;68] Although DRB1*0803 was higher in both sets of siblings compared with unrelated controls, there was no difference between peanut allergic and peanut tolerant siblings. [68] Thus, at present, there is conflicting evidence as to whether variations in HLA Class II genes are responsible for the development of PA. However, there are variations in other genes which have been associated with PA.

1.7.2 Genetic polymorphisms

1.7.2.1 Background

Genetic risk factors can be determined by assessing variations at a specific deoxyribonucleic acid (DNA) sequence that is known to code for a gene. Coding DNA sequences (genes) are transcribed into ribonucleic acid (RNA) and then translated into proteins. Variations in the sequence of DNA are called polymorphisms if the less common allele (an alternative form of a given gene) occurs in more than 1% of the individuals in any population. [(69)] Variations in DNA sequence may consist of a substitution of one of the bases (such as arginine (A), thiamine (T), cytosine (C) and guanine (G) which are the building blocks of nucleotides and thus DNA), deletions or insertions of one or more bases. Over 90% of DNA polymorphisms are single nucleotide polymorphisms (SNPs) which occur when a single base differs between members of paired chromosomes in an individual. The frequency of SNPs are approximately one every thousand base pairs. [(69)] A SNP may occur within a gene resulting in altered gene expression and thus protein formation. This is referred to as a mutation. However, the majority of SNPs are silent (do not lead to altered gene expression and proteins) and the functional significance of these is not yet understood. The 1000 Genome Project Consortium has provided a haplotype (groups of closely linked alleles that tend to be inherited together) map of 38 million SNPs and 1.4 million short insertions and deletions. [(70)]

1.7.2.2 Genetic polymorphisms of the innate immune system

Two SNPs in the glycoprotein CD14, which is expressed on APCs and acts as a receptor for lipopolysaccharide (bacterial cell wall) and other pathogen associated molecular patterns (PAMPS), are associated with PA. [(71)] Homozygous (both alleles) polymorphisms for CD14 (TT) are 4 times more common in food allergic individuals than non-atopic controls. [(72)] CD14 has also been shown to modulate the effect that exposure to bacterial products (such as in farming environments) [(73)] has on the development

of Th2 responses. In a similar manner, variations in genes that code for other APC receptors (Toll-like receptors) that recognise PAMPS modify the association between day care attendance and atopic wheeze at follow-up between the ages of 1 to 11 years. [74] CD14 has a key role in the innate immune system and provides one example of the way that innate immunity, the microbial environment and atopy may interact. [75]

1.7.2.3 Genetic polymorphisms of transcription factors

Variation in APC receptors may alter the way allergens are processed and presented to T cells, but additional mutations within genes that code for downstream signal transduction within the T cell may also influence whether atopy or tolerance ensues. In a study by Amoli et al. (2002), SNPs in the gene encoding for the Th2 transcription factor STAT6 were approximately three times more frequent in 71 peanut or tree nut allergic individuals versus 184 blood donors controls. [76] However, there was no difference in the rate of STAT6 mutations between the same 71 nut allergic individuals and 45 atopic individuals without nut allergy which suggest that the STAT6 SNPs increased the risk of atopy rather than nut allergy per se. [76] Given the role of STAT6 in Th2 differentiation under the influence of IL4 (see Section 1.6) variations in the gene that codes for STAT6 may inhibit or promote Th2 differentiation to allergens in general rather than peanut or tree nut allergens. Polymorphism in interleukins such as IL10 [77] and IL13 [78] have also been associated with food allergy but not PA specifically. Variations within genes important for skin barrier function will be discussed inSection 4.2.

1.7.3 Microarray analysis for hypothesis generation

The previously described gene analysis method described is hypothesis driven: i.e. researchers already need to have a hypothesis as to which genes might affect atopic disease or modify an environmental exposure. Given that there are at least 25,000 human genes there are potentially many as yet undiscovered genes which

may be important biomarkers of allergic disease or tolerance. The use of gene microarrays, evaluates all genes expressed in affected versus non- affected individuals which allows the discovery of novel genes and the generation of hypotheses on the basis of these findings.

Holt's research group identified novel Th2-associated genes in 'recall' responses to HDM (HDM) using gene microarray analysis in HDM allergic vs. non-atopic controls.[79] CD8+ and CD4+ T cells were isolated from HDM-stimulated peripheral blood mononuclear cells (PBMCs) by positive selection using Dynabeads. RNA was extracted from CD4+ and CD8+ T cells and screened for allergen-induced gene activation using Affymetrix microarray technology. Genes identified were then validated using independent panels of subjects by real time quantitative polymerase chain reaction (RT-qPCR). Kinetic analysis revealed an early 12-24hr wave of atopy-associated genes involved in signalling (such as *DACT1*, *NDFIP2* and *GNG8* see Glossary for further description) followed by a later 24-48hr wave of genes encoding the Th2 effector cytokines (such as *IL5, IL13* and *IL9*). This group subsequently performed a network analysis on the genes activated in the CD4+Th cells. They used pair-wise gene-gene co-expression in response to HDM stimulation and activation to determine modules of interconnected genes which were defined as 'atopy associated modules'.[49] They found that blocking 'hubs' of connectivity within an 'atopy associated module' collapsed the whole module thus providing possible target genes for therapy.

Gene expression microarrays have been used to elucidate the mechanisms underlying peanut oral immunotherapy,[80] and for the prediction of efficacy for venom immunotherapy. [81] Of interest in peanut allergic children undergoing oral immunotherapy, T-cell microarrays showed down-regulation of apoptosis related genes (such as BCL2L11 and GADD45A in p53-dependent apoptosis and TNFSf8 in the TNFs/NF-κB/IAP anti-apoptosis). This suggests a novel role for apoptosis in inducing oral tolerance and may not have been considered without the hypothesis generating gene microarray analysis.

Chapter 2 Routes of peanut sensitization

To devise effective PA prevention strategies it is important that the route of PS is understood. In the next section I will discuss the literature pertaining to various routes of peanut exposure and the public health recommendations that have arisen from this.

2.1 In utero peanut allergen exposure

The data surrounding maternal peanut consumption and PS in the unborn child are conflicting. From a mechanistic point of view, IgE in cord blood was thought to be produced by the foetus as foetal cells are capable of producing IgE from the second trimester; [82] furthermore total IgE [83-85] and allergen sIgE (to HDM) [86] in cord blood have been previously used to predict atopy in the child. The ALSPAC (Avon Longitudinal Study of Parents and Children) study is a large birth cohort consisting of over 14,000 pregnant mothers recruited from 1991-1992. Children of mothers recruited to the ALSPAC study that went on to develop PA (confirmed by double blind placebo controlled OFCs) had their cord blood assessed retrospectively. There was no detectable peanut-sIgE (using a cut-off of 0.35kU/L) in the cord blood of peanut allergic children which suggested that antenatal PS in these children was unlikely. [49]

Cord blood may not actually be reflective of foetal IgE production but of maternal IgE production.[87] In the Copenhagen Studies on Asthma in Children (COPSAC) cohort Bonnelykke et al. (2008, 2010) demonstrated that total and sIgE levels in cord blood were frequently the result of maternofoetal IgE transfer. [87;88] Maternofoetal IgE transfer was assessed by comparing levels of total IgE or sIgE in cord blood, maternal blood and in the infant 6 months after birth. Additionally the authors correlated cord blood IgE levels with cord blood IgA as this is used as a measure of maternal blood contamination of cord blood (IgA is not produced by the foetus). On the basis of this method, Bonnelykke et al. (2010) showed that total IgE in cord

blood was maternal in origin in 46% of positive (defined as total IgE >0.5kU/L) cord blood samples. [87] SIgE to inhalant and food (cow's milk and egg) allergens was present in 14% of cord blood samples but the corresponding sIgE was not found at 6 months of age in the infant, [88] which suggests that sIgE in cord blood was either maternally derived or not of clinical significance. Cord blood IgA was highly correlated with total and sIgE levels thus both total and sIgE in cord blood may reflect maternal atopy instead of the infant's atopic status.

2.2 Peanut allergen exposure via breast milk

Cow's milk (β-lactoglobulin) and egg (OVA) proteins have been measured in breast milk. [89;90] A study by Vadas et al. (2001) [91] used the Neogen 'Veratox' peanut polyclonal sandwich ELISA to quantify peanut protein in the breast milk of 23 lactating women. Within one to three hours of eating 50g of dry roasted peanuts, proteins with molecular weights corresponding to the major peanut allergens Ara h 1 (66 kDa) and Ara h 2 (17 kDa) were detected in breast milk using SDS-Densitometry (gel electrophoresis used to separate out proteins according to their molecular size). On the basis of this the authors postulated that Ara h 1 and Ara h 2 were excreted in their whole form in breast milk. However, Vadas et al. (2001) did not confirm their findings with a Western blot using antibodies directed against Ara h 1 and 2, thus could not confirm whether these proteins were Ara h 1 and 2 or different proteins with similar molecular weights. More recently Bernard et al. (2014) measured the major peanut allergen Ara h 6 in breast milk using anti-Ara h 6 monoclonal antibodies.[92] Ara h 6 in maternal breast milk was detectable within 10 minutes of maternal peanut consumption and reached peak levels of 550pg/ml around 30 minutes following ingestion followed by a reduction over time but was still present at 26 hours post ingestion. Ara h 6 in breast milk was able to bind IgE from peanut allergic patients using a competitive Enzyme AllergoSorbent Test (EAST); this assay showed inhibition of labelled Ara h 2 and 6 binding to immobilized IgE from peanut allergic patient serum by increasing the amount of the breast milk containing peanut antigen. This finding suggests that the 2S

albumins Ara h 2 and Ara h 6 are the main peanut allergens found in breast milk. This may be because these 2S albumins are less susceptible to gastric and protease digestion than other peanut allergens, such as Ara h 1 which is less resistant to digestion.[93] The authors also found native non-processed Ara h 6 in breast milk suggesting that this allergen undergoes very little processing in the gastrointestinal tract. Bernard et al. (2014) subsequently showed that IgE in breast milk was able to cause mast cell degranulation in vitro thus would be potentially capable of inducing allergic reactions in peanut allergic infants. Finally, Ara h 6 immune complexes with IgG and IgA were also found in breast milk and were secreted independently of free Ara h 6 a little later, within 40 minutes, and reaching peak concentration at 220 minutes. There is therefore now evidence that major peanut allergens in breast milk are present in their whole form following maternal peanut consumption and could therefore pose a risk for PS in the breastfeeding child.

2.3 Maternal peanut consumption during pregnancy and lactation

2.3.1 Animal studies

In animal studies maternal gastrointestinal exposure to peanut and/or hens' egg during pregnancy and / or lactation, protected the offspring from developing peanut and/or egg allergy respectively. [94;95] Female mice were sensitized orally to peanut using cholera toxin (CT) adjuvant then were mated with naïve male mice.[95] When these peanut sensitized female mice were fed low dose peanut feeds with adjuvant during pregnancy and lactation (beneath the threshold to trigger an allergic reaction), 28% of their offspring developed anaphylaxis on subsequent intragastric exposure to peanut. However, if maternal mice did not receive low dose peanut feeds during pregnancy and lactation all of their offspring developed severe anaphylactic reactions on subsequent intragastric exposure to peanut. Anaphylaxis in the offspring mice was confirmed by a rise in plasma histamine and mouse mast cell protease 1 (mMCP-1) which are markers for anaphylaxis in mice.[96] In the offspring whose mothers had *not* received low dose oral peanut administration during

pregnancy and breastfeeding, peanut specific IgG1, (a mouse specific allergic antibody) was detectable before their peanut challenge; this must therefore have been maternally derived via the placenta as these mice had not had any other form of peanut exposure. In the offspring whose mothers *had* received low dose oral administration during pregnancy and breastfeeding, those protected against peanut anaphylaxis had higher IgG2 levels and lower peanut sIgE production to active (intragastric + CT) PS. [95]

More recently, peanut antigen present in human breast milk was shown to prevent PS when fed to mouse pups prior to weaning.[92] Peanut protein in human breast milk following maternal peanut consumption was shown to be biologically active by demonstrating binding of free Ara h 6 to peanut sIgE and subsequent mast cell degranulation. Pre-weaned mice were fed human breast milk containing no peanut or only free Ara h 6 (earlier time points following maternal peanut consumption) or containing both free Ara h 6 and IgG- and IgA-Ara h 6 immune complexes (later time points following maternal peanut consumption). Mice were then sensitized to peanut using repeated intragastric gavages of peanut and CT and subsequently underwent a peanut OFC. Following administration of peanut-free breast milk there was no reduction in mouse Th1 (IgG2a) and Th2 (IgG1) responses to peanut, but these were significantly reduced following administration of human breast milk containing free Ara h 6 and with breast milk containing free Ara h 6 plus Ara h 6 in IgG and IgA immune complexes. There was also a trend towards a reduction in mMCP-1 in mice fed human breast milk with free Ara h 6 and free Ara h 6 plus Ara h 6 immune complexes, but this did not reach statistical significance when compared with mice fed breast milk without Ara h 6 or controls (not treated with breast milk) and there was no decrease in peanut sIgE. There was surprisingly no significant additional benefit of IgA- and IgG-Ara h 6 complexes (although this was associated with lower mMCP-1 levels than mice administered free Ara h 6 in breast milk); this may suggest that the presence of peanut allergen with the intrinsic immunoregulatory properties of breast milk itself are the most important aspect in inducing

tolerance, rather than any tolerogenic effects of antigen bound to IgA or IgG. In support of the tolerogenic effects of breast milk alone, this group showed higher regulatory (IL10) and Th1 (IFNγ) cytokine secretion from peanut cultured spleen cells in mice fed human breast milk whereas peanut cultured spleen cells in controls secreted Th2 (IL5) and Th17 (IL17) related cytokines.[92] On the basis of these findings there does seem to be some animal data in support of exposure to peanut through breast milk preventing PS; however results for peanut exposure in utero are less clear.

2.3.2 Human studies

2.3.2.1 Evidence for an association between maternal peanut consumption and PS/PA in the child

A number of observational studies have assessed the impact of maternal peanut consumption during pregnancy and breastfeeding; however, these studies have shown conflicting associations. There are three studies I will present in this section which provide evidence for maternal peanut consumption during pregnancy (and in some cases lactation) being a risk for PS in the offspring.

1) In a small study from South Africa with 25 peanut sensitized individuals (defined as peanut sIgE >0.35kU/L) and 18 control subjects, there was a trend towards PS (OR 3.97 95% CI: 0.73-24 *P*=0.063) if mothers ate peanut more than once a week during pregnancy.[97] There was no significant association between maternal peanut consumption during breastfeeding and PS in their children.

2) The CoFAR study (a prospective US cohort of 512 infants with egg or cow's milk allergy or moderate to severe eczema with cow's milk or egg sensitization) assessed the association between maternal peanut consumption during pregnancy and PS/likely PA (defined as a peanut sIgE

≥5KU_A/L) in their children. Peanut consumption during pregnancy was defined as 'avoided',' <2 times weekly', '>2 times weekly', 'daily' or 'not known'. A dose response relationship between maternal peanut consumption during pregnancy and a serological diagnosis of likely PA was reported. Furthermore, in a subgroup analysis of 71 infants who were never breastfed, peanut consumption >2 times weekly during pregnancy increased the risk of peanut IgE ≥5kU/L 5-fold (OR 4.99, 95% CI, 1.69-14.74; *P*=0.004). [98] There was no significant association between maternal peanut consumption during breastfeeding and likely PA in children recruited to the CoFAR study.

3) A Canadian study by DeRoches et al. (2010) included 202 children less than 18 months old who had been referred to an allergy clinic for a presumed peanut allergic reaction in the last month. [99] Controls were children under 18 months of age without a history of food allergy being seen in other paediatric clinics (n=201). Maternal peanut consumption was assessed by a non-validated dietary food frequency questionnaire (FFQ) where respondents were asked to specify the frequency with which they had eaten peanut containing foods during pregnancy and lactation (at least daily, 5-6 times weekly, 2-4 times per week, once a week or 1-3 times a month or never). In the case of peanut butter they were also asked to report the average portion size, however, they were not asked this information for other peanut containing foods thus the amount of peanut protein consumed could not be quantified. Peanut consumption during pregnancy was found to be higher in peanut allergic cases (196/202=97%) than controls (176/199=88%) with an adjusted OR of 4.22 (1.57-11.30). Peanut consumption during breastfeeding was also higher in the peanut allergic cases (154/162=95%) than controls (124/157=79%) with an adjusted OR of 2.28 (1.31-3.97). Multivariate logistic regression (LR) analysis was adjusted for age, sex, family history of atopic diseases, and duration of breastfeeding.

DeRoches et al. (2010) therefore concluded that the route of PS was likely to be via maternal peanut consumption in utero or via lactation. However, the questionnaires were administered after PA was confirmed in clinic. This could have resulted in recall bias of maternal peanut consumption during pregnancy and lactation because, at the time, this was considered a risk factor for PA and was recommended against in atopic families;[100] thus mothers could have overestimated the amount of peanut they consumed during pregnancy and lactation once they knew that their child had PA. The authors also acknowledged that using non-atopic children as controls was a limiting factor in the study design as these children would have had a lower rate of PA regardless of the level of maternal peanut consumption during pregnancy or lactation.

Despite these limitations there is some observational data described in these three studies which points towards an association between maternal peanut consumption, particularly during pregnancy and later PA in the their offspring. In the next section I present evidence to the contrary and in particular will focus on a study, similar in many ways to the DesRoches et al. (2010) study which showed different results once they included household peanut consumption (HPC) in their analysis.

2.3.2.2 Evidence against maternal peanut consumption during pregnancy / lactation and PS / PA

Three large cohort studies (BAMSE study n=4089, [101] ALSPAC study n= 13,971, [49] and the Isle of Wight study n=1456) [102;103] did not find an association between maternal peanut consumption during pregnancy or lactation and PS or PA in their children.

Fox et al. (2009),[104] recruited peanut allergic children under 24 months (n=133) and high-risk egg allergic controls (n=150) who had been referred to a paediatric allergy clinic, predominantly due to eczema. Children without atopy were recruited from general paediatric clinics (n=150). A validated peanut FFQ was used to assess maternal peanut consumption during pregnancy and lactation and HPC during the child's first

year of life (average peanut protein consumption in grams/week). Dietary questionnaires were performed before the diagnosis of peanut was ascertained therefore reducing the risk of recall bias. Although maternal peanut consumption during pregnancy and lactation was higher in peanut allergic cases than high-risk controls on univariate LR analysis, once HPC in the first year of the child's life was entered into the model maternal peanut consumption during pregnancy and lactation was no longer significantly associated with PA. Maternal peanut consumption during pregnancy and lactation and HPC were measured using the same dietary questionnaire and was quantified in g/week. Thus peanut consumption could be assessed using continuous rather than categorical variables (thus had a similar power to determine effects).

To separate out the different routes of peanut exposure involved, the authors evaluated families where there was disparity between maternal peanut consumption during pregnancy and lactation and HPC in the first year of life. In families where the only peanut consumption was due to HPC (because mothers excluded peanut during pregnancy and lactation) the relationship between peanut consumption (household but not maternal) and the likelihood of PA persisted. In families where the only peanut consumption was by the mother during pregnancy or lactation but with no other household members eating peanut then the association between peanut consumption (maternal) and likelihood of PA was lost. [105] There was a positive albeit weak correlation between maternal peanut consumption during pregnancy (Spearman Rank correlation coefficient r_s=0.45) and lactation (r_s=0.51) and HPC during the first year of the child's life. These results, taken together suggest that the apparent association between maternal peanut consumption during pregnancy and lactation and PA was attributable to the link between maternal peanut consumption and HPC and that HPC was the predominant risk factor. The impact of HPC on PA will be discussed in more detail in Section 2.4.

2.3.3 RCTs assessing impact of maternal dietary avoidance during pregnancy / lactation

The literature described above provides conflicting observational data on whether maternal peanut consumption during pregnancy and/or lactation is a risk factor for the development of PA. To assess a causal relationship a randomised controlled trial (RCT) where maternal diet during pregnancy and lactation was controlled would be required. There are several RCTs which have attempted to reduce prenatal and postnatal exposure to allergenic foods by manipulating the maternal diet in a bid to prevent the development of eczema, food and respiratory allergies in the child. The RCTs performed did not, however, limit dietary restriction to just peanut but included a range of allergenic foods; they also did not focus on peanut allergic outcomes but on atopic sensitization and disease in general.

The Cochrane Collaboration reviewed the evidence available up until 2012 from RCTs comparing maternal dietary antigen avoidance during pregnancy and lactation (in women with atopic family histories) but excluding trials with additional interventions such as manipulation of the infant's diet. [106] Maternal dietary restriction of allergenic foods during pregnancy was not associated with a reduction in eczema in five RCTs, involving a total of 952 participants. [107-111] Maternal dietary restriction during lactation was not associated with a reduction in eczema or SPT sensitization to cows' milk, egg or peanut in two RCTs involving a total of 523 participants.[109;111] Furthermore, the authors found that mothers who underwent dietary restrictions gained less weight during pregnancy and there was an increased risk of preterm birth and reduced birth weight in the infant. [107;111] The authors therefore advised that there was insufficient data to recommend maternal dietary restriction during pregnancy and lactation and this may in fact incur risks for the mother and child due to nutritional insufficiencies.

There are two publications from prospective RCTs from the US (Zeiger et al. 1989)[112] and the Isle of Wight (Hide et al. 1992/1996, Arshad et al. 2007)[113-115] which reported a protective effect of combined

maternal and infant dietary restrictions on atopic disease which were not included in the Cochrane review cited in the paragraph above (as these studies included manipulation of the child as well as the maternal diet). In the study by Zeiger et al. (1989) high-risk children were recruited on the basis of at least one family member having atopic disease and evidence of sensitization by sIgE or SPT analysis. The intervention arm dietary measures included maternal avoidance of all dairy, egg, peanut and concentrated forms of soya (e.g. tofu) plus limited wheat intake in the last trimester of pregnancy and during lactation. If formula fed, the infants were given Nutramigen (an extensively hydrolysed casein formula) until 12 months of age. Parents were asked to avoid giving their infants dairy, egg, soya, peanut and wheat until 12 months of age, when cow's milk could be introduced followed by soya, wheat, and then egg at 24 months and peanut at 36 months. Compliance with this dietary regimen was ascertained by maternal self-reporting and daily diaries.

At 12 months follow-up there was a reduction in cumulative 'atopy' defined as prevalence of food associated eczema, urticaria and /or gastrointestinal disease (5.1% in the intervention arm versus 16.4% in the control arm, P=0.007). At 24 month follow-up the intervention group showed a reduction in food SPT sensitization at16.5% (16/97) versus 29.8% (50/168); P= 0.019, predominantly due to fewer positive cow's milk SPTs (1% versus 12.4%; P= 0.001) but also due to lower egg SPT (12.4% versus 17.3%). At 24 months peanut SPT was slightly higher in the intervention arm at 5/97 (5.1%) versus the control arm at 7/168 (4.2%). There was no difference detected in inhalant SPTs at 24 months. [112] At 7 years of age there was no difference between the intervention and control group for rate of atopy, asthma, eczema or food allergy. [116]

Although the study by Zeiger et al. (1989) seemed to show an early protective effect of dietary manipulation, the reduction in cumulative 'atopy' at 12 months is likely to have been due to lack of dietary exposure to the foods, as infants in the intervention arm were not supposed to be having any allergenic foods. Additionally other household members may have continued to have reduced intake of dietary foods

which may have decreased environmental exposure to these foods (this information was not available in the study). The reduction in egg SPT sensitization could be explained by the fact that infants were only advised to start egg introduction at 24 months; however, the reduction in milk SPT at 24 months cannot be explained in the same way as parents were advised to introduce dairy into the infant's diet at 12 months, thus lack of cow's milk sensitization was not just due to delayed exposure.

There were certain limitations to the study by Zeiger et al. (1989). The patient population were recruited from the Kaiser Permanente Health plan, a private healthcare system that may have resulted in selection bias due to the skewed socioeconomic status of the population recruited. In the first year there was a 38% rate of drop out in the intervention arm of the study, predominantly due to inability to comply with the dietary intervention. These participants were excluded from the study and therefore the results obtained were based only on a per protocol analysis rather than an intention to treat analysis, which would have included outcomes from all children whose mothers' were assigned to the intervention arm, even if their mothers had not complied with the dietary interventions. This study was additionally limited by a 43% loss to follow-up in both the intervention and control arm of the study bringing down the number of children assessed at age 7 years to only 59 children in the intervention arm and 106 children in the control arm. The study is likely to suffer from several sources of bias: selection bias from the population recruited, bias due to the high drop-out rate due to failure to comply with the study intervention (who were subsequently excluded from the study rather than included in an intention to treat analysis) and bias due to loss to follow-up.

The Isle of Wight RCT (Arshad et al. 2007, n=120) reported a protective effect of combined maternal and infant dietary restriction in high-risk infants in conjunction with HDM reduction measures which persisted up to 8 years follow-up for eczema (OR 0.23 95% CI: 0.08-0.64; $P<0.01$), positive SPT to at least one allergen (OR 0.13 95% CI: 0.05-0.32; $P<0.001$), asthma (OR 0.24; 95% CI: 0.09-0.66; $P<0.01$) and rhinitis

(OR 0.42; 95% CI 0.19-0.92; *P*=0.03).[114] High-risk infants were defined by allergic disease (asthma, eczema, or allergic rhinitis) in two immediate family members and high [>0.5kU/L] cord blood IgE levels. The intervention consisted of strict elimination of common allergenic foods (dairy, egg, fish, wheat, unhydrolysed soya and nuts) until 9 months of age,with breastfeeding mothers followed the same regimen (except wheat exclusion). Formula fed infants received a soya based hydrolysate (Aptimal HA, Milupa, UK). Cow's milk was introduced at 10 months, followed by egg at 11 months then other foods were introduced. Maternal compliance with the dietary intervention was assessed by random testing of breast milk for cow's milk proteins; however, this information was not presented in the manuscript and very little data on dietary compliance assessment was provided. Additionally this study also excluded infants and mothers who found the dietary restrictions too difficult to manage, rather than including these children in an intention to treat analysis. Nevertheless, this cohort study benefited from no drop-outs as all 120 children were followed up at 1, 2, 4 and 8 years.

HDM reduction measures (including infant anti-allergy mattress covers and acaricide sprays) successfully reduced HDM levels in the home as quantified by ELISA analysis of dust samples from the infants' bedroom carpet, living room and upholstered bedroom. The addition of anti-allergy mattress covers in this study to the intervention may have contributed to why this study was effective in reducing atopy; food allergens may have been reduced in the infant bed thus leading to lower levels of environmental food allergen exposure.

Following publication of the Cochrane review a large prospective cohort study (Growing Up Today Study 2) found that peanut and tree nut allergy was less common in the children of mothers who consumed more peanut or tree nuts respectively around the time of birth of the child.[117] In non-atopic mothers the reduction in peanut and tree nut allergy in their offspring was significant (OR 0.31, 95% CI 0.13-0.75, *P*=0.004) when

mothers increased their peri-pregnancy peanut and tree nut consumption from less than once a month to at least 5 times a month. There were certain limitations to this study, however, as the authors used a non-validated FFQ and peanut and tree nut allergy was not confirmed by OFC, but by review of medical notes and allergy tests by two paediatricians (one of whom was an allergist). Although the authors stated that they assessed peri-pregnancy maternal peanut consumption, in 76% of cases this was actually after birth and up to the child's first birthday. The authors did not adjust for maternal peanut consumption during breastfeeding nor did they adjust for the child eating peanut, which would be more common in a peanut eating family and could therefore have led to oral tolerance induction.

Overall, the literature in humans does not support maternal dietary restriction of allergenic foods (including nuts) during pregnancy and breastfeeding to reduce PS / PA or other atopic conditions. The only studies which did suggest a positive association between maternal peanut consumption during pregnancy and lactation and PS/PA (or benefit from dietary restrictions) were in high-risk children, thus future research to evaluate this further would be more appropriate in high-risk children. Although not directly related to peanut consumption and exposure, duration of breastfeeding is also frequently discussed as a potential modifier for allergic disease therefore I will discuss this briefly in the next section.

2.3.4 Duration of breastfeeding and PS / PA

The association between duration of breastfeeding and food sensitization/ allergy is fraught with the confounding effect of reverse causality i.e. that mothers breastfeed longer because their child is atopic or because there is a risk of atopy in the family. This was demonstrated in the ALSPAC study where on univariate analysis, duration of breastfeeding was associated with PA with a 2.6-fold risk if the infant was breastfed over 6 months (P=0.03); however, when entered into a multiple regression analysis which

included maternal history of atopy and eczema, duration of breastfeeding was no longer associated with PA. Of note, the ALSPAC cohort obtained data on duration of maternal breastfeeding prospectively. [49] This may, in part, explain the reason why studies that have evaluated the role of breastfeeding on the risk of food allergy have ranged from reducing the risk of food allergy, [118] to neutral effects [119] to increasing the risk of atopic disease.[120] Nonetheless, this is an important covariate to consider when evaluating risk factors for allergic disease.

2.4 HPC during the child's first year of life.

Three studies to date have assessed whether 'household' exposure to peanut during the first year of life is a risk factor for the development of PS and PA. In the high-risk CoFAR study there was no association between household exposure to peanut and PS or likely PA.[98] However, the CoFAR study used a crude measure of household exposure to peanut defined as: 'Were/are there peanuts or peanut products in the house while breastfeeding?'. Although Sicherer et al. (2010) tried to ensure that this question would capture household exposure to peanut during infancy by qualifying the questions with 'while breastfeeding' this question did not assess whether the peanut products were eaten and thus handled or whether the peanut products had just stayed in the cupboard.

A second study by DesRoches et al. (2010) (methodology discussed in Section 2.3.2.1) assessed potential environmental exposure to peanut by quantifying the presence of peanut containing foods in the child's home and day-care centre. [99] Their study population included children < 18 months either referred for a presumed peanut allergic reaction in the last month (n=212) or non-atopic controls in other general paediatric clinics (n=201). Peanut–containing products in the home environment or day-care were classified into peanut butter, peanuts in chocolate, peanuts in cookies or whole peanuts; thus household consumption of savoury peanut containing foods which often have a high peanut protein contact (peanut soup, satay

sauce, Chinese, Thai and Indonesian food) were not included. They found less peanut butter, peanuts in chocolate and peanuts in cookies in the homes of the peanut allergic cases versus non-atopic controls, but no significant difference in the presence of peanut containing foods in day-care (with the exception of whole peanuts which were present more in day-care centres with peanut allergic children).

A limitation of this study is that PA was already suspected in the month leading up to their assessment, thus it is highly likely that the parents of peanut allergic children would have removed many peanut products from the home in the weeks leading up to their clinic assessment. This would also explain why there was no difference between numbers of peanut containing foods in day-care between peanut allergic cases versus controls as they are unlikely to have removed all peanut-containing foods from the day-care, particularly before the child had a formal diagnosis of PA. Other limitations in this study, as in the CoFAR study, were that the measurement of household exposure to peanut was the presence of peanut-containing foods in the home rather than actual HPC; thus peanut butter could have been in the home but the frequency of use and, therefore, potential transfer into the environment or onto the child's skin is not known. Furthermore, comparing peanut allergic cases against non-atopic controls may have also influenced the presence of peanut containing foods in the home: non-atopic families might be more likely to have peanut in their home as they are not concerned about the potential of peanut allergic reactions in any of their family members.

The third study by Fox et al. (2009) [104] addressed several of the shortcomings in the previous two studies:

1) Comparing peanut allergic cases (n=133) against both atopic egg allergic controls (n=160) and non-atopic controls from general paediatric clinics (n=150). This allowed the study to tease out factors and peanut consuming behaviours which might be associated with atopy per se rather than PA.

2) Asking parents to complete the dietary questionnaire before their child was diagnosed with PA. If parents already knew their child was peanut allergic they were excluded from participation to prevent recall bias in the dietary questionnaires. After completing the questionnaire, they were assessed for PA by SPT (>8mm) or sIgE >15 kU/L, on the basis of previously validated 95% PPV, [18] or a double blind placebo controlled OFC. One must, however, consider that just being in an allergy clinic might invoke a higher index of concern about the possible role of household or personal nut consumption on the child's allergic status.

3) Quantifying HPC (by combining peanut consumption of all household members in the child's first year of life), rather than just stating the presence of peanut-containing foods in the home (as in the DesRoches et al.(2010) and Sicherer et al. 2010 studies described) is likely to have been a better representation of the amount of peanut containing food being eaten / handled thus more likely to lead to EPE.

4) The study by Fox et al.(2009) assessing HPC using a validated semi-quantitative peanut FFQ with a large range of savoury and confectionery/bakery peanut containing foods. Validation of this FFQ for maternal recall over 2 years [121] and against a 7-day food diary over 6 months for the mother and infant [122] made this dietary questionnaire suitable for this study design.

5) The FFQ quantified the amount of peanut protein in each of the peanut containing foods and thus obtained an average level of peanut protein consumption (g/week) by combining the different types of peanut containing foods into one continuous variable. Using a continuous variable will usually have more power to show an association than a categorical variable.

In the study by Fox et al. (2009) HPC was ten times higher in peanut allergic children (18.8 gram/week) than high-risk egg allergic children without PA (1.9 grams/week) ($P<0.0001$) and three times higher than non-atopic children without PA (6.9 grams/week) ($P<0.0001$). Peanut butter consumption versus other

'covered' peanut containing foods such as chocolates were more likely to be associated with PA. The authors postulated that peanut butter, being stickier, was more amenable to transfer by hands; this could potentially be transferred onto the infant's skin directly or onto their bedding or play-area. A limitation of this study is that the authors did not directly measure peanut protein levels in the environment (such as in dust or surface wipe samples). Fox et al. (2009) also quantified peanut protein consumption by the child in their first year of life, and found that the dose response between increasing HPC and PA was abrogated in children who had eaten peanut within the first year of life. The counteracting influences of early oral peanut consumption versus exposure to peanut via other routes (such as through the environment via the epicutaneous route) is a central theme to this PhD. In the next Section I will discuss the evidence surrounding the impact of oral peanut consumption on the development of PA vs. tolerance in both animal and human studies.

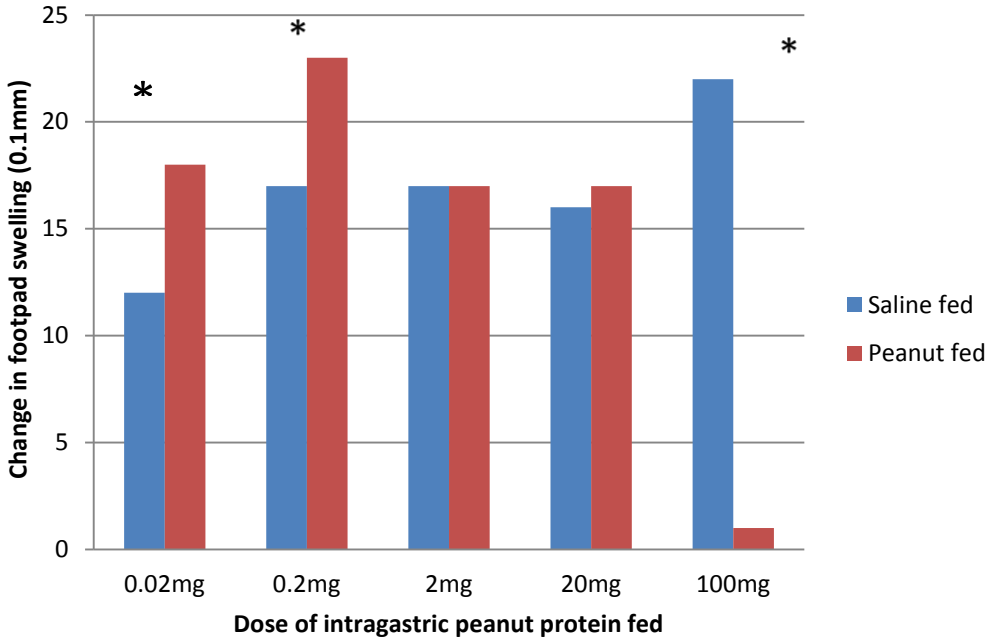
2.5 Animal studies of oral peanut and/or ovalbumin exposure

The majority of food allergy research in mice is performed using ovalbumin (OVA) rather than peanut allergen. I have therefore included certain important animal studies which have assessed OVA sensitization and allergy where it is relevant to the argument being constructed. Yamashita et al. (2012) showed that OVA sensitization (using IP injection with alum) and subsequent anaphylaxis could be prevented in BALB/c mice through oral pre-treatment with 1mg OVA every day for 5 days; [(123)] this supports the concept of oral tolerance induction. In another study researchers were able to achieve oral tolerance or allergy to OVA or peanut depending on the dose employed.[(124)] BALB/c mice were fed a single intragastric feed of peanut or OVA, ranging from 0.02mg to 100mg (0.001-5 mg/gram bodyweight) versus phosphate buffered saline (PBS). One week later mice were immunized at the base of the tail with 100 µg peanut or OVA with complete Freund's adjuvant (heat killed and dried Mycobacterium tuberculosis). To assess the immunological response mice were administered 100µg peanut or OVA into their left hind footpad after 3

weeks and the swelling was recorded (Figure 4). Mice were sacrificed a week later and proliferation of peanut specific T cells in the spleen and peripheral lymph nodes (LNs) from the tail base immunisation site was quantified by tritiated (H^3) thymidine uptake by T cells cultured in peanut protein for 90 hours.

There was a bell-shaped curve for clinical allergic reactivity dependent on the dose of intragastric OVA or peanut protein administered prior to sensitization (Figure 4). At low doses up to 0.02 mg of OVA or up to 0.2 mg for peanut protein there were increased allergen specific T cell proliferative responses, Th2 cytokine production and footpad swelling on challenge compared to PBS fed mice. Thus low-dose oral peanut consumption increased allergic responses in these mice. However, in mice fed ≥ 2 mg OVA (≥ 0.4 mg/gram bodyweight) or 100 mg peanut protein (5 mg/gram bodyweight) there was minimal footpad swelling following hind foot pad injection and significantly reduced T cell proliferation and cytokine responses vs. controls, demonstrating a protective effect of high-dose oral exposure against allergic reactions.

Figure 4: Clinical reactivity to hind foot pad challenge in peanut sensitized mice dependent on dose of previously administered intragastric peanut feed. Created from data from Strid et al. (2004). [124]



2.6 Human studies on oral peanut exposure

Similar themes emerging in animal data with regard to oral tolerance induction have been investigated in humans, such as the timing of peanut introduction into the diet and peanut protein dosage required to prevent PA as described below.

2.6.1 Age of introduction of peanut into diet and PS / PA

A small case control study (n=43) by Frank et al. (1999) found that children who were sensitized to peanut had introduced peanut into their diet at an earlier age (12.5 vs. 17.3 months). However, participants did not undergo diagnostic OFCs to establish PA or tolerance. [97] PS was also anecdotally linked to earlier peanut consumption in an observational study of 62 consecutive peanut allergic children by Ewan et al. (1996) although age of introduction of peanuts was not described in this study. [125] In the study by DesRoches et al. (2010) (see Section 2.3.2.1 for description of study design) age of introduction of peanut into the child's diet was similar between peanut allergic cases and non-allergic controls (11-12 months). Conversely, a large cross-sectional study by Du Toit et al. (2008) demonstrated that early high-dose peanut consumption during infancy was associated with a lower rate of PA. [17] In this study, over 5000 Jewish primary school aged children from Israel and North London (UK) were assessed for PA by initial questionnaire for reported PA. Diagnostic OFC or allergy tests above the 95% PPV (peanut SPT ≥ 8mm or peanut sIgE ≥15kU/L) were used to confirm or refute this self-reported diagnosis. Peanut consumption during infancy was assessed by a validated peanut FFQ. [121;122] PA prevalence was 10 times higher in the UK (1.85%) than Israel (0.17%) ($P< 0.001$). Median peanut protein consumption during infancy (in grams/week) in UK Jewish children was 0 grams versus 7.1 grams in Israeli Jewish infants ($P<0 .001$) and Israeli Jewish children ate peanut on average 8 times a week during infancy. The authors concluded that high and frequent peanut consumption during infancy might protect against the development of PA. [17]

There were certain limitations to the study by Du Toit et al.(2008); the authors adjusted for relevant covariates such as atopy (asthma, eczema or allergic rhinitis), other food allergies, age and sex and selected children of similar genetic background. However, the authors did not assess maternal peanut consumption during pregnancy or lactation, which may have influenced the development of peanut sensitization and allergy in these children. The authors postulated that the differences observed in PA rates in the UK versus Israel were likely to be due to specific weaning practices (high versus low peanut consumption during infancy). There were, however, also lower rates of other food allergies in Israel such as sesame, tree-nut allergy and egg allergy ($P<0.05$). The authors argued that this might be due to earlier and more frequent sesame seed consumption in Israel (this was not quantified in the publication) and due to cross-tolerance induction by peanut for sesame and tree nut allergy given the homology between amino acid sequences of these allergens. The higher rate of egg allergy was not explained and as such the possibility that other factors were also driving the reduction in PA in Israel cannot be excluded (such as for example Vitamin D), thus the need for an RCT to confirm these findings.

The Learning Early About Peanut (LEAP) Study (www.leapstudy.co.uk), is an RCT which has evaluated whether early peanut consumption leads to a reduction in PA in high-risk children (of which some were already sensitized to peanut at baseline). In the LEAP study 640 infants at high-risk of developing PA (due to egg allergy or moderate–severe eczema) were randomised to either high-dose (2 grams peanut protein), frequent (3 times a week) peanut consumption from infancy until 5 years of age or peanut avoidance. [(9)] PA was assessed by OFC at 5 years of age to determine whether early high dose, frequent oral peanut consumption led to a reduction in PA in high-risk children. The LEAP study structure is displayed in displayed in Figure 5.[(126)] There was a 98.4% retention rate. The outcomes were divided into children with negative peanut skin prick tests (SPT) (n=530) at baseline as a model for primary prevention of peanut allergy and children with peanut sensitization (SPT between 1-4mm) (n=98) as a model for secondary

prevention. In the intention to treat analysis, early regular peanut consumption reduced the rate of peanut allergy from 35.3% to 10.6% (a 70% risk reduction) in SPT positive children and reduced the rate of peanut allergy from 13.7% to 1.9% in the SPT negative group. In the per protocol analysis children with positive SPT reduced their peanut allergy from 34.0% to 0.0% (see Figure 6). These findings provide a strong basis for the role of early high-dose peanut consumption leading to a reduction in PA; however, the authors did not comment on whether children who ate a lower dose of peanut protein were protected against PA. Thus it may not be possible to set a cut-off for the minimum amount of peanut consumption required to prevent PA and it is still possible that children eating very low doses of peanut might increase their risk of PA.

Figure 5: LEAP study design

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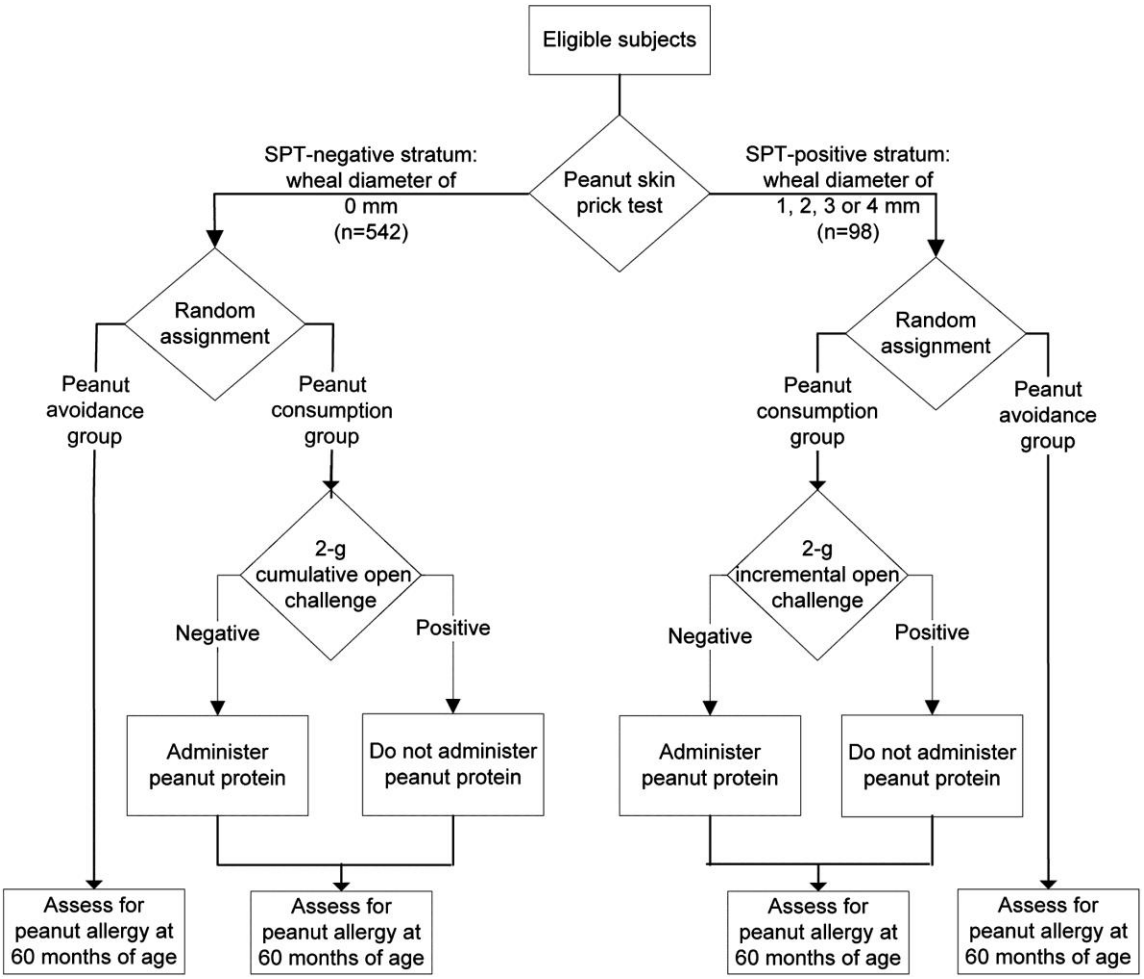

Figure 6: Results from the LEAP study

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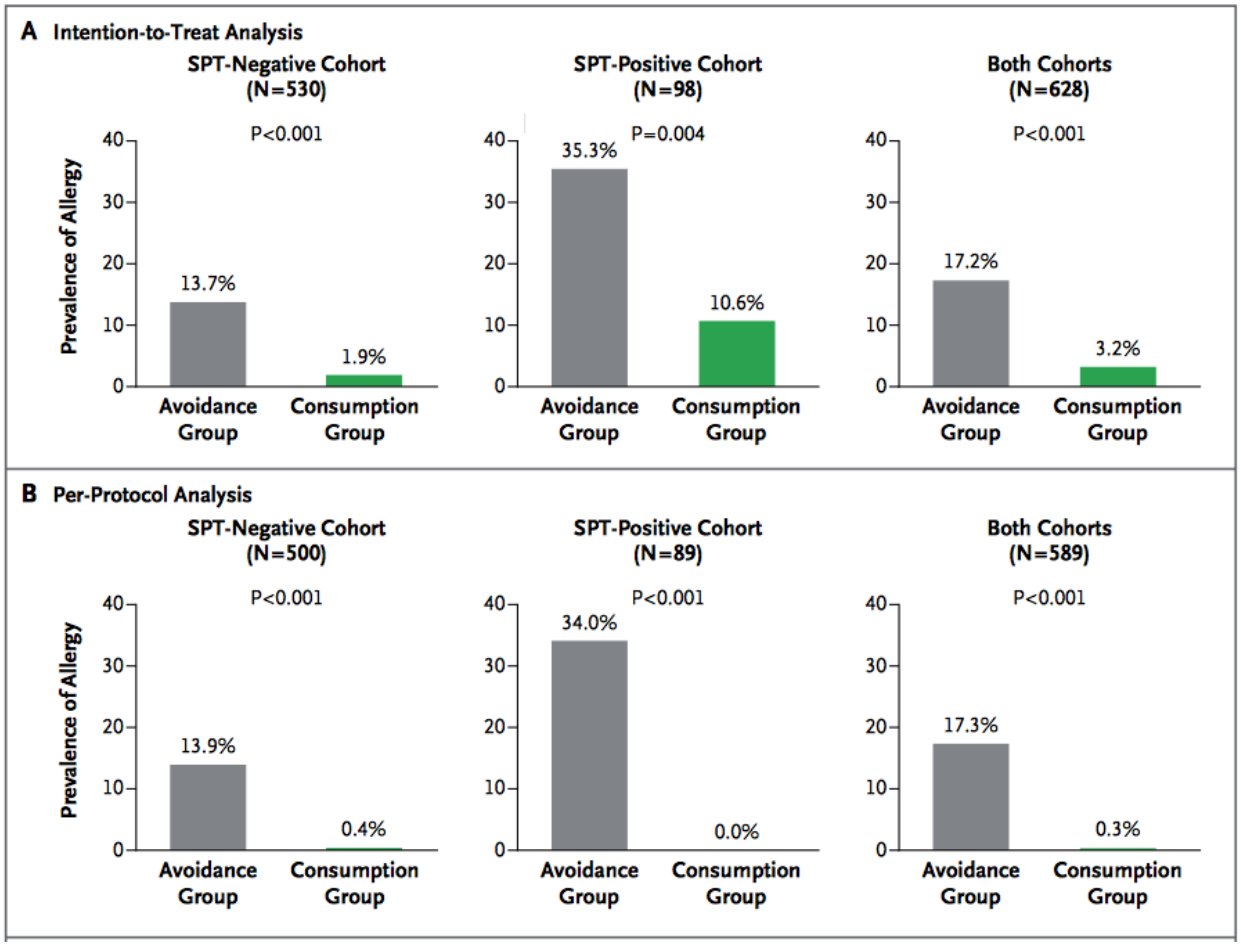


2.6.2 Dose of peanut oral exposure

Given that low-dose oral peanut consumption increased allergic responses to peanut in animal studies, one could hypothesise that parents could inadvertently sensitize their infants to peanut by feeding them low quantities of peanut protein in foods, or Arachis (peanut) oils in milk formulae [127] and vitamin supplements (e.g. Abidec). Previous literature raised concerns about the implications of low dose exposure to Arachis Oil in infant vitamin supplements. De Montis et al. (1993) found a higher rate of PS in children administered vitamin D which contained Arachis oil in the first two years of life (daily administration - PS rate: 9/28= 32.1%, weekly administration - PS rate: 11/40=25%) versus children who received vitamin D without

peanut oil in the first two years of life (PS rate: 2/40=5%). [128] However, the study was limited by a small sample size and lacked adjustment for other confounding factors (such as eczema or parental atopy). Subsequently, a Swedish group refuted this finding through retrospective analysis of their large observational cohort study (BAMSE) in which 98% of children received vitamin A and D in the first year of life. [129] They found no difference in PS rates (defined by sIgE $\geq$ 0.35kU/L) at 4 years of age in children who took oil based vitamin supplements (containing Arachis Oil) (n=2288) versus those who took water based vitamin supplements (without Arachis Oil) (n=159). [129] These results were adjusted for parental atopy, maternal age, maternal smoking, fish consumption and breastfeeding. In fact, children who had received oil based vitamin D supplements (containing Arachis Oil) had a lower risk of asthma, food hypersensitivity and sensitization to egg white, birch pollen, cat and horse allergen.

Current data thus indicates that it is unlikely that Arachis oil in medication is a risk factor for developing PS or PA. However, peanut oils are refined in vitamin supplements and other medication thus are unlikely to contain any peanut protein. This has been demonstrated by the lack of SPT reactivity [101] and lack of clinical reactivity in peanut allergic patients [130] to refined peanut oil. To date, no study in humans has assessed the impact of low dose peanut consumption during infancy and the development of PA; thus, as yet, there is no clear evidence as to the role of low dose peanut oral exposure in infancy and PS or PA.

2.7 Public health recommendations

In 1998 the Department of Health Working Group for the Committee on Toxicity (COT) of Chemicals in Food, Consumer products and the Environment issued a report recommending that mothers whose unborn children had a first degree relative with atopic disease ‘may wish’ to avoid peanut during pregnancy and breastfeeding. This document also stated that children ‘*should*’ avoid peanut and foods containing peanut

products in the first 3 years of life as this *'may increase the risk of sensitization and should be avoided'* (p.29). [131]Since the introduction and dissemination of these recommendations in 1998 the prevalence of PA has not significantly reduced, [15] and may have increased (between 2001-2005 prevalence of PA diagnosed in primary care doubled in England according to QRESEARCH national health database). [132]Although this suggests that peanut avoidance in pregnancy, breastfeeding and early life is not effective at reducing PA, there are several explanations as to why this may not have been effective. Data suggests that the COT 1998 advice was only implemented correctly by a low percentage of the atopic population and mainly by families without an atopic history. [16] There are also other factors such as a rise in the incidence of eczema from when this recommendation was introduced (QRESEARCH UK national database showed a 42% increase in eczema diagnosis between 2001-2005)[133] which may have negated any protective effect this dietary recommendation may have had.

The 2007 House of Lords Science and Technology Committee report on Allergy highlighted that these recommendations may have resulted in an increase in the incidence of PA and thus recommended that *'this advice should be withdrawn immediately'* (key recommendation 6.57). [1] A systematic review of studies published in this field was thus funded by the Food Standards Agency and performed by Thompson et al. (2008) ten years after the 1998 COT recommendations; [134]they found no evidence that maternal consumption of peanuts during pregnancy or breastfeeding increased the risk of PS or PA in the child. The findings from that review, in addition to the Cochrane review performed in 2006 [135] (updated in 2012 see Section 2.3.2.2), led to a revision of the recommendations from The Working Group for the Committee on Toxicity of Chemicals in Food, Consumer products and the Environment in 2009.

The 2009 COT recommendations no longer advocate the avoidance of peanut during pregnancy, lactation or early childhood, rather they provide the following more general recommendations:

'(i) In common with the advice given for all children, infants with a parent or sibling with an atopic disease should be breast-fed exclusively for around 6 months;

(ii) Infants and children who are allergic to peanuts or peanut products, should not consume them or foods that contain them;

(iii) Those who are allergic to peanut should seek advice from medical professionals about avoidance strategies.' (p. 25-26) [100]

For both the 1998 COT recommendations and 2009 COT recommendations, there was very little advice provided to medical professionals on how to advise their patients. This may have been in part the reason why the original 1998 COT recommendations were incorrectly implemented and may also lead to unforeseen complications in implementing the 2009 COT recommendations.

The 1998 COT report did state that sensitization to peanut might occur through the skin, '*particularly abraded or damaged skin'* (p.11) through the application of peanut oil containing creams. However, they then stated that *'given such creams contain refined oil, dermal sensitization is unlikely*' (p.11).[131] Consideration was also given as to whether inhalational or cutaneous exposure (via hand to hand transmission) led to sensitization, but no further scope or recommendations were made on the basis of this. Epicutaneous exposure was addressed in the Committee on Toxicity statement on the review of the 1998 COT recommendations on peanut avoidance. [100] They stated that although mouse data supported the role of epicutaneous sensitization, there were not enough human data, as yet, to make Public Health recommendations. They highlighted that further studies were needed in humans: '*to determine whether and to what extent the skin and respiratory tract are important routes of sensitization to peanut and other food allergens, and if they are, to determine the importance of timing and dose, and the underlying mechanisms*' (p.26).[131]

In the next theory section I will review the literature on the role of epicutaneous exposure in the development of PS and PA. However, first I will discuss eczema and its relationship with food allergy as the consistent association between early onset, severe eczema and PA is one of the arguments used to support the hypothesis of children becoming sensitized through inflamed and broken skin (as is found in eczema).

Chapter 3 Epicutaneous sensitization

3.1 Eczema – nomenclature and prevalence

Eczema is a chronic skin condition characterised by an itchy scaly rash. Atopic eczema is associated with the production of IgE against common food and aeroallergens. [136] The prevalence of eczema is increasing. In a longitudinal cohort study on the Isle of Wight, UK the population questionnaire based 'current' eczema rate was 11.9% (145/1214) at 4 years of age in 1993 and 14.3% (194/1358) at 10 years of age in 1999. The rate of 'eczema ever' at 10 years of age was 563/1373 (41%). [137] The ISAAC study (International Study of Asthma and Allergies in Childhood), assessed questionnaire based allergic conditions in over 100 different countries and found an increase in 'current' eczema over a 12 month period from 13% (1994-1995) to 16% (2002-2003) in 6-7 year old UK children. Evaluation of GP records in England from 2001 to 2005 (QRESEARCH database) found that the diagnosis of eczema had increased by 42%, predominantly in children, [133] although this may be secondary to increased recognition of eczema in primary care.

3.2 Eczema pathogenesis

The pathogenesis of eczema was originally thought to be a consequence of an immunological abnormality leading to an inflammatory dermatosis (the inside–outside pathogenesis hypothesis). [138] Acute eczematous skin lesions are characterised by spongiosis (epidermal intracellular oedema) and cellular infiltrates (particularly CD4+ activated Th cells). This inflammatory process was thus thought to lead to an impaired skin barrier. More recently, research groups have argued that the permeability barrier abnormality in eczema is not merely a secondary consequence of disease activity but rather the driver of disease activity (the outside-inside pathogenesis hypothesis). [139] The outside-inside pathogenesis hypothesis gained significance, in part, due to the discovery of the filaggrin (*FLG*) gene (see Section 4.1), and many other

genes responsible for the integrity of the skin barrier (see Section 4.2) which were shown to be important risk factors for the development of eczema. In additional support of the outside-inside pathogenesis hypothesis, eczema severity is associated with skin barrier permeability; uninvolved skin in patients with known eczema still displays barrier function abnormalities and treatments which improve skin barrier function by targeting lipid abnormalities (which lead to barrier abnormality) have anti-inflammatory effects.[140] It is thus feasible that, in the presence of an already impaired skin barrier, irritants, pathogens and allergens (such as peanut) might penetrate the skin leading to an inflammatory milieu which predisposes to allergic sensitization and subsequent allergy.

3.3 IgE mediated food sensitization in children with a history of infantile eczema

Eczema is often cited as the first step in the allergic march.[141;142] One of the seminal papers evaluating the link between eczema and IgE mediated food sensitization was the Melbourne Atopy Cohort (MAC) study.[143] In the MAC study, 620 children were recruited after birth based on a first degree relative with eczema, asthma, allergic rhinitis or IgE mediated food allergy. The study coordinators selected an additional group of 70 infants referred consecutively to their Paediatric Allergy Clinic with severe eczema unresponsive to emollients and topical steroids. Eczema was defined according to the criteria of Hanifen and Rajka which had been modified by a UK working party; [144] this included a history of eczema, or visible flexural dermatitis, a history of dry skin and/or pruritis, onset under 2 years and a personal history of asthma. 'IgE mediated food sensitization' in the MAC study was defined as a SPT twice the size of the histamine control to cow's milk, egg or peanut. This usually meant an SPT wheal size of at least 6mm, which for children under 2 years of age would be highly predictive of food allergy (in one case series all OFCs were positive in children <2 years of age with SPT wheal diameters of 6mm for cow's milk, 5mm for egg, and 4mm for peanut).[145]

At one year of age children in the MAC study with mild eczema had an approximately 3-fold increased risk of 'IgE food sensitization' (44/121 = 36%) compared with infants without eczema (44/394 = 11%). Children with severe eczema had an approximately 6-fold increased risk of 'IgE food sensitization' (19/29 = 65%) compared with infants without eczema (44/394 = 11%). Thus, this study not only showed that eczema was a risk factor for food sensitization (and likely allergy given the SPT ≥ 6mm definition of sensitization), but also that severity of eczema conferred an additional risk for the development of food sensitization.

The same group then assessed the association between eczema and food allergy using screening data from the EPAAC (Early Prevention of Asthma in the Atopic Child) study. [146] The EPAAC study was a multicentre international study with 2218 children (mean age 17 months) that had active eczema and a positive family history of atopy and/or asthma. The EPAAC study was designed to assess whether regular use of second-generation long-acting antihistamines might prevent the development of asthma in atopic children. At the screening visit eczema was assessed using a Scoring Atopic Dermatitis (SCORAD) index. IgE mediated food allergy was assessed using PPV for ImmunoCAP previously established by diagnostic OFCs for cow's milk (90% PPV under 1 year ≥2.5kU/L),[147] egg (94% PPV under 2 years ≥0.35kU/L) [148] and/or peanut (95% PPV aged 3.8 years ≥14kU/L). [149] Using these predictive cut-offs, the rate of IgE mediated food allergy was associated with early onset (Figure 7**)** and more severe eczema (in early onset eczema) (Figure 8).

Figure 7: Onset of eczema and IgE mediated food allergy in the EPAAC study.

Created using data obtained from Hill et al. (2007) [146]

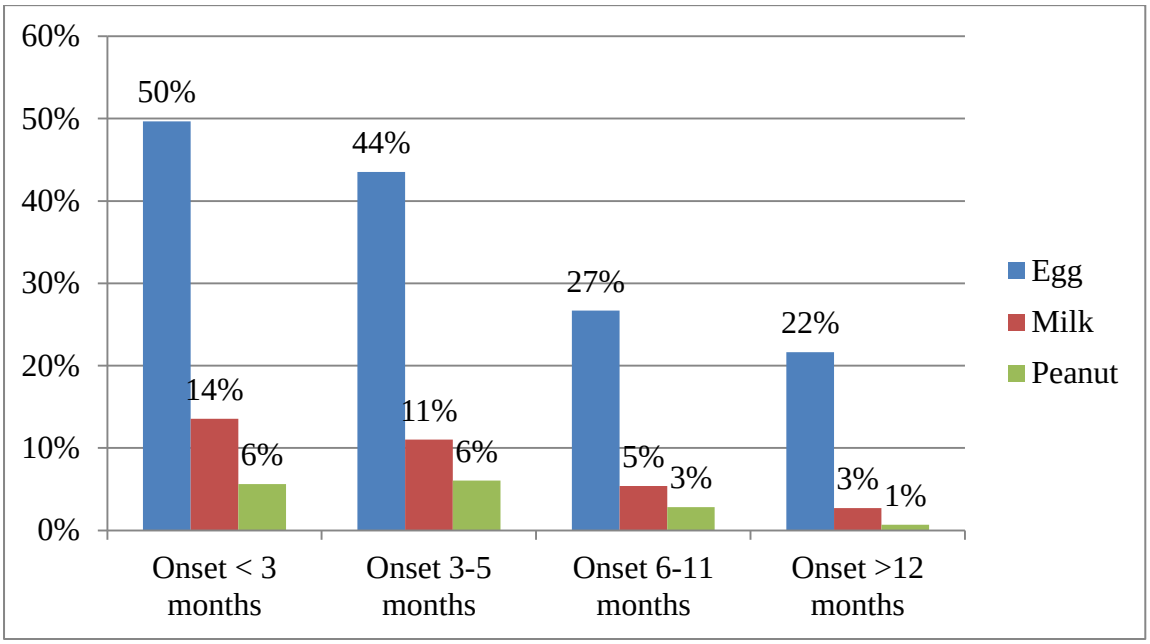
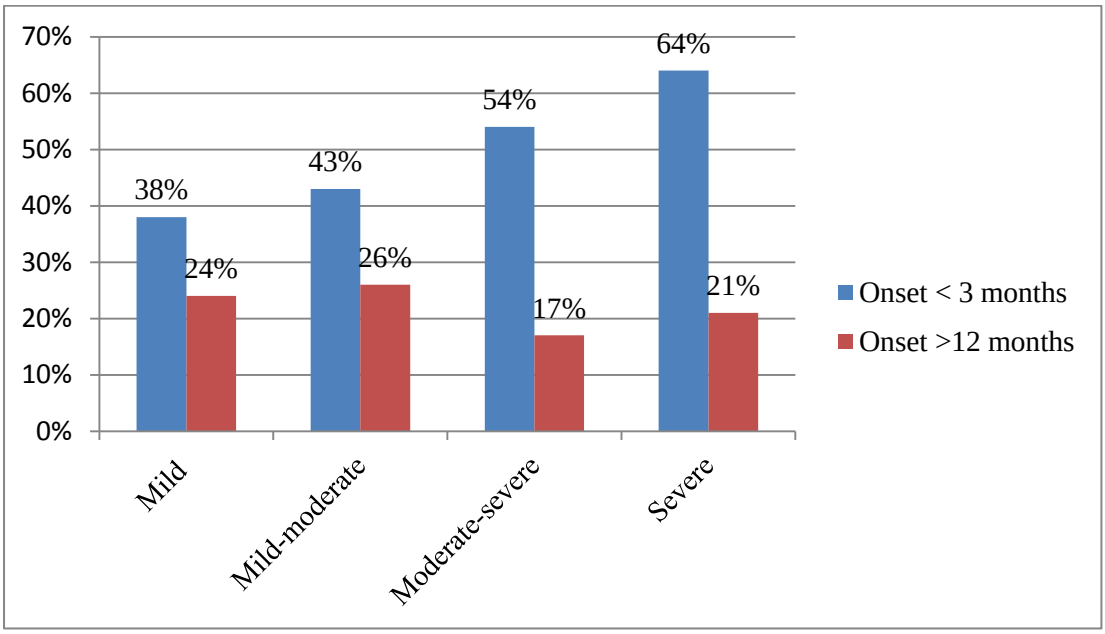


Figure 8: Eczema severity and IgE mediated food allergy (defined by 90-95% PPV) in the EPAAC study.

Created from data obtained from Hill et al. (2007) [146]



3.4 IgE mediated food allergy in children with a history of infantile eczema

In the general population, the rate of confirmed food allergy in children ranges between 5-8%;[2;3] however this is much higher in children with atopic eczema. The European Academy of Allergy and Immunology (EAACI) published a position paper on eczematous reactions to food in atopic eczema.[150] The prevalence of food allergy in children with eczema ranged from 33-81% in 8 studies (Table 1). [151-158] I performed a random effects statistical analysis on the rate of clinically confirmed IgE mediated food allergy in children with eczema in the studies published in this review, including the prevalence of food allergy and sample size of each study. The pooled rate of food allergy in children with eczema (ranging from mild to severe) was 50% (95% CI: 37-62%) (Figure 9, p83). This calculation was obtained using random effects by incorporating the sample size and percentage of children with IgE mediated food allergy. The EAACI position paper incorrectly stated that in the study by Niggemann et al. (1999) the rate of IgE mediated food allergy in children with eczema was 51%; this is because they used the number of positive challenges 51% (131/259 challenges) rather than the number of children with at least one positive challenge which was 87/107 (81%). [150]

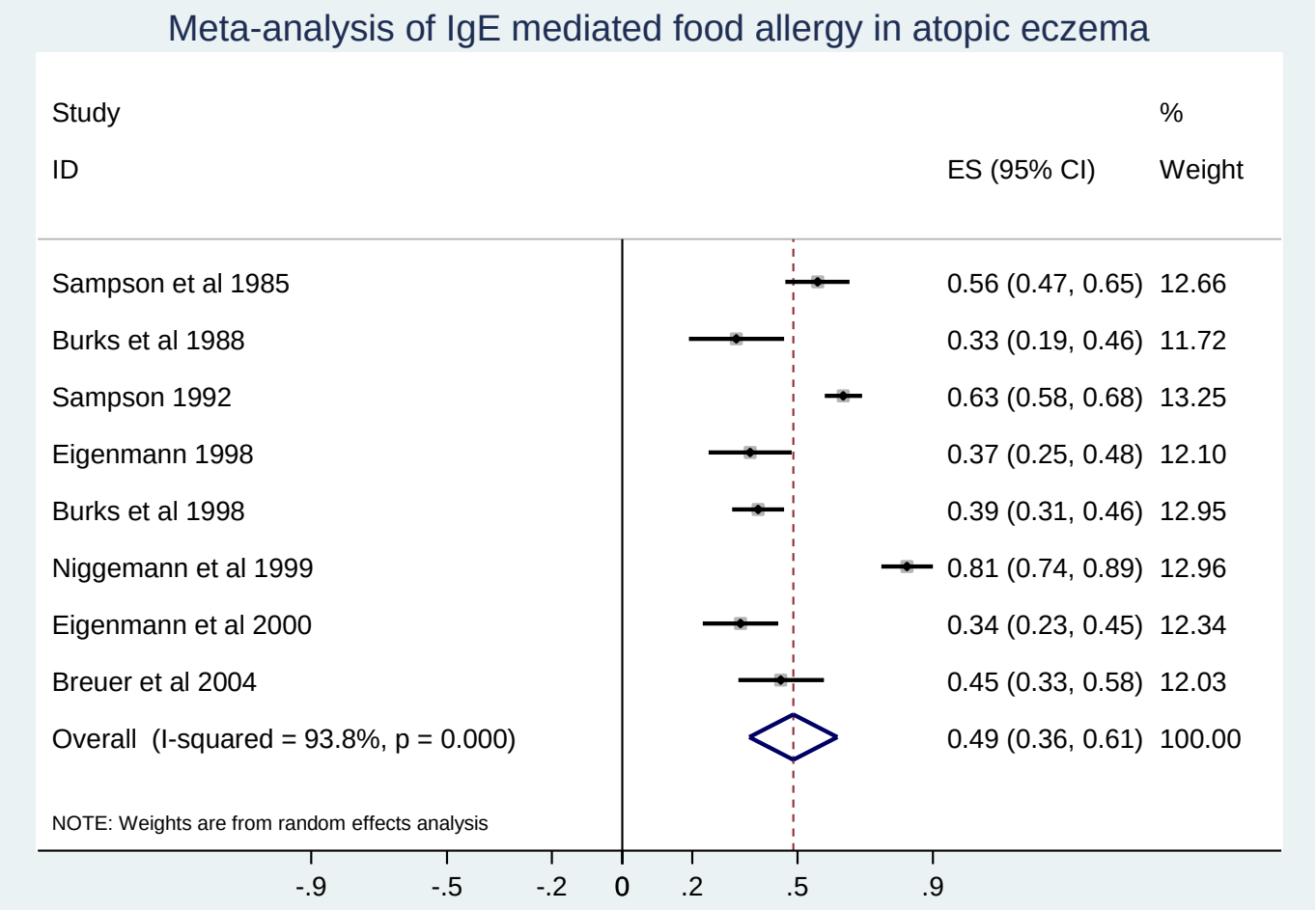
Eczema within the first 6 months of life was specifically shown to be a risk factor for PA (confirmed by diagnostic OFC) in the ALSPAC study, a longitudinal birth cohort study (see Section 2.1).[49] There was an association between PA and a rash over joints and skin creases in the first 6 months of life (OR 2.6; 95% CI: 1.4-5.0), and an oozing, crusted rash in the first 6 months of life (OR 5.2; 95% CI: 2.7-10.2). Additionally, there was a dose-response for PA with increasing severity of eczema within the first 6 months of life (the most severe eczema category had an OR 43.5, 95% CI: 5.79-327.13). Questionnaires to determine rashes observed in the child and their severity were collected prospectively at 1, 6, 15 and 16 months of age.

Table 1: Challenge proven IgE mediated food allergy in children with eczema

Study[150]	Severity of eczema	% IgE food allergy	Method of IgE mediated food allergy assessment	Foods responsible (in order of frequency)
Sampson et al. J Pediatr.1985 [151]	Severe	56%	Prospective analysis of DBPCFC	Egg, peanut, milk
Burks et al. J Pediatr. 1988 [152]	Mild to severe	33%	Prospective analysis of DBPCFC	Egg, peanut, cow's milk, fish, wheat, cashew, soy
Sampson Act DermVen1992[153]	Moderate-severe	63%	Prospective analysis of DBPCFC	Egg, peanut , cow's milk
Eigenmann et al. Pediatr 1998[154]	Moderate-severe	37%	OFC, ImmunoCAP >95% PPV, reaction + sensitization	Egg, cow's milk, wheat, peanut, barley
Burks et al. J Pediatr 1998[155]	Mild-severe	39%	Prospective analysis of DBPCFC	Egg, cow's milk, wheat, soya (peanut not tested)
Niggemann et al. CEA. 1999 [156]	Moderate-severe	81%	Retrospective analysis of DBPCFC	Egg, milk, peanut and soy
Eigenmann et al. PAI 2000.[157]	Mild to severe	34%	OFC, ImmunoCAP >95% PPV, reaction + sensitization	Eggs, peanut, milk, wheat, fish, soy, tree nuts.
Breuer K et al. CEA. 2004[158]	Mild to severe	45%	Retrospective analysis of DBPCFC	Cow's milk, egg, wheat and soy

*Double-blind placebo-controlled food challenge (DBPCFC)

Figure 9: Confirmed food allergy in children with eczema using random effects



On the basis of this and other studies there are now several guidelines which encourage clinical assessment of food allergy in young children with severe eczema. For example, the 2007 National Institute of Clinical Excellence (NICE) guidelines for the management of atopic eczema in children recommends that:

'*Healthcare professionals should consider a diagnosis of food allergy … in infants and young children with moderate or severe atopic eczema that has not been controlled by optimum management* (p. 7).'[159]

3.5 Epicutaneous exposure to Arachis (peanut) oil

Among peanut allergic children with eczema in the ALSPAC study (see Section 2.1), 90% had been topically exposed to creams containing Arachis (peanut) oil in the first 6 months of life.[49] The authors argued that exposure to peanut through the skin may have led to PS and subsequent PA in these children. The ALSPAC study also assessed maternal use of Arachis oil-containing nipple creams whilst breastfeeding, which would imply oral exposure as well as cutaneous exposure and found no difference in maternal Arachis oil-containing nipple cream application comparing children who went on to develop PA versus those who did not develop PA. Questionnaires on creams used for the infant or nipple creams were obtained retrospectively; however they were obtained prior to the diagnosis of peanut allergy. Parents were asked to list the creams used and the researchers subsequently evaluated whether the creams contained Arachis oil. Thus although the retrospective nature of this information may have resulted in errors due to lack of recall, as parents are highly unlikely to have been aware of the Arachis oil content of these creams thus this is unlikely to have led to recall bias.

In 2003 the Committee on the Safety of Medicine from the Medicines and Healthcare products Regulatory Agency (MHRA) reviewed the potential issue of Arachis oil in topical medicinal products and concluded that, because pharmaceutical grade oil is refined during the manufacturing process and only very small amount of peanut protein remain in refined peanut oil, there was '*insufficient evidence to conclude that peanut oil leads to sensitization to peanut protein*' (p. 5). [160] Hourihane et al. (1997) performed DBPCFC with refined peanut oil on 60 individuals with known PA, and none reacted.[130] However, a previous study showed that refined peanut oils used in food trademarks Ready, Imperial, Beffroi, Lesieur, Amphora and Homa contained 100-200 ng protein per gram of oil (by Protein Reagent Assay); using Western blot analysis the authors demonstrated the presence of a 18kDa molecular sized protein (similar to Ara h 2) which was recognised by the serum of two peanut allergic patients.[161]

Most eczema creams no longer contain Arachis oil, although Dermovate NN Cream (Chemidex Pharma), a 'very potent' topical steroid which includes antibiotics and antifungal medication contains Arachis Oil but has been discontinued in the UK. However, creams used for nappy rash, such as Zinc and Castor Oil Ointment (L.C.M Ltd, UK) and Siopel barrier cream (DermaUK, UK), and cream used for broken skin (Naseptin, Alliance Pharmaceuticals, UK) still contain Arachis oil. [162;163] Roaccutane, a cream currently used to treat moderate to severe acne also contains Arachis oil. There is a case report (in abstract form only) of a 3 year old child with peanut allergy and eczema who developed eczematous flare following application of a topical steroid (Flucinolone Acetonide 0.01%) which contained Arachis oil.[164] The abstract stated that peanut protein was measured in this cream but did not specify the quantity in the abstract. Personal communication with one of the authors (Dr Soheila Maleki, US Department of Agriculture / Agricultural Research Service, February 2015) informed me that they measured between 10-20ng/ml peanut protein in this cream. Other historical uses of Arachis oil are in the intramuscular adrenaline preparations that were used for status asthmatic (no longer in use) and rectal paraldehyde for use in status epilepticus which used to be made up in Arachis oil but now is pre-prepared in olive oil instead of Arachis oil.

3.6 Animal models of epicutaneous allergen exposure

In order to prove a causal relationship between epicutaneous allergen exposure and the induction of various allergic sequelae, several mouse models have been employed to date. In the sections below I will describe the mouse models that have been employed to prove the causal link between epicutaneous allergen application and the induction of eczema, allergen specific sensitization and anaphylaxis, allergic airway responses and eosinophilic gastrointestinal disease in mice.

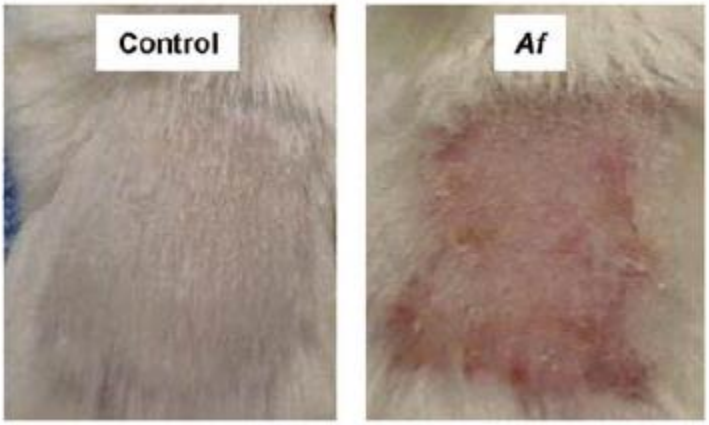
3.6.1 Animal models of epicutaneous allergen application leading to eczema

In several murine models, epicutaneous exposure has been shown to lead to local eczema at the site of antigen application. The eczema at the site of allergen application was characterised by spongiosis, infiltration by CD4+ T cells, eosinophils, mast cells and local expression of mRNA for the cytokines IL4, IL5 and IL13. [165;166] This mimics the immunohistological analysis of affected acute eczematous lesions in humans where there is a significant increase in the number of cells expressing IL4, IL5 and IL13 mRNA and protein, suggesting preferential accumulation of Th2 cells. In mouse models, local eczema eruptions following epicutaneous exposure resolved with treatment with topical steroids. [166] Figure 10 depicts the eczematous reaction of murine skin which was shaved with an electric razor and then had a patch applied with Aspergillus fumigatus (a mould allergen which is commonly used to induce inhalant sensitization) versus skin which was shaved but had only phosphate buffered saline (PBS) applied as a patch to the skin as a negative control. [167] This experiment demonstrated that it was the application of Aspergillus fumigatus rather than just the skin shaving or patch application that led to eczema.

Spergel et al. (1999) showed that eczema following epicutaneous allergen application was Th2 dependent using mice genetically modified to be deficient in IL4 and IL5. [168] In their study murine skin was shaved with an electric razor and a patch of OVA was applied. OVA-patched skin from IL5 and IL4 deficient mice had no or significantly reduced eosinophils, reduced epidermal thickening and no thickening of the dermis in contrast with control mice. These results suggest the presence of a Th2 pathway for eczema induced by epicutaneous application of antigen onto shaved skin.

Figure 10: Skin of BALB/c mouse following epicutaneous exposure to Aspergillus fumigatus vs. PBS.

Reprinted from Akei et al. (2010) with permission from Elsevier.[167]



3.6.2 Epicutaneous sensitization and allergen specific systemic reactions in mice

In murine models, epicutaneous sensitization to food allergens has been shown to lead to systemic reactions, including fatal anaphylaxis, on subsequent oral exposure. This is relevant as oral exposure to an allergen (to which the individual is allergic) is the usual means by which humans have more severe allergic reactions. In an experimental model Hsieh et al. (2003) shaved the skin on the back of BALB/c and C57BL/6 mice and applied a 100 µg OVA impregnated patch for a week on three consecutive occasions. [169] Seven days following the last patch application, these mice developed high OVA-sIgE and IgG1 (capable of inducing mouse mast cell degradation and anaphylaxis). Following epicutaneous sensitization, mice were challenged with OVA (50mg) via an intragastric feed and anaphylaxis was confirmed by symptomatology, raised histamine levels in their serum and histological changes in the gut and lungs. Mice developed anaphylaxis at either Grade 3 (wheezing, laboured respiration, and cyanosis around the mouth and tail) or Grade 4 (slight or no activity after prodding, or tremor and convulsion) in 7 out of 8 (88%) cases. This allergic reaction was Th2 dependent, as when BALB/c mice were injected intraperitoneally with anti-IL4 antibodies, epicutaneous application of OVA failed to provoke an IgE mediated allergic reaction on subsequent oral challenge. Several murine studies have further

evaluated different aspects of epicutaneous sensitization, such as the need for an impaired skin barrier to achieve sensitization, the form of peanut protein more likely to induce epicutaneous sensitization and the comparison of epicutaneous sensitization with other forms of sensitization.

3.6.3 Epicutaneous sensitization dependent on type of epidermal allergen application

Animal models have shown that epicutaneous exposure to 100 µg of peanut protein or OVA induces a potent allergic Th2-type response, associated with IL4 secretion by T cells from draining LNs and high levels of peanut or OVA sIgE respectively. [165;170-173] In these studies mice only developed epicutaneous sensitization if the skin was abraded before application of peanut. This was achieved by removing the stratum corneum (or cornified layer) from the ear with 6-8 repetitions of adhesive tape or the back by shaving in order to mimic the desquamation of skin in eczema and other dermatoses with an impaired skin barrier. Strid et al. (2004) stated that if the cornified layer was not disrupted, there was no antibody response following antigen application. [170] Strid et al. (2004) also showed that when 100µg of peanut protein or OVA was injected subcutaneously (into the dermal layer) rather than epicutaneously (onto the cornified layer) this gave rise to a predominantly Th1 non-allergic response. [170]

Conversely, a recent study by Tordesillas et al. (2014) showed that repeated applications of defatted crude peanut extract from roasted peanuts onto intact skin of C3H/HeJ and BALB/c mice induced PS to the major peanut allergens Ara h 1 and Ara h 2 and anaphylaxis upon IP peanut rechallenge.[174] Mice underwent hair removal using a depilatory cream or direct application of peanut onto the ear without skin stripping and the cornified layer was confirmed to be intact on histology. These authors postulated that peanut protein was able to sensitize through the skin without the need for cornified layer disruption or an adjuvant because peanut protein

itself had adjuvant properties. In support of this, Tordesillas et al. (2014) showed that peanut antigen led to bystander sensitization for the milk allergen α-lactalbumin. Having previously shown that application of milk allergen α-lactalbumin onto intact skin did not lead to sensitization or allergy on rechallenge, the authors then showed that concomitant application of peanut protein in addition to milk allergen α-lactalbumin onto intact skin led to anaphylaxis on subsequent oral exposure to milk allergen α-lactalbumin.

3.6.3.1 Epicutaneous sensitization is dependent on the form of peanut applied to the skin

Recently, Moghaddam et al. (2014) showed differential effects of topical application of dry roasted peanut antigen versus raw peanut antigen onto eczema-like skin lesions.[175] On subsequent oral gavage with roasted peanut, there was significantly higher peanut sIgE, Th2 cytokines from mesenteric LNs and eosinophilic infiltration of the lamina propria in mice that had received epicutaneous sensitization with dry roasted peanut than raw peanut. The authors postulated that dry roasting conferred greater immunogenicity to peanut antigen due to oxidation-driven generation of advances glycation end (AGE) products, however little information was provided on how this might come about. Nevertheless, differences in peanut preparation may also be important for epicutaneous sensitization. Dry roasted peanut have also been shown in in vitro work to bind higher levels of IgE and enhance some of the functional properties of the allergen (such as increasing the trypsin inhibitor function of Ara h 2 thus providing more resistance against digestive enzymes).[25;176]

3.6.3.2 Epicutaneous versus systemic (IP) sensitization and subsequent anaphylaxis on oral challenge

Bartnikas et al. (2013) explored the mechanisms behind epicutaneous sensitization leading to subsequent anaphylaxis on oral challenge.[177] Epicutaneous OVA and/or peanut application onto tape stripped skin of BALB/c mice led to intestinal mast cell expansion, increased serum IL4 and food-induced anaphylaxis. Following epicutaneous sensitization, systemic anaphylaxis was induced following a single oral challenge with

the respective allergen with an associated reduced core body temperature and raised MMCP-1 compared with mice sensitized with PBS. Where sensitization was achieved via intraperitoneal (IP) injection, anaphylaxis was achieved only after multiple oral challenges; thus epicutaneous sensitization seemed to prime the gut in a more effective manner for subsequent allergic reactions than systemic sensitization (IP). Researchers were unable to elicit anaphylaxis to an oral challenge with OVA after sensitising via the oral route with 5mg OVA plus adjuvant (CT), although they did find a rise in OVA sIgE levels. Mast cells numbers in the jejunum increased after OVA epicutaneous sensitization (2.4 fold) but not after OVA oral sensitization, which may explain the resistance to anaphylaxis in the orally sensitized mice. When researchers performed epicutaneous sensitization in mice bred to be deficient in IgE, there was no intestinal expansion of mast cells, no rise in serum IL4 and no features of anaphylaxis on intragastric challenge, which suggested that the effects arising from epicutaneous sensitization are IgE dependent.

3.6.4 Epicutaneous sensitization and allergic airway responses

There are murine studies which suggest that epicutaneous sensitization may also lead to allergic responses in the airway. [165;169;171;173] In a study by Spergel et al. (1998) 100μg of OVA was applied to the shaved skin of BALB/c mice, which resulted in localized eczema at the site of application. [165] Subsequent aerosolized OVA induced eosinophilia in the bronchoalveolar lavage fluid and airway hyper-responsiveness to methacholine which was 10 times greater than in PBS sensitized mice. In another study, epicutaneous sensitization followed by a single nasal challenge to Aspergillus fumigatus induced clinical nasal symptoms (sneezing and itching), airway hyper-responsiveness and sneezing to methacholine, systemic raised IL4 and Aspergillus fumigates specific IgG1 and nasal mucosal eosinophilia. [167] Mice deficient in STAT6 (a Th2 transcription factor: see Section 1.6) who underwent the same procedure were completely protected from nasal mucosal eosinophilia, clinical symptoms and nasal/ airway hyper-responsiveness. This also implies a Th2 dependent mechanism for epicutaneous sensitization leading to allergic responses in the upper and lower airway.

Unfortunately, no lung-homing markers for Th cells have as yet been identified, thus the mechanism of sensitization via the respiratory tract cannot be assessed by isolating these Th cells to assess recall responses to peanut.

3.6.5 Epicutaneous sensitization and eosinophilic eosophagitis

Epicutaneous exposure onto abraded skin in mice does not only lead to eczema, respiratory and systemic allergic responses but also primes the mouse for developing eosinophilic eosophagitis [178] via a Th2 dependent method. BALB/c mice were shaved and allergen (100 µg of OVA or 100 µg of Aspergillus fumigatus mould) was repeatedly applied using a patch onto abraded skin. Eosinophilic inflammation of the oesophagus was only achieved if epicutaneous sensitization with OVA or Aspergillus preceded an airway challenge with OVA or Aspergillus respectively. [178] Using mice deficient in IL4, 5, 13 or STAT6 (a Th2 transcription factor) Akei et al. (2005) found that IL5 was the most important Th2 mediator for eosinophilic eosophagitis but that IL4, IL13 and STAT6 also contributed towards its pathogenesis. IL13 has also been shown to directly decrease *FLG* expression in the eosophagus of humans with eosinophilic eosophagitis. Eosophageal expression of *FLG* mRNA is downregulated in humans with eosinophilic eosophagitis, and normalizes after successful eosinophilic eosophagitis treatment. [179] However, *FLG* mutation 2282del4 is also overrepresented in patients with eosinophilic eosophagitis, thus may have a role in its aetiology. *FLG* is expressed in the oral mucosa (mRNA and protein), [180] and may be expressed in the upper esophageal mucosa (mRNA but not protein) [181] and stomach (mRNA only) [182] but is not present in the small intestine.[182] (see Table 15, p264).

3.6.6 Epicutaneous exposure prevents oral tolerance acquisition

BALB/c mice underwent epicutaneous sensitization to peanut which induced peanut specific T cell proliferation from LNs and spleen, and serum peanut sIgE, IgG1 and IL4. [183] When these mice were fed 100mg of peanut

protein 20 days after epicutaneous sensitization (which has been previously been shown to induce oral tolerance in Section 2.5), this led to even higher levels of peanut sIgE/IgG1 and IL4 from draining LNs, peanut specific T cell proliferative responses and hind foot pad swelling (following peanut injection) than PBS fed control mice. Additionally, epicutaneous peanut exposure disrupted previously established oral tolerance to peanut, and led to peanut specific T cell proliferation, IL4 and IL10 (a Th2 cytokine in mice)[184] production and hind foot-pad swelling (following peanut injection) greater than control mice. Thus epicutaneous peanut exposure onto abraded skin was both able to prevent oral tolerance induction and partially switch off pre-established peanut oral tolerance. This may explain why some children develop certain food allergies after many years of tolerating these foods in their diet, and why some children are resistant to oral tolerance induction. Epicutaneous exposure to peanut in a child with a disrupted inflamed skin barrier could lead to immunological changes via facilitated antigen presentation (see section 3.10) and lead to a Th2 predominant response to peanut; this could both initiate a de novo allergy (usually in a child no longer regularly consuming peanut) or prevent the acquisition of tolerance during both the normal weaning process in infancy and in oral peanut immunotherapy trials.

3.6.7 Summary of animal evidence and limitations of extrapolation to humans

There is a significant body of animal evidence that indicates that epicutaneous exposure onto abraded skin primes the mouse for allergen specific systemic, airway and gastrointestinal allergic responses via a Th2 dependent mechanism. More recently allergen specific systemic reactions were replicated after epicutaneous sensitization to peanut on intact skin, as peanut was found to have adjuvant properties that obviated the need for skin abrasion or concomitant adjuvants.[174] However, it is important to highlight the differences between mouse models and allergic processes in humans. Wild mice do not naturally exhibit food or aeroallergen sensitization and related allergies, [185] whereas these are reasonably common in humans and very common in humans who have a history of infantile eczema. Thus the mice used in the experiments described previously have been bred

to mimic humans with a genetic predisposition for atopy. BALB/c mice are bred to be good IgE responders, [186] and C3H/HeJ mice have a point mutation in Toll-like receptors and defect in their innate immune system and LPS-induced cytokine production;[187] thus this influences how these mice respond to specific antigens. Allergen specific IgG1 is bound to the cell surface of mast cells in mice and may leads to degranulation when cross-linked by specific allergens, [188] whereas IgG1 does not lead to mast cell degranulation in humans. IL10 is considered to be a Th2 cytokine in mice, whereas in humans Th1, Th2 and Treg cells secrete IL10, but IL10 is considered to be a predominantly tolerogenic cytokine. [189] Having highlighted only some of the differences between mice and humans in allergic models of disease, it is evident that, although they provide good in vivo mechanistic models of disease, the responses obtained must be taken in context and, where ethically possible, be assessed in humans.[190]

3.7 Epicutaneous immunotherapy

Given the body of literature supporting the concept of epicutaneous sensitization, it is important to briefly consider the recent studies which suggest that is it also possible to desensitize through the skin. Although this might seem contradictory, on further review this body of work provides further understanding as to how antigen may present to immune cells via the skin. In mouse studies there is compelling data that topical application of pollen, HDM, OVA and peanut may desensitize mice. [191-193] Specifically, peanut epicutaneous immunotherapy (EPIT) was shown to be as effective in desensitising mice as subcutaneous immunotherapy (SCIT) in reducing the IgG1/IgG2 ratio, methacholine airway hyperresponsiveness and IL4, IL5, IL13, eotaxin and eosinophils in the bronchoalveolar lavage of mice. [193] In the Arachild pilot study (a multicentre double blind placebo controlled clinical trial), 'Viaskin' patches containing peanut protein (100 μg) versus placebo were applied onto the skin of children with proven peanut allergy.[194] After 18 months 67% of children aged 5-11 years of age were able to eat ten times the amount of peanut that they had tolerated before or at least 1000mg peanut protein

(equivalent of approximately 5 peanuts). A reduction in peanut sIgE and an increase in IgG4 correlated with the response rate of individuals.

In order to better understand the mechanism by which topical application of antigen was supporting a tolerogenic response this research group showed that EPIT only works when the allergen patch is applied onto healthy intact skin. [(195)] Application of peanut onto intact skin led to reduced IgE and a rise in IgG2a (a marker of tolerance induction in mice). Using the same concentration of allergen, timing of application and patch methodology that induced desensitization, stripping the skin of the mouse before applying the patch reinforced allergen sIgE and did not modify IgG2a. In further mechanistic work they showed that, in skin which had been abraded, there was free passage of allergens through the skin into the bloodstream and lymphatics. [(195)] Free passage of allergens through the skin did not occur following EPIT onto intact skin; the powdered form of the allergen was solubilized by perspiration and disseminated into the cornified layer. When allergen (OVA) was placed onto intact skin of BALB/c mice, processed allergen was transported to LNs by migratory Langerhans cells in the epidermis rather than reaching the LNs through the blood stream.[(196)] Additionally they showed that dendritic cell maturation differed in stripped versus intact mouse skin; following skin stripping and application of OVA, migrating dendritic cells were skewed towards higher production of IL5 and lower IFNγ. OVA application onto intact skin in mice led to dendritic cells expressing mixed IL5 and IFNγ responses from draining LNs. [(197)]

These findings therefore conflict with the study by Tordesillas et al. (2014)[(174)] where application of peanut protein using patches onto intact skin led to PS and clinical PA in mice. Tordesillas et al. (2014) argued that the adjuvant properties of peanut antigen were due to peanut inducing an innate immune response from keratinocytes and altering the skin draining APC phenotype via the IL33 receptor which subsequently led to

Th2 cytokine production. Another potential adjuvant property of peanut antigen could be to have proteolytic activity on tight junctions as does Der p 1 (see Section 1.6); however, none of the known peanut allergens have been shown to have proteolytic activity (personal communication, Dr Soheila Maleki, US Dept of Agriculture, New Orleans, March 2015). Peanut lectins (also known as peanut agglutinins) are proteins that bind specifically to carbohydrates and were previously used as an adjuvant in mechanistic assays (personal communication Professor Jonathan Hourihane Feb 2015). However, in the literature when mice were fed peanut agglutinins this led to oral tolerance rather than allergy.[198;199] On the other hand, there are peanut glycans (oligosaccharides) that do enhance the Th2 immunogenicity of peanut.[200;201] Helminths are rich in glycosylated proteins that are recognised by pathogen recognition receptors on dendritic cells leading to Th2 skewing; the glycan of the Ara h 1 glycoprotein binds to the C-type lectin receptor on dendritic cells called dendritic cell (DC)-specific intercellular adhesion molecule-grabbing nonintegrin (SIGN). Binding of Ara h 1 glycan to DC-SIGN facilitates antigen uptake, activation and increased capacity for Th2 skewing in the dendritic cell. [200;202] Thus Ara h 1 may have intrinsic adjuvant properties, other than protease activity that could facilitate epicutaneous sensitization.

3.8 Mechanisms underlying epicutaneous sensitization versus oral tolerance induction.

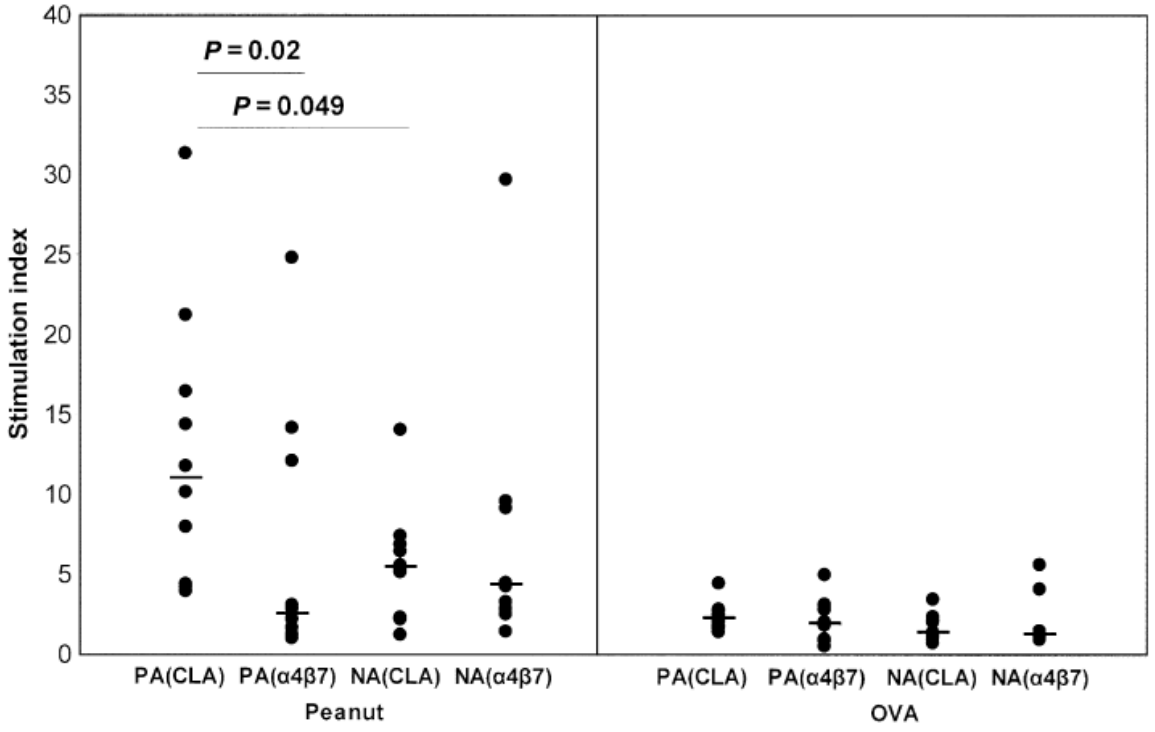
Previous in-vitro work has used homing markers on CD4+ Th cells for the skin (Cutaneous Lymphocyte Antigen: CLA+) and gut-associated-lymphoid tissue (integrin α4β7+) as markers for the route of initial route of sensitization. [203-205] CLA is an adhesion molecule that binds to E-selectin in skin capillaries, ensuring the preferential homing of CLA+ T cells in the skin. Integrin α4β7+ binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed in venules of the mesenteric LNs in the gastrointestinal lamina propria and Peyer's patches. In patients with contact allergy secondary to epicutaneous sensitization to nickel, the nickel specific Th cell proliferation responses were largely confined to CLA-expressing Th cells. [204] In another study,

participant volunteers were orally or parenterally immunized with keyhole limpet haemocyanin (KLH), a respiratory protein isolated from a mollusc that is known to be highly immunogenic in humans. Following sensitization, immunomagnetic selection was used to deplete circulating Th cell with α4β7+ homing markers. In participants that had been orally immunised, depletion of α4β7+ Th cells reduced KLH proliferative responses by a mean of 71%, whereas in parenterally immunised participants, this made no different to proliferative responses to KLH. [(205)] This demonstrated that sensitization to KLH had occurred predominantly through α4β7+ Th cells in humans orally immunised with KLH.

In recent research assessing Th cell memory response to peanut in children with PA there was differential peanut specific Th cell proliferation in peanut allergic (n=10) versus peanut tolerant (n=10) children.[(203)] Peanut specific Th cell proliferative responses were higher in CLA+ Th cells in peanut allergic children than peanut tolerant children. There was mixed CLA/ α4β7 Th cell proliferation in peanut tolerant children (Figure 11). The specificity of Th cell responses was confirmed by also culturing cells from peanut allergic and non-allergic donors with OVA which showed no differential CLA/β7 stimulation index. Cytokine responses from CLA+ peanut-specific Th cells in peanut allergic children showed a trend towards Th2-polarisation (IL13 and IL4 secretion), whereas cytokine responses from α4β7+ peanut-specific Th cells in peanut tolerant children showed a trend towards Th1-polarisation (IFNγ and TGFβ).

Figure 11: Ratio of Stimulation Indices for CLA/β7 Th memory cells in peanut and OVA.

Reprinted from Chan et al. (2012) with permission from Copyright Clearance Center, Inc.[203]



To assess cytokine responses in the peanut allergic versus non allergic children the authors used the Th1/Th2 cytokine ratio (e.g. IFNγ/IL4) to correct for responses from unstimulated cells. Cytokine responses from CLA+ peanut-specific Th cells in peanut allergic children showed a trend towards Th2-polarisation (IL13 and IL4 secretion). Cytokine responses from α4β7+ peanut-specific Th cells in peanut tolerant children showed a trend towards Th1-polarisation (IFNγ and TGFβ). These findings therefore support the hypothesis that peanut exposure through the skin induces sensitization and peanut exposure through the gut induces tolerance.

3.9 Mechanism of epicutaneous sensitization

3.9.1 Langerhans cells

Human skin is exposed to millions of different antigens. The Langerhans cell (LC) is the predominant antigen presenting cell (APC) in the skin and has an important role in initiating Th2 immune responses to antigens. [185] T cells grown with cultured Langerhans cells from mouse ears secrete IL4 and stimulate IgE production by B cells consistent with a Th2 bias. [206] Researchers have demonstrated, through the use of LC depleted mice, that LCs are a prerequisite for the polarization of CD4+ Th cells required for the development of atopic eczema. [207] In mouse models LCs are activated by tape stripping leading to skin barrier impairment; in one study tape stripping alone was sufficient to drive LC migration from the epidermis to skin draining lymph node (LN), and LC precursors from the bone marrow to the epidermis. [208] Another study showed that LC morphology in mice began to mature within 2 hours of tape stripping; the LC dendrites pulled in and the cell body widened until, by 24 hours, the LC were completely oval.[170] The LCs also increased cell surface expression of MHC Class II (see Section1.7.1) consistent with the process of maturation of dendritic cells.[209] Although tape stripping alone did not promote LC emigration to LNs in this study, two hours after application of antigen (peanut or OVA) the majority of LC had migrated away from the epidermis to skin draining LNs. [170] T cells from skin draining LNs produced large amounts of IL4 after epicutaneous immunization but very little IFNγ and IL10. [170] IL4 has been shown to make LC more efficient in taking up and processing antigens. [210]

LC express three receptors likely to be involved in the pathogenesis of eczema and allergic sensitization through the skin: the high affinity receptor for IgE (FCεRI), the low affinity receptor for IgE (FCεRII/CD23) and the thymic stromal lymphopoietin (TSLP)-receptor. [211] The FCεRI on LCs differs from those on effector cells like mast cells and basophils as it is not constitutively expressed. Rather, when it is expressed the efficacy of antigen

uptake and presentation to T cells increases up to 1000 fold [212] via a mechanism now understood as facilitated antigen presentation. [213] This is very important in the context of allergen exposure through the skin as, through facilitated antigen presentation, even tiny amounts of allergen on the surface of the epidermis could lead to activation of allergen specific T cells, especially in children with an impaired skin barrier. In the next sections I will discuss other important mediators of epicutaneous sensitization and their interplay with LC and Th cells.

3.9.2 Thymic Stromal Lymphopoietin

One of the main cytokines responsible for epicutaneous sensitization through an impaired skin barrier is thymic stromal lymphopoietin (TSLP). TSLP is increased in the cornified layer of patients with atopic eczema compared with patients with healthy skin and also positively correlates with eczema severity (using SCORAD).[214] TSLP is highly expressed by the keratinocytes of children with eczema[215] and by the epithelial cells of asthmatics.[216] TSLP deficient mice are protected from developing allergic skin and airway inflammation following antigen exposure,[217-219] which highlights the importance of this cytokine in allergic sensitization. Pro-inflammatory cytokines TNF-α and IL1-α, secreted in response to skin stripping, [220] induce TSLP secretion from human keratinocytes. [221] TSLP levels also increase following topical application of OVA onto tape stripped skin. [177] TSLP expression correlates with LC maturation, upregulation of the TSLP receptor on LCs, migration of LC cells to skin draining LNs where they promote the differentiation of naïve Th cells to Th2 cells and Th2 proliferation. [215;218;222] TSLP has also been showed to induce Th2 skewed APCs to the mesenteric LNs following tape stripping, thus providing evidence of a skin to gut migration. [223] These findings therefore link TSLP to the early stages of epicutaneous sensitization: by increased secretion by skin disruption or epicutaneous allergen exposure and interacting with LCs to prime Th cells.

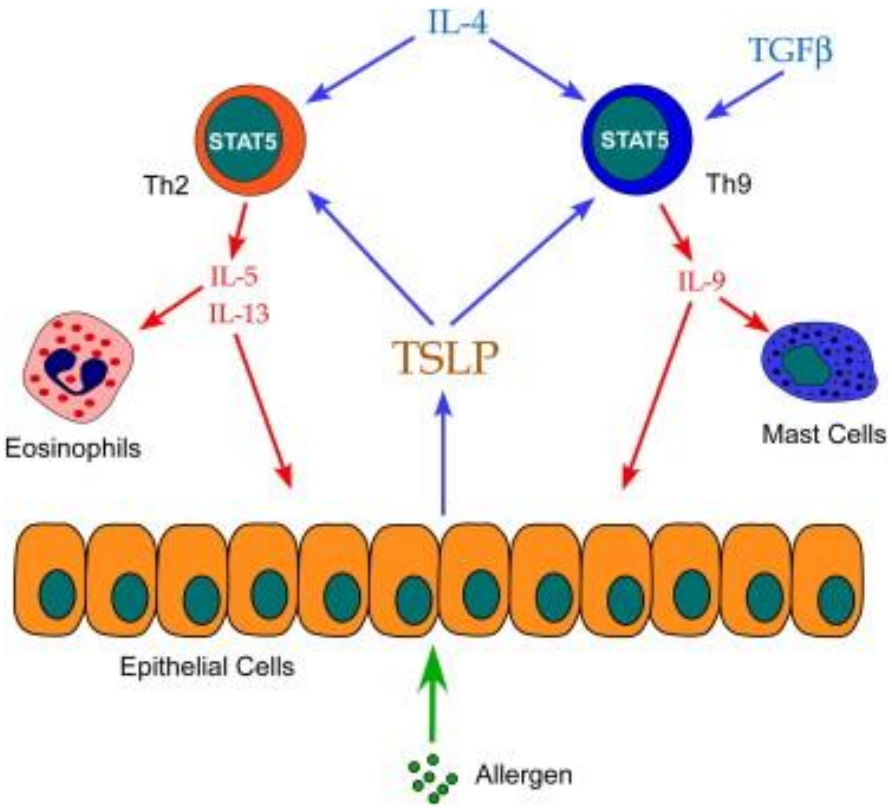
3.9.3 TSLP and IL9 in allergic skin and lung inflammation

As well as TSLP's effects on Th2 differentiation, proliferation and cytokine secretion, TSLP is also linked to the newly discovered Th9 cell (see Section 1.6) and its signature cytokine Interleukin 9 (IL9). TSLP promotes Th9 differentiation and function in human naïve T cells and mice by inducing greater STAT5 binding to IRF4 and GATA3 promotors which are required for Th9 cell development.[224] TSLP also increases *IL9* expression and IL9 production in Th9 cell cultures through a Th9 TSLP receptor.[224] There are greater numbers of TSLP receptors on Th9 cells than Th2 cells, suggesting that TSLP may be even more important for the activation of Th9 than Th2 cells.[225]

TSLP and IL9 are both found in inflamed eczematous skin. Although, no murine model to date examined the interdependency between these two cytokines for epicutaneous sensitization, Th9 cells are required for TSLP-induced allergic inflammation in the lungs. Intranasal challenge with either OVA alone or OVA plus TSLP was performed after adoptively transferring OVA-specific Th9 cells to mice. [224] The addition of TSLP to the OVA intranasal challenge enhanced airway inflammation, IL9 and Th9 cell-mediated eosinophilia in the bronchoalveolar lavage and mucous production.[224] Neutralizing IL9 antibodies in this model decreased eosinophil counts, IL9 and Th2 cytokines in the bronchoalveolar lavage. In contrast using IL13 blocking antibodies reduced airway inflammation and Th2 cytokines but had little effects on IL9 gene and protein levels in the lung. In mice bred to have overexpression of TSLP in their lungs, IL9 was induced and contributed to airway inflammation. In the OVA and TSLP co-administration model, allergic airway inflammation was reduced in $SfpiI^{ck-/-}$ mice that had a T cell PU.1 deletion (Th9 lineage specific transcription factor in mice); this demonstrated the requirement for Th9 cells in mice for TSLP-induced allergic inflammation in the lungs. [224] The relationship between TSLP and IL9 on allergic airway inflammation is depicted in Figure 12.

Figure 12: Interleukin-9 is required for allergic airway inflammation mediated by the cytokine TSLP

Reproduced with permission from Elsevier from Yao et al. 2013.[224]



3.9.4 IL9 and mast cells in epicutaneous sensitization and clinical reactivity

IL9 is raised in the skin[226] and serum[227] of patients with atopic eczema versus patient without atopic eczema; it is even further raised in the serum of children with severe atopic eczema.[227] In mouse models of epicutaneous sensitization, IL9 is induced by tape stripping, [228] potentially via TSLP as described in Section 3.9.3 and Figure 12. One of the main functions of IL9 is to promote mast cell differentiation and proliferation in response to an antigen (see Section 1.6 for function of mast cells in allergic reaction). In the presence of antigen specific IgE and antigen, IL9 also activates mast cells and promotes the secretion of pro-allergic cytokines IL13, IL4, IL5, IL9 and IL10 (a Th2 cytokine in mice) from mast cells in-vitro. [229;230] To date there is no study which has assessed the role of Il9 in mast cell migration from the skin to skin-draining LNs following epicutaneous

antigen exposure; however, there is evidence that IL9 is necessary for mast cells to migrate from the kidney tissue to draining LNs.[231] In this mouse model, infiltration of mast cells in the LNs was abrogated when an anti–IL9 monoclonal antibody was administered. IL9 could similarly play a role for epicutaneous sensitization through mast cell activation, trafficking to draining LNs and induction of pro-allergic cytokines. Once primed antigen specific Th2 cells interact with B cells to promote IgG class-switching to IgE in the presence of IL4; this IgE production is also potentiated by IL9. [232;233] In addition, the effects of IL9 on mast cell proliferation and migration may have an important role in clinical reactivity. The literature supporting the role of mast cells in Ps and PA is described below.

3.9.4.1 Role of mast cell activation and trafficking in epicutaneous sensitization

It is known that mast cells rapidly migrate to the lung mucosa and degranulate after antigen exposure in human asthmatics.[234] In a mouse model of epicutaneous sensitization by Wang et al. (2014), [235] topical application of dinitrofluorobenzene (DNFB) led to mast cell activation and degranulation in the skin. Following epicutaneous antigen application mast cell migrated from the skin to skin-draining LNs; [235] this was demonstrated using two experimental findings: (1) there was a 50% reduction in mast cell density in the skin and 5-fold expansion of mast cells in the skin-draining LNs and (2) fluorescent labelled mast cells injected into the left or right footpads were found in the popliteal LNs of the respective leg following epicutaneously antigen exposure (not in controls). Following migration to LNs, mast cells recruit Th cells to the LN by producing large amounts of the chemokine CCR4 also known as macrophage inflammatory protein (MIP)-1β. In the study by Wang et al. (2014), the primary source of MIP-1β was from mast cells and increases in MIP-1β occurred in parallel with an expansion of T cells in the LNs and was inhibited by 50% with the use of anti-MIP-1β antibodies.[235] Activated mast cells are an important source of pro-allergic cytokines,[236] thus could lead to subsequent Th2 skewing of Th cells within the LN.

Mast cells have furthermore been shown to induce LC migration from the skin to skin-draining LNs. Jawdat et al. (2004) showed an increase in mature LC in mouse skin draining LNs following intradermal injection of anti-trinitrophenyl (TNP) IgE followed by intravenous challenged with TNP-conjugated to bovine serum albumin.[237] Mast cells were necessary for LC migration in this model as mice deficient in mast cells had no migration of LC to the skin-draining LNs. The mechanism by which mast cells induce LC migration is dependent on local mast cell degranulation and histamine release as treatment with anti-histamine abrogated the migration of LCs. Histamine alone however was not sufficient to lead to LC migration as mice deficient in mast cells who had intradermal histamine injections did not demonstrate LC migration. [237] Thus the following mast cell functions: activation (and pro-allergic cytokine secretion), migration to skin-draining LNs, recruitment of Th cells to LNs via MIP-1β and local degranulation and histamine release (leading to LC migration to LNs) provides a multitude of ways in which mast cells are important in bringing together the elements required for induction of a primary immune response in the LN: LCs (carrying antigen), Th cells and a Th2 cytokine milieu.

3.9.4.2 Role of mast cell migration and proliferation in allergic elicitation following epicutaneous sensitization

In the study by Bartnikas et al. (2013) epicutaneous sensitization to OVA and / or peanut through tape stripped skin led to OVA and /or peanut anaphylaxis respectively following oral exposure; this was prefaced by a proliferation of mast cells in the gastrointestinal submucosa. [177] In the mouse model of epicutaneous sensitization by Wang et al. (2014), [235] mast cells were detected in the spleen 24 hours after epicutaneous antigen exposure, which is consistent with subsequent migration from LNs to the blood stream. [235] Thus it is plausible that activated mast cells in the skin could migrate to the gastrointestinal submucosa. The mechanism by which mast cells could lead to allergic elicitation is as follows: in murine oral antigen challenges, antigen in the intestine is first transported in small quantities transcellularly in a mast cell independent manner; however,

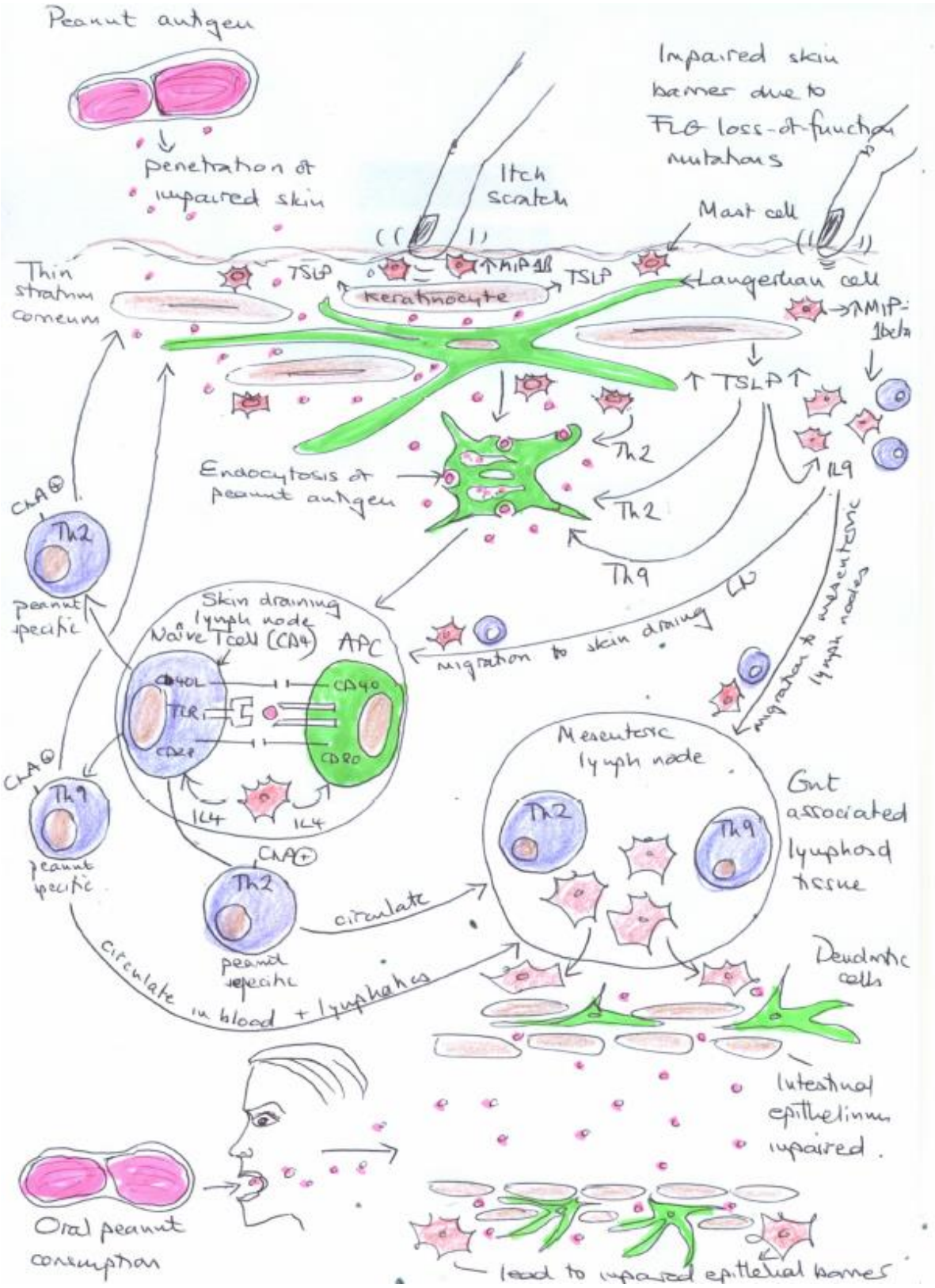
this is followed by massive antigen transport dependent on mast cell activation. [238] Mast cell activation leading to degranulation results in the release of various pro-inflammatory mediators (histamine, prostaglandins and mast cell proteases) that would increase intestinal permeability and could therefore allow large passage of antigen across the intestine, cross-linking the FcεRI receptor on mast cells and leading to allergic manifestations.[238] Moreover, routes of mast cell migration, together with local IL9 induced proliferation at local tissue sites could potentially explain the different manifestations of allergic symptoms; such as predominantly respiratory or gastrointestinal allergic symptoms in addition to cutaneous symptoms.

3.10 Summary of mechanism of epicutaneous sensitization

The interplay between skin barrier function, EPE, LC, TSLP, mast cells and Th2 and Th9 cells proposed in this PhD is depicted in Figure 13 (p106). To summarize, skin barrier impairment through *FLG* loss-of-function mutations and atopic eczema predisposes to mechanical injury of the epidermis, through irritants and allergens in the environment and the itch scratch cycle of eczema. Mechanical injury to the skin results in TSLP secretion from keratinocytes; this leads to LC maturation and migration to skin-draining LNs and mesenteric LNs. When LCs encounter peanut allergen passing through an impaired skin barrier, they present this to naïve Th cell in skin draining LNs and induce Th2 differentiation of CD4+ naïve Th cells. These peanut-specific skin-homing Th2 cells migrate back to the skin and secrete Th2 cytokines (IL4, IL5 and IL13) which attract eosinophils and other pro-inflammatory cells leading to the typical eczematous flare and further skin barrier impairment. Through facilitated antigen presentation on mature LCs (by FCεRI expression) very tiny amounts of peanut allergen might be enough to activate and differentiate naïve peanut specific CD4+ Th cells into a Th2 biased phenotype (see Section 1.6) in the skin-draining LNs.

IL9 may also play an important role in inducing PS following epicutaneous peanut exposure through its effects on mast cell activation, trafficking, cytokine secretion and its effects on B cell IgE class-switching in the presence of IL4. IL9 additionally enables allergic reactions to occur since the main allergy effector cell – the mast cell – requires IL9 to proliferate. Activated mast cells migrate to different areas in the body following epicutaneous sensitization, and gastrointestinal mast cells expansion is required for allergic elicitation oral challenges in mice. Thus the hypothesised pathway for IL9 mediated PS and PA following EPE is as follows (Figure 13): TSLP (secreted following skin barrier disruption) enhances Th9 differentiation and IL9 secretion in the skin which can then exert its effects on mast cells. Thus IL9, stimulated by skin barrier disruption activates mast cells, thereby initiating migration of mast cells to LNs and subsequent Th2 skewing of naïve Th cells within the LN. Activation and local degranulation of mast cells following epicutaneous peanut exposure could also lead to migration of the LC APC to skin-draining LNs thereby facilitating interaction between Th cells and LCs, leading to PS. Once Th2 skewed, the secretion of IL4 drives IgE class switching by B-cells which is enhanced by IL9. In mouse models, peanut applied onto disrupted skin leads to mast cell expansion in the gut and induces peanut anaphylaxis on subsequent oral exposure to peanut. Following this model in humans, EPE could lead to epicutaneous PS, and on subsequent oral exposure to peanut, peanut specific mast cell expansion and degranulation in the intestine could lead to increased intestinal permeability and IgE mediated allergic reactions.

Figure 13: Interaction between skin barrier function, EPE, LC, TSLP, mast cells and Th2 and Th9 cells



Chapter 4 The skin barrier

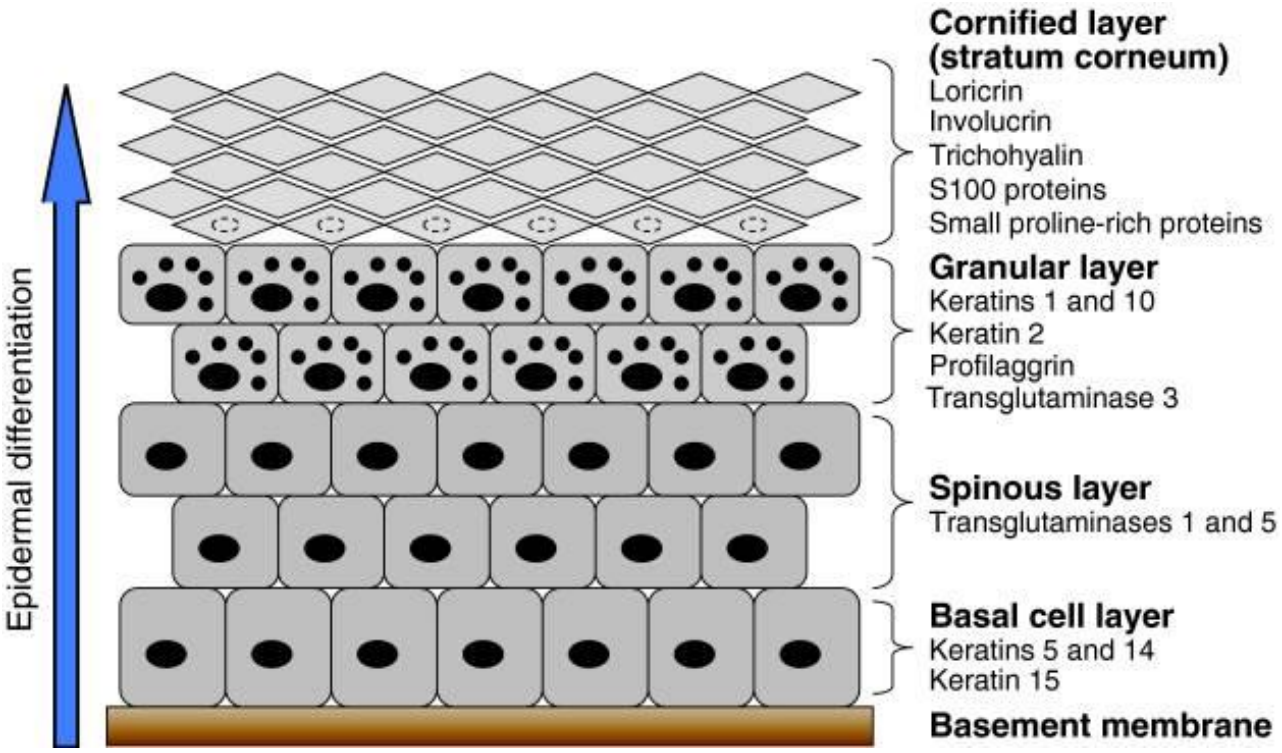
In order to better understand how sensitization may be occurring through the skin it is important to understand the skin structure and function of the proteins and enzymes within the skin. Human skin has three layers, starting with a layer of fat and connective tissue, followed by the dermis and then the outer layer of the epidermis. In most regions of the body, skin thickness ranges between 1-2mm, but can range from 0.5mm around the eyes to 5mm between the shoulder blades. The difference in thickness of the skin is generally due to variation in the thickness of the dermis.

The dermis is a layer of fibrous and elastic tissue comprising sweat glands and sebaceous glands, hair follicles, blood vessels, nerve endings, collagen and elastin. The epidermis acts as a barrier to prevent infectious and antigenic material gaining entry into the body and is usually around 0.5 mm in thickness. The epidermis is in a constant cycle of desquamation (skin shedding) and regeneration by terminally differentiated keratinocytes so that the skin remains the same thickness. Each keratinocyte has a life span of 30-40 days as it makes its way through the layer of the epidermis through a process called keratinization. The bottom layer of the epidermis is the basal layer where keratinocytes divide and push upwards to renew the epidermis. The next layer is the spinous layer where cells start to synthesise keratin. Above this is the granular layer where keratinocytes lose their nuclei and are filled with cytoplasmic material. Lamellar bodies are formed within the keratinocyte in the granular layer; these are released in the cornified layer (stratum corneum) as lipid lamella (ceramides, cholesterol and fatty acids). The lucid layer is only present in areas of thick skin (such as soles of the feet) and its role is to reduce shear forces between the cornified layer and granular layer. The outermost cornified layer comprises up to 30 layers of flattened cells which comprise keratin (corneocytes) and are bound together by corneodesmosomes and surrounded by the lipid lamella. This has been compared to a bricks and mortar structure where the corneocytes are the bricks, the lipid lamella are the cement and the corneodesmosomes are

the masonry ties between the bricks. [239] Figure 14 displays the normal anatomy of the epidermis and skin barrier proteins which are expressed in the epidermal differentiation complex (EDC) (discussed in Section 4.2). [240]

Figure 14: Structure of the epidermis and EDC proteins

Epidermal differentiation is characterised by the expression of specific proteins, which are listed in this Figure. The smaller black dots in the cells of the granular layer represent keratohyalin granules. Reprinted from Sandilands et al.(2009) with permission from Copyright Clearance Centre.[240]



The cornified layer acts as a barrier to prevent water loss and prevents the reduction of skin lipids. The cornified layer and spinous layer are the thickest layers of the epidermis. In the infant the cornified layer from the arm

and thigh area, measured using fluorescence spectroscopy, videomicroscopy and confocal laser scanning microscopy, [241;242] ranges between 7-35µm, whereas it is slightly thicker in adults. Desquamation of the cornified layer is promoted by degrading proteases which break down corneodesmosomes.[243] The most intensely studied protease family in the skin are the kallikrein family of which there are at least 8 in the human cornified layer. Other proteases include trypsin-like serine proteases like pro-urokinase and plasminogen, cysteine and aspartic proteases and matrix metalloproteases.[239] Proteases are tightly regulated by protease inhibitors which include metal ions, cholesterol sulphate, the lympoepithelial Kazal-type 5 serine protease inhibitor (LEKTI-1), secretory leukocyte protease inhibitor (SLPI) and elafin, α2-macroglobulin-like-1 (A2ML1), Plasminogen activator inhibitor-2 (PAI-2) and cystatin protease inhibitors.[239] Proteolytic activity is higher in facial skin, which would account for the cornified layer being thinner on the face than elsewhere on the body. In acute atopic eczema lesions there is an increase in serine proteases[244] and matrix metalloproteases [245] which results in thinning of the cornified layer.

4.1 Role of filaggrin in skin barrier maintenance

*Fil*ament *agg*regating prote*in* (filaggrin) is a complex polypeptide which is vital for the strength and integrity of the cornified layer of the epidermis. Much progress has been made in identifying the mechanisms by which filaggrin facilitates epidermal differentiation and maintains the skin barrier. [243;246;247] The *FLG* gene codes for a ~ 400kDa profilaggrin polyprotein which is found in the keratohyalin granules of keratinocytes within the granular layer of the epidermis. Profilaggrin undergoes cleavage to yield 10, 11 or 12 tandem repeats of the filaggrin peptide during the process of terminal differentiation of keratinocytes as they migrate towards the outermost later of the epidermis (cornified layer).

As keratinocytes undergo apoptosis (programmed cell death) in the granular layer and cornified layer, filaggrin forms an aggregate with keratin intermediary filaments and facilitates collapse and flattening of the keratinocyte to become cornified cells. This layer of collapsed corneocytes is then enclosed within a structure of proteins cross-linked by transglutaminases and surrounded by a lipid envelope, called the cornified cell envelope. [243] Using immunoelectron microscopy Manabe et al. (1991) demonstrated the function of filaggrin on filament aggregation in mice and humans cells. They showed that filaggrin peptides in the lower cornified cells which correlated precisely with the formation of aggregated keratin filaments, and a reduction in filaggrin levels in the upper cornified cells was associated with loosening of keratin filaments. [246]

In the top layer of the cornified layer, filaggrin is degraded into free amino acids that form major components of natural moisturizing factor (NMF). [247] NMF has hygroscopic effects (drawing in water) and reduces skin pH. Low skin pH reduces the activity of serine proteases which in turn reduces skin shedding by desquamation. [248] Low skin pH also promotes the action of lipid converting enzymes, which produce lipid factors such as sphingosine and ceramides which maintain the cornified layer. [33] Ceramide-dominant, physiologic lipid–based emollients (Triceram, Osmotics, US and Epiceram PuraCAP) have been shown to accelerate the restoration of barrier function after tape stripping in a murine model and have comparable efficacy to moderately potent steroids in children with moderate to severe eczema. [249;250]

4.2 *FLG* and the epidermal differentiation complex (EDC)

Profilaggrin (which is cleaved into filaggrin) is encoded by the *FLG* gene which resides in the EDC on chromosome 1q21. Although *FLG* has received the most attention since its successful genotyping in 2007,[251] the EDC comprises is a cluster of three gene families which are all involved in the terminal differentiation of keratinocytes (Table 2):

(1) Precursor proteins of the cornified envelope: involucrin, loricrin and small proline-rich proteins and late cornified envelope proteins

(2) Calcium binding proteins (S100)

(3) Fused gene proteins include profilaggrin *(FLG*), trichohyalin, hornerin and repetin. [243;252]

Although how the *FLG* gene relates to eczema is now well understood (see Section 4.1 and 4.4), the biological function of other EDC gene variants and their relation to eczema is less well understood.

Table 2: Genes involved in terminal differentiation of the human epidermis

Name of gene	Epidermal expression	Structural role	Phenotype with mutations in the EDC gene
Involucrin	Upper spinous and granular layer	Cross-linked to membrane proteins by transglutaminase enzyme to form the cornified envelope. Involucrin forms the outermost protein layer of the cornified envelope to which lipid ceramides attach.	Minor component of cornified envelope. Mice lacking involucrin have normal cornified envelopes
Loricrin	Cornified layer Late granular layer	Cross-linked to membrane proteins by transglutaminase enzyme to form the cornified envelope. Represents more than 70% of epidermal cornified envelope	Loricrin deficient mice are born with erythroderma but normalizes after compensation by other EDC proteins
Small proline-rich proteins (SPRRs)	Cornified layer	Cross-bridges receivers and donors of amino groups during cornified envelope formation by transglutaminase enzymes	SPRR polymorphisms involved in atopic eczema
Transglutaminases 1,3 and 5	Spinous and granular layer	Enzyme which crosslinks keratin intermediate filaments to form the	Lamellar icthyosis (transglutaminase 1)

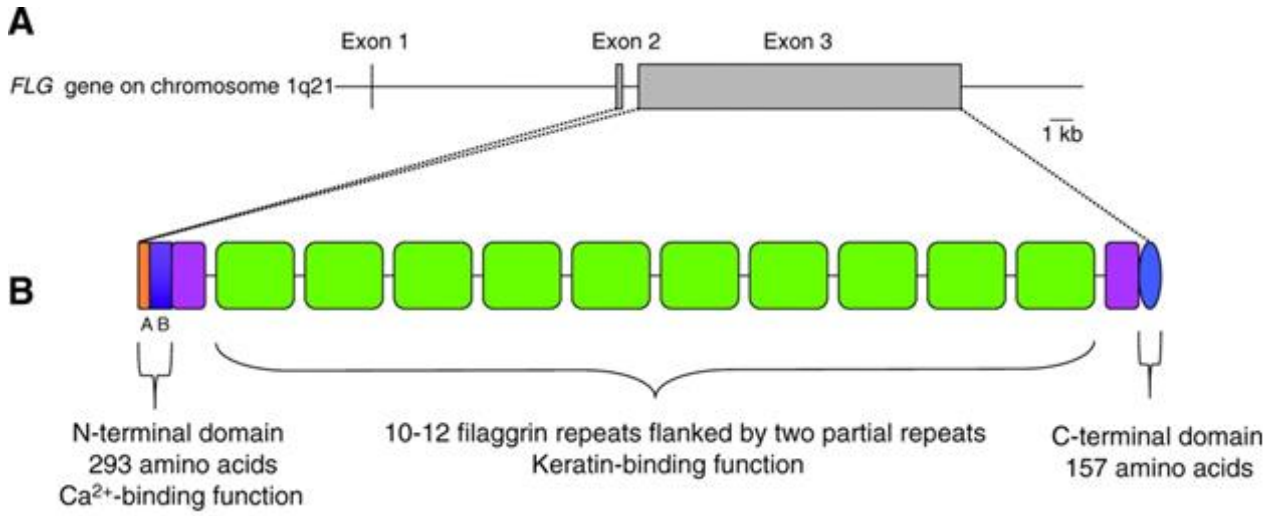
		keratin matrix	
Late cornified envelope	Late upper granular layer	Protein crosslinking functions similar to SPRR	Polymorphisms involved in psoriasis
S100A family	S100A7 in differentiated keratinocytes / S100A15 present in all epidermal layers	Regulatory proteins involved in calcium signal transduction pathways regulating cell shape, cell cycle progression, and differentiation. S100A7 and S100A15 are antimicrobial peptides	S100A7 and S100A15 are present in chronic eczema
Profilaggrin (*FLG*)	Granular and cornified layer	Profilaggrin is cleaved into filaggrin subunits which aggregate keratin filaments into tight bundles and promote collapse of the keratinocyte into flattened corneocytes. Filaggrin is then degraded in the cornified layer to free amino acids (NMF).	Ichthyosis vulgaris and atopic eczema
Trichohyalin	Component of keratohyalin granule of hair follicle / granular and cornified layer	Cross-linked to membrane proteins by transglutaminase enzyme to form cornified envelope.	Involved in alopecia areata, molluscum contagiosum
Hornerin	Granular and cornified layer	Cross-linked to membrane proteins by transglutaminase enzyme to form cornified envelope.	Involved in psoriasis and atopic eczema
Repetin	Granular and cornified layer	Cross-linked to membrane proteins by transglutaminase enzyme to form cornified envelope.	Unknown function

4.3 *FLG* gene variations and impact on filaggrin protein expression

The *FLG* gene comprises three exons and two introns. The first exon is non-coding and the protein translation initiates within exon 2 but the bulk of the profilaggrin protein is encoded by exon 3 (Figure 15). Exon 3 comprises repetitive sequences called tandem repeats and individuals have between 10-12 tandem repeats.

Figure 15: Structure of *FLG* gene on chromosome 1q21

(A) Profilaggrin gene structure (B) Profilaggrin protein structure contains 10, 11 or 12 tandem repeats of filaggrin which are flanked on either side by partial imperfect repeats. Reprinted from Sandilands et al.(2009) with permission from Copyright Clearance Centre, Inc.[240]



4.4 *FLG* loss-of-function mutation phenotypes

Loss-of-function mutations in the genes which code for profilaggrin were originally identified as the underlying genetic cause for the most common form of ichthyosis: ichthyosis vulgaris. [251] Ichthyosis vulgaris is characterised by dry, flaking skin appearing in the postnatal period followed by hyperkeratosis pilaris (roughening of skin around hair follicles predominantly on extensor surfaces) and hyperlinearity of the palms and soles. Homozygous loss-of-function mutations of *FLG* lead to complete loss of filaggrin expression and

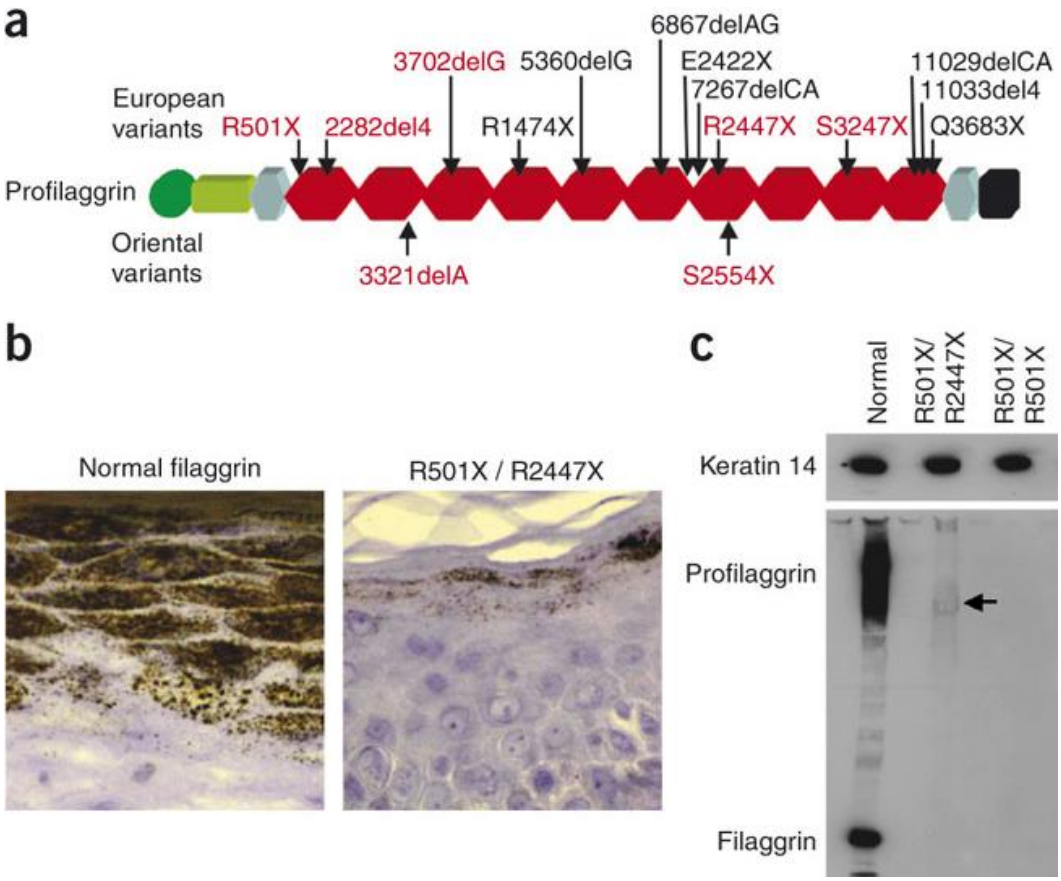
absence of epidermal keratohyalin granules, whereas heterozygous (i.e. one allele) mutations lead to approximately 50% less filaggrin expression. [240] Individuals with homozygous or compound heterozygous *FLG* loss-of-function mutations have 100% penetrance for the severe ichthyosis vulgaris phenotype whereas heterozygotes have a milder phenotype with 90% penetrance.[253]

Approximately 37-50% of patients with ichthyosis vulgaris have eczema, which is characterised by dryness but not hyper-linearity or hyperkeratosis pilaris and has an itchier inflammatory phenotype. The estimated penetrance of *FLG* mutations, i.e. the likelihood of a person with at least one *FLG* mutation developing eczema, is between 42-79%; [254-256] this may explain, in part, why not all children with *FLG* mutations develop eczema. *FLG* was assessed as a candidate gene for eczema due to the co-existence of these two dermatoses. Since then *FLG* loss-of-function mutations have been replicated in multiple candidate gene studies for eczema in both children and adults in the US, UK, Ireland, France, Germany, Italy, Sweden, Denmark, South Asian, China, Singapore and Japan. [255;257-259] In the 21 studies from the above mentioned countries, *FLG* gene mutations were implicated in 14-56% of patients with eczema with a 1.2 to 13.0 fold increased risk of eczema.[259]

Two *FLG* mutations, R501X (a SNP) and 2282del4 (a 4 base pair deletion) are present in 6.1% to 9.6% of Caucasian European populations. [251;260-263] Less common SNPs leading to *FLG* loss-of-function mutations include R2447X, S3247X, and 3702delG; these were found in 188 Irish paediatric eczema patients (Figure Figure 16a). [251] A further study found these less common *FLG* mutations plus 3673delC in 186 adult eczema patients from London and Newcastle Upon Tyne. [264] Other *FLG* loss-of-function mutations, such as 441delA, 1249insG, 3321delA, Q2147X, E2422X, S2554X and 7945delA, are associated with ichthyosis vulgaris and eczema in South-East Asian populations.[265;266] In children with wild-type *FLG*, there may be reduced filaggrin expression depending on the number of tandem repeats of filaggrin in exon 3 (see Section 4.3); the intragenic copy number variation (from 10-12 tandem repeats) reduces the odds ratio of atopic eczema by 0.88 (95% CI: 0.78-0.98) for each filaggrin unit copy number increase. [267]

Figure 16: Profilaggrin molecule (a), skin immunohistochemistry (b) and Immunoblot (c)

Reprinted from Sandilands et al. (2007) with permission from Copyright Clearance Centre, Inc). [268]



Legend:

(a) Schematic of profilaggrin molecule (from 10 tandem repeat allele) showing positions of known loss-of-function *FLG* mutations. Variations in red are common in European or Oriental populations. Variations in black are family specific.

(b) Immunohistochemistry staining of skin biopsy for filaggrin with keratohyalin granules in normal epidermis and in R501X/R2447X compound heterozygote.

(c) Immunoblot shows reduced truncated profilaggrin molecules (arrow) in the R501X/R2447X compound heterozygote and no processed filaggrin compared to healthy control and a R501X homozygote showing neither profilaggrin nor filaggrin staining. Keratin 14 was used as a loading control.

4.5 Filaggrin deficient mouse models

The flaky tail (*ft*) mouse has the autosomal recessive frameshift mutation (5303delA) analogous to the common human *FLG* loss-of-function mutations within the murine *FLG* gene. [269] The homozygous (*ft/ft*) flaky tail mouse phenotype comprises dry, flaky skin, matted hair and disorganised scales on the tail. These mice have truncated profilaggrin which is not cleaved into tandem repeats of the filaggrin peptide; immunoblot analysis reveals virtually absent filaggrin protein within the cornified layer of the epidermis.[270] Topical application of OVA to intact skin in homozygous (*ft/ft*) mice leads to a significant cellular infiltration (predominantly lymphocytes and eosinophils) and increased transepidermal water loss at the site of allergen exposure after 24 hours.[269] In fact several studies using the homozygous (*ft/ft*) flaky tailed mouse have shown that antigen permeation (such as OVA and/or haptens) through the skin results in an inflammatory infiltrate. [269;271;272] Following epicutaneous exposure to OVA, the homozygous (*ft/ft*) flaky tailed mouse's spleen cells produce OVA specific Th2 cytokines (IL4, IL5 and IL13) but also Th1 (IFN-γ), regulatory/Th2 (IL10) and Th17 (IL17) cytokines. Fallon et al. (2012) reported that the homozygous (*ft/ft*) flaky tailed mouse had similar antibody responses to application of peanut onto intact skin;[273] however, immune responses were not seen in the heterozygous (*ft/wt*) or wild type (*wt/wt*) mice following epicutaneous allergen application.

To confirm that OVA sensitization in the homozygous (*ft/ft*) flaky tailed mouse was due to skin barrier impairment rather than general altered immunity, Fallon et al.(2012) used IP injections of OVA and alum to both *wt/wt* and *ft/ft* mice and found that both groups developed comparable OVA specific Th2 biased OVA specific cytokine and antibody responses. Surprisingly, *ft/ft* mice sensitized to OVA via the skin did not develop increased airway resistance when subsequently challenged with OVA aerosol into their lungs and there was no significant increase in eosinophils in their lung histology. One argument for this would be that filaggrin in not

expressed in the lung (see Section 8.4) in humans; however there are significant differences between mouse models and humans (see Section 3.6.7).[274]

More recent murine studies have shown that the flaky tail (*ft*) mouse contains both mutations in the *FLG* gene as well as the *Tmem79* (also known as *Matt)* gene. Tmem79 is a transmembrane protein that encodes lamellar granules required for processing of filaggrin, lipids and proteases (Elias et al Mechanism of abnormal lamellar body secretion and the dysfunctional skin barrier in patients with AD. 2014). The *Matt* gene derives its name because mutations thereof also lead to the matted hair phenotype in the flaky tail *(ft)* mouse. A mouse genetics approach was used to separate the Matt and *FLG* mutations to produce congenic single-mutant strains; authors demonstrated that it was the *Matt* gene mutation rather than the *FLG* gene that led to spontaneous dermatitis over time in pathogen free conditions. (Saunders et al. Tmem79/Matt is the matted mouse gene and is a predisposing gene for atopic dermatitis in human subjects jaci 2013, Sasaki et al. A homozygous nonsense mutation in the gene for Tmem79, a component for the lamellar granule secretory system, produces spontaneous eczema in an experimental model of atopic dermatitis2013). A SNP in the *Matt* gene in humans has also been associated with eczema in humans (Saunders et al. 2013).

4.6 Filaggrin deficiency and inflammation

Based on current understanding of filaggrin's role in the cornified layer, it makes sense that *FLG* loss-of-function mutations are associated with the xerosis (dryness) and barrier defect found in eczema. The inflammatory component of eczema is less well explained by filaggrin deficiency; as for example filaggrin deficient mice do not exhibit spontaneous atopic eczema lesions;[275] however, it may be due to a reaction to the entry of irritants, pathogens and allergens.[271;276] Thus skin barrier impairment may itself lead to a maladaptive immune system with a predisposition towards atopy: the so-called 'allergic march'. *FLG* loss-of-function mutations are associated with inhalant allergic sensitization, allergic rhinitis and asthma only in children with

preceding eczema.[254;277-279] This highlights the role of the epidermal barrier in the pathogenesis of allergic sensitization and disease and the basis of this thesis is that it also holds for food-, and in particular, peanut allergy.

The local cytokine milieu has also been shown to affect filaggrin expression. Filaggrin protein expression was assessed in the skin of 69 patients with at least one *FLG* loss-of-function mutation. Researchers found that acute eczematous lesions in these patients had even lower levels of filaggrin protein than non-inflamed skin. Using punch biopsies of acutely inflamed eczematous skin, keratinocytes were cultured in the presence or absence of IL4, IL13 or IFNγ for 5 days. IL4 and IL13 reduced expression of filaggrin in differentiating keratinocytes and down-regulated filaggrin in keratinocytes which were already differentiated.[280] IFNγ increased filaggrin protein expression. Thus Th2 inflammation (IL4 and IL13) of the skin of eczematous patients, reduced filaggrin expression, whereas the Th1 cytokine IFNγ increased filaggrin expression. [280] The mechanism for filaggrin downregulation by Th2 cytokines was two-fold; downregulation of keratinocyte differentiation modulated the calcium sensitive protein S100A11, and increased protease activity broke down filaggrin.[281] Taken together these studies show that skin barrier abnormality due to *FLG* loss-of-function mutations can enhance allergen penetration thus favouring Th2 inflammation, however, on the other hand, Th2 inflammation can impair skin barrier function via a reduction in filaggrin expression. Thus in children with a heterozygous *FLG* mutation and actively inflamed eczema there could well be significantly less than 50% filaggrin expression in the skin due to a vicious circle whereby lower filaggrin promotes allergen entry, further Th2 inflammation and further reduction in filaggrin expression.

4.7 *FLG* loss-of-function mutations and food allergy

There have been conflicting results for the association between *FLG* loss-of-function mutations and food allergy. In a sample of 3471 Caucasian Danish adults invited to participate in a general health examination, *FLG* loss-of-function mutations represented a significant risk for self-reported egg, cow's milk, fish and wheat IgE-mediated allergy.[282] Other studies, however, found a less clear association between *FLG* loss-of-function mutations and food allergy. In infants recruited to the HealthNuts population study, carriage of a *FLG* loss-of-function mutation was associated with sensitization to food (defined as SPT ≥2mm to egg white, peanut, sesame, shrimp or cow's milk) even after adjusting for eczema (OR 3.0, 95% CI: 1.0-8.7; *P*=0.04, n=428). *FLG* loss-of-function mutations were associated with challenge proven food allergy on univariate analysis (OR 3.2, 95% CI: 1.2-8.5, *P*=0.02, n=321); however, after adjusting for eczema this association was no longer significant (OR 2.9, 95% CI: 1.0-8.6; *P*=0.06, n=321). This may have been due to sample size as the OR were very similar to those obtained for food sensitization.[283] In the Enquiring About Tolerance (EAT) population based cohort study, *FLG* loss-of-function mutations were not associated with sensitization to one of six foods (cow's milk, egg, wheat, peanut, sesame, cod fish) in infants at 3 months of age (OR 1.27, 95% CI: 0.48-3.38, *P*=0.7 n=619), which is surprising given previous findings. [284] This may have been due to the low rate (34/619=5.5%) of sensitization to one of six foods at 3 months, which is likely to increase at their 1 and 3 year assessments.

Venkataraman et al. (2014) found that *FLG* loss-of-function mutations were likely to be associated with food allergy in later childhood (10 and 18 years) rather than earlier childhood (1, 2 and 4 year). [285] They showed that *FLG* loss-of-function mutations exerted their action on food sensitization and allergy through eczema, rather than having a direct effect on food allergy itself. The impact of *FLG* loss-of-function mutations on food allergy in later childhood, may be because of the propensity for *FLG* loss-of-function mutations to predispose towards more persistent forms of food allergy (such as peanut and tree-nut allergy) rather than generally more

transient food allergies (such as cow's milk and egg allergy). In the paper by Venkataraman et al. (2014), the prevalence of peanut allergy was only 0.08% at one year's assessment but reached 1% at 18 year's assessment. Tree nut allergy was not present at one year and was 0.5% at 18 years assessment. Thus at 18 years peanut and tree nut allergy made up 37.5% of all food allergies whereas at 1 year they made up only 1.5% of all food allergies. The hypothesis for this PhD is that *FLG* loss-of-function mutations (as a marker of skin barrier impairment) increases the risk of peanut allergy, thus the publication by Venkataraman et al. (2014) also supports this hypothesis.

Data supporting an association between *FLG* loss-of-function and challenge proven IgE-mediated PA were assessed retrospectively in several populations by Brown et al. (2011)[(286)] Records of 71 white European children with PA (confirmed by diagnostic OFC) from England (ALSPAC birth cohort) (n=35), Netherlands (n=20) and Ireland (n=16) were compared against 1000 non sensitized controls from the ALSPAC study or controls from their respective population (Dutch and Irish) controls. The authors performed a replication study in 383 white Canadian patients with PA (confirmed by diagnostic OFC or history of allergic reaction on peanut exposure and >95% PPV for peanut sIgE ≥15 kU/L or SPT ≥8mm) against 891 non sensitized controls. Genotyping for *FLG* loss-of-function mutations was performed for R501X, 2282del4, R2447X, and S3247X in all cohorts except for the ALSPAC cohort where genotyping was only performed for mutations R501X and 2282del4.

There was a strong and significant association between *FLG* loss-of-function mutations and PA (OR 5.3, 95% CI: 2.8-10.2, $P=3x10^{-6}$) in the 71 patients from the UK, Netherlands and Ireland. The authors adjusted for eczema in the LR model as eczema is associated both with *FLG* loss-of-function mutations and with PA, and thus could be an important confounder. Even after adjusting for eczema, a significant association between *FLG* loss-of-function mutations and PA remained (OR 3.8 95% CI: 1.7-8.3, $P=0.0008$). The association between

FLG loss-of-function mutations and PA was replicated in the Canadian cohort (OR 1.9 95% CI: 1.2-2.6, $P=5.4x10^{-5}$), although no adjustment was made for eczema, as this information was not available for the control participants.

On the basis of the data described above it seems that *FLG* does have a role to play in food sensitization and allergy, but that this effect may be more relevant in older children. The positive association between *FLG* loss-of-function mutations and PA in the number of cohorts reviewed by Brown et al. (2011) is very convincing, thus it may be that *FLG* loss-of-function mutations are more closely related to PA than food allergy in general. This would fit with this association being stronger in older children who are less likely to have outgrown their PA than egg or cow's milk allergy.

4.8 Phenotypic markers of impaired skin barrier

Although the study by Flohr et al. (2010) did not find an association between *FLG* loss-of-function mutations and food sensitization in infants, they found that a functional assessment of the integrity of the epidermis using transepidermal water loss (TEWL) was associated with food sensitization even after adjusting for atopic eczema and *FLG* mutation status (OR 2.23, 95% CI: 1.04-4.76, $P<0.05$). [(284)] TEWL is measured using the non-invasive Aquaflux® closed chamber tewameter [(287)] which measures evaporation of water from the skin. This measure incorporates the spectrum of genetic and environmental factors leading to barrier impairment, such as water hardness, frequency of washing and detergents. If the skin barrier is impaired more water can evaporate and also allergens, irritant and bacteria can penetrate the skin. [(248)] A small French cross-sectional study in children aged 3 to 12 months showed that the higher the TEWL, the higher the prevalence of sensitization to aeroallergens both in children with and without eczema. This suggests that skin barrier dysfunction, even in the absence of overt eczema, contributes to the risk of allergic sensitization to aeroallergens.[(287)]

A cross-sectional study of 59 children and 40 adults found a trend towards an association between increased TEWL and *FLG* mutations; [288] however, more recently a study of 88 infants found that carriage of a *FLG* mutation was significantly associated with increased TEWL at three months of age, even in the absence of eczema. [284] The first study [288] did not separate out adults and children in their analysis which may have accounted for the lack of significance as, by adulthood, skin barrier function may have been affected by many other factors (such as long term inflammation or lichenification).

4.9 Summary of evidence on skin barrier function and epicutaneous sensitization

Taken together, the evidence outlined in Chapter 3 and Chapter 4 demonstrates a good theoretical basis for epicutaneous sensitization to peanut especially via an impaired skin barrier. The association between eczema and PA in cohort studies, the link between topical peanut exposure on eczematous skin in humans and on abraded skin in mice and PA, and the preponderance of CLA+ memory Th cells proliferating in response to PA in PA allergic individuals all support the concept that peanut antigen may sensitize via the cutaneous route, predominantly where there is concomitant skin barrier dysfunction and inflammation. The discovery of *FLG* loss-of-function mutations and their association with skin barrier dysfunction and eczema, provides a novel genetic screening tool to identify children potentially at an increased risk of PS and PA through epicutaneous sensitization.

The median onset of PA is 14 months, [289] therefore sensitization is likely to occur during infancy. Although direct epicutaneous peanut exposure during infancy would be very hard to measure in humans, environmental levels of peanut antigen may reflect epicutaneous exposure. Infants spend most of their time in the home and so it would seem appropriate to look for the possible means of sensitization in the home environment. Publication 3 resulting from this PhD thesis is the first study to directly assess the impact of EPE in household dust on the

development of PA. However, Fox et al. (2009) used HPC as an *indirect* marker for EPE and found an association with PA (see Section 2.4); [104] in their study peanut butter consumption versus other 'covered' peanut containing foods such as chocolates were more likely to be associated with PA. They postulated that peanut butter, being stickier, was more amenable to transfer by hands; this could potentially be transferred to the infant's skin directly or onto their bedding or play-area. In the next theory section I will discuss the role of environmental exposure in allergic sensitization, starting with aeroallergens for which there is a large body of evidence.

Chapter 5 Environmental exposure and allergic sensitization

5.1 Environmental exposure to inhalant indoor allergens

Environmental exposure to inhalant allergens has been evaluated as a risk factor for sensitization and development of allergic disease (in particular asthma) in several studies. Data from the Isle of Wight cohort study suggested that HDM sensitization was the most potent predictor of respiratory and cutaneous allergic disease. Overall 68.4% of children aged 4 years, who were sensitized to HDM, had asthma, eczema or allergic rhinitis.[102] In a landmark paper from the Poole cohort, Sporik et al.[290] showed that early environmental exposure (at 1 year) to >10μg/g HDM was associated with the development of asthma at 11 years with a relative risk of 4.8 (P=0.05). Subsequent studies have investigated the association between early HDM exposure and later sensitization and asthma revealed contradictory findings; some studies indicated a positive relationship between HDM exposure and asthma, particularly in high-risk children; [291;292] however, other studies negated this association. [293-295] Implementation of HDM reduction measures have, for the most part, not reduced the incidence of HDM sensitization, wheeze or asthma. [296;297]

In the Childhood Asthma Prevention (CAP) study, high-risk children (at least one family member with a diagnosis of asthma) were randomised before birth to HDM reduction measures plus high omega 3 oil supplementation (tuna fish oil supplementation and canola oil for cooking) or no HDM reduction measures and low omega 3 supplementation (Sunola oil supplementation with polyunsaturated oils for cooking). [298] HDM reduction measures included impermeable mattress covers and an acaricide wash which resulted in a sustained low HDM level in the child's bed in the intervention group at 18 months (6.1mcg/g) whereas in the control group this increased from 5.2mcg/g at 1 month to 22.2mcg/g at 18 months. [299] These interventions did not

reduce HDM sensitization at 18 months or 5 years, [300] and did not reduce the incidence of asthma, wheeze, or atopy at 5 years of age. [297]

In the Manchester Asthma and Allergy Study (MAAS) (one of the collaborating cohorts for this PhD) rigorous HDM reduction measures were applied prenatally (by the 16th week of pregnancy) to the parent's bedding and from 36 weeks pregnancy to the infant's home environment. HDM reduction measures included pillow, mattress and quilt mite-proof bedding to the parental and infant bed, HEPA filter vacuum cleaners, fortnightly hot-washing of bed-linen, carpet removal in the infant's room with vinyl replacement and acaroside application to carpets and sofas. The MAAS study showed a significant reduction in HDM levels in the parental mattress, living room floor, child's mattress and floor; [301] however, children randomised to the HDM intervention arm did not have a reduced rate of HDM sIgE sensitization (intervention arm 23.3% versus control arm 8.2%; RR 2.85; 95% CI: 1.02-7.97; $P = 0.05$) and SPT sensitization to any allergen (intervention group 34.7% vs control arm 21.5%; RR 1.61; 95% CI: 1.02-2.55; $P = 0.04$).[302]

One randomised prospective study, the Isle of Wight study, [114] did show a protective effect of HDM reduction measures on preventing HDM sensitization and allergic disease. One hundred and twenty pregnant mothers whose unborn children were at high-risk of developing atopic disease due to family history were recruited. HDM reduction measures included impermeable mattress covers for the infant's cot and acaricide treatment. Infants in the intervention group also underwent dietary restrictions (see Section 2.3.3 for further details). Follow-up at 1, 2, 4 and 8 years of age showed a reduction in allergic disease and sensitization (both food and HDM) at each time point.

One reason for the conflicting evidence surrounding indoor allergen exposure is that the use of the allergy mattress, duvet and pillow covers, not only reduces exposure to HDM but also to endotoxin, which may be protective against atopy. In a high-risk US prospective birth cohort study by Celedon et al. (2007) there was an association between postnatal environmental HDM exposure (≥10 mcg/g) and asthma at 7 years of age (OR 3.0 95% CI: 1.1-7.9).[303] Conversely, early exposure to endotoxin was protective against atopy (defined as sensitization to ≥1 allergen and or physician diagnosis of allergic rhinitis or eczema) at 7 years of age (OR 0.5 95% CI: 0.2-0.9), but was a risk factor for wheeze (OR 1.23; 95% CI, 1.07-1.43);[303] these data suggest that interactions between different types of exposure and genetic background may result in the development of atopy and asthma. I will discuss this further in the next section.

5.2 Gene-environment interactions for inhalant allergen exposure

It has long been known that atopy is hereditary and that the impact of environmental allergen exposure is modified by parental atopy. For example, The UK based Ashford birth cohort study (n=625) found that the impact of environmental HDM and cat exposure at 8 weeks of age on sensitization to HDM or cat (respectively) and atopic wheeze at 5 years of age was modified by paternal atopy and birth order. [304] High-risk, first-born children were most susceptible to HDM and cat allergen exposure. They postulated that reductions in indoor allergen exposure would be unlikely to have a major impact on decreasing atopy and asthma if modifying factors were not taken into consideration. Other studies have also shown a selective effect of indoor allergen exposure and allergic disease depending on parental atopy. [291;292;305] Defining risk on the basis of parental atopy alone is not very precise and thus the search for more specific predictors or biomarkers of disease risk began.

Since the mapping of the human genome was achieved in 2001 a large number of studies have assessed the interaction between genetic variations and environmental exposure.[306] In particular, associations between SNPs and allergic disease have been extensively assessed (see Section 1.7.2). The German Multicentre Atopy Study found that variations in the gene encoding for IL4 (C-590T) modified the response of HDM exposure (Der p 1 allergen in carpet dust) on HDM sensitization, and there was a significant interaction between HDM exposure and IL4 genotype (OR 8.56, P=0.002). [78] This may, in part, account for the discrepant study findings described for HDM exposure and sensitization in the previous Section (5.1); as the effect of early HDM exposure could be dependent on the IL4 genotype, and potentially other genotypes.

The MAAS group assessed the relationship between a promoter polymorphism in the CD14 gene (innate immune system gene) and environmental endotoxin exposure as the effect of endotoxin is mediated through the LPS receptor (CD14). Overall CD14 genotype was not associated with sensitization or eczema; however, in children with CD14 genotype CC, increasing endotoxin exposure was associated with a marked reduction in the risk of sensitization and eczema. Conversely, there was an increased risk of non-atopic wheeze with increased endotoxin exposure in children with the CC genotype.[307] Thus the CC genotype could explain the discrepant findings in the study by Celedon et al. (2007) where environmental endotoxin exposure reduced the risk of endotoxin sensitization but increased the risk of wheeze in high-risk US children. [303]

Custovic et al. (2011) assessed day-care attendance on the development of allergic outcomes in the MAAS and Tucson Infant Immune (IIS) birth cohorts. [74] They hypothesised that the discrepancy found in previous studies as to whether day-care attendance protected or was a risk factor for allergic outcomes was due to variations (SNPs) in the Toll-like receptor (TLR) family genes. [74;308;309] TLRs are pattern recognition receptors (PRRs) bound to APCs which recognise microbial pathogen associated molecular patterns (PAMPs) and initiate activation of the immune system. TLRs form part of the innate immune system and have important effects on

the development of allergic disease (see Section 1.7.2). In the study by Eder et al. (2004) children of farmers who carried a T allele (AT genotype) for the Toll-like receptor 2 (TLR-2) were significantly less likely to have asthma, atopic sensitization (positive SPT or sIgE to common aeroallergens and/or egg, milk, peanut) and allergic rhinitis than children with an AA genotype (where there was no protective effect of a farming environment). [310] The interaction between a farming environment and the TLR-2 genotype on asthma was independent of other markers of atopy. The TLR genotype could thus be expected to modify the way the immune system responds to a higher microbial load in day-care and /or farming environments. Indeed, in children carrying the TLR2-AT or TT genotype, day-care attendance reduced atopic sensitization over 4 time points (3, 5, 8 and 11 years) in both the MAAS and IIS birth cohort and reduced atopic wheeze in the IIS; however, children with the TLR2 AA genotype trended to have an increased risk of sensitization and atopic wheeze.

Another example of a gene environment interaction comes from the MAAS and COPSAC birth cohort studies where the impact of *FLG* loss-of-function mutations and environmental cat allergen exposure on the likelihood of developing eczema was assessed.[311] In both cohorts, an interaction between *FLG* loss-of-function mutations and environmental cat exposure on the development of eczema in the first year of life was demonstrated (COPSAC: OR 11.11, 95% CI: 3.79-32.60, $P < 0.0001$ and MAAS: OR 3.82, 95% CI: 1.35-10.81, $P = 0.01$). Environmental HDM exposure was not related to eczema or *FLG* genotype. This may reflect a different mechanism of sensitization.

Apart from Publication 3 in this PhD (see Section 7.7) there have been no studies to date assessing the interaction between food proteins in dust and variation in specific genes involved in skin barrier function (e.g. *FLG* loss-of-function mutations or SPINK5) or the innate immune system (e.g. CD14, TLRs). This is predominantly because there is little data on food allergen exposure in dust. In the following section I will

review the available evidence on environmental food exposure in dust and surfaces and discuss the validation process that I previously employed to quantify peanut protein levels in the home environment as part of my MSc thesis for the University of Southampton.[312]

5.3 Food proteins in household dust

Two studies in 1995 demonstrated quantifiable levels of food proteins in household dust using ELISA based methods. Witteman et al. [313] detected egg (ovomucoid) and milk (β-lactoglobulin) proteins in vacuumed samples of settled dust on carpeted and smooth floors in the home; [313] they found a range of 170 to 6,280 ng ovomucoid and 16 to 71 ng β-lactoglobulin per gram of dust. These levels were high enough to give a positive radio-allergosorbent test (RAST) with the serum of egg and milk-allergic patients; thus they were considered high enough to sensitize patients. Dybendal et al. [314] also detected ovomucoid and cod-fish protein in the vacuumed settled dust of smooth and carpeted homes. They measured percentage inhibition of the same allergen added after incubation of patient sera with lyophilized dust extract solution. They found sIgE percentage inhibition of 7-74 per metre^2 for ovomucoid and 32-93 per metre^2 for cod-fish.[314] Ara h 2 has been quantified in settled dust from classrooms and cafeterias of (14/159=8.8%) inner city schools with a median concentration of 0.76 µg/g (range 0.47-6.59µg/g) and in bedroom dust of 18/77 (23.4%) student's homes with a median concentration of 1.13 µg/g (range 0.4-9.79µ/g). [315] More recently there seems to be resurgence in interest in food proteins in household dust. In 2013-2014 two articles were published measuring fish, egg, milk and peanut allergens in the mattress dust of 13 year old Norwegian adolescents, [316] and in different areas of the home in Germany. [317]

Using a polyclonal ELISA against whole peanut protein (Veratox, Neogen, Europe), I have previously shown that peanut levels increase on bed-sheets (on which participants have slept) the day after a single peanut-

containing meal (peanut butter sandwich). [318] Median peanut protein level increased from below the lower limit of quantitation (LLQ) on clean bed-sheets to a median levels of 2.31µg/m^2 (range 0-8.5µg/m^2) on the following day (n=10, *P*=0.017). In other pilot work, which was submitted as part of my Allergy MSc at the Univserity of Southampton, I assessed where peanut butter was most likely to be found in the bed the day after peanut butter consumption. Peanut levels were highest was in the middle third of the bed (1.05µg/m^2), followed by the top (0.88µg/m^2) then the bottom of the bed (0.53µg/m^2). This gives important information as to the likely means of transfer (hands for the middle of bed and saliva for the top of the bed), which I will mention further in the discussion. The persistence of peanut protein on bedding following peanut protein consumption over 5 days was also assessed. Peanut protein levels were present for 48 hours after peanut butter consumption after which dust samples returned to below the LLQ for peanut protein. [312]

5.4 Peanut proteins on surfaces

In previous work, Perry et al. (2003) [319] assessed the amount of peanut allergen remaining on tables after 'spiking' surfaces with peanut butter and subsequently applying different methods of cleaning. They used their own commercial monoclonal ELISA against *Ara h 1* (range of detection, 30-2000 ng/ml) as a marker for peanut protein in the environment as previously described. [320] Arachis hypogaea 1 (Ara h 1) is a major peanut allergen which belongs to the vicilin family of seed storage proteins and is recognized by serum sIgE of over 90% of peanut-allergic patients. [321;322] However, Ara h 2 is considered to be the most important peanut allergen for the diagnosis of PA on the basis of IgE binding and cell activation, [323;324] intracutaneous testing and diagnostic oral peanut challenges. [324;325] Peanut butter (5ml) was placed onto table surfaces and then wiped/washed off with water, cleaners with bleach, sanitizing wipes, liquid soap or a bar of soap. Glass-fibre filter wipes were moistened with extract solution (PBS and 1% Tween) to sample the area before cleaning. The wipes used were extracted in 1.5mls of extract solution, so the units were expressed per millilitre (ml). The authors showed that

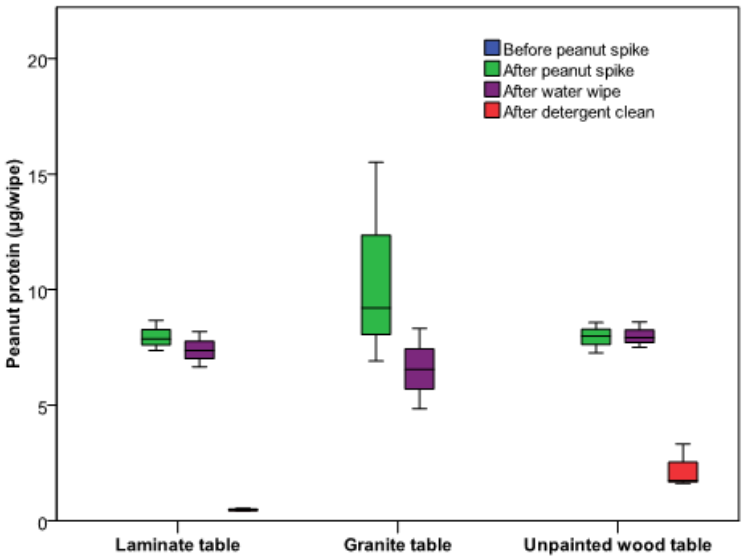
on table surfaces peanut protein levels decreased from 0.7-6.4µg/ml to 40-140ng/ml in 25% of tables (post liquid soap). Other methods of cleaning rendered Ara h 1 undetectable. [312]

I have shown that peanut protein levels increase on kitchen surfaces immediately after and 18-24 hours after a single peanut containing meal. [312] Using a polyclonal ELISA against whole peanut protein (which measures both Ara h 1 and 2 as well as other peanut allergens), peanut protein increased from below the lower limit of quantitation to 3.95 (range 0-29.3) µg/wipe on taps (P=0.043); to 4.42 (range 0-223.4) µg/wipe on fridge handles (P=0.018); to 5.58 (range 0-42.5) µg/wipe on dishwasher handles (P=0.018) and to 134 (range 0-816) µg/metre2 on kitchen tables (P=0.043). Peanut protein levels remained elevated on kitchen table surfaces with median 8.4 (range 0-116) mcg/m2 18-24 hours after exposure but were not significantly raised on other kitchen appliance handles and surfaces. This is probably because most of the peanut antigen had been removed with the first wipe I had performed to sample peanut levels immediately after peanut consumption. On the kitchen tables, which were often wood, it is likely that there was still some residual peanut remaining even after obtaining a wipe sample.

On the basis of finding persistent peanut protein on tables 24 hours after peanut butter consumption, as part of my Allergy MSc project I proceeded to explore residual peanut protein after usual detergent cleaning of three types of table surfaces following spiking of peanut protein.[326] I chose to spike table surfaces with only 0.5ml of smooth Sunpat peanut butter which was 10 times less than the study by Perry et al. (2003) to provide a more realistic representation of environmental contamination of peanut that was likely to occur.[319] Kitchen laminate, unpainted wood and granite table surfaces (n=3) were 'spiked' with 0.5ml of peanut butter, then wiped with water, then vigorously cleaned with dishwasher detergent. [327]

Wipe samples were taken before, after peanut spiking and following cleaning. I also spiked surfaces with plain butter to determine any false positives or contamination of samples. Prior to spiking with peanut butter there was no detectable peanut on any table surface. After spiking with 0.5ml smooth peanut butter median levels of peanut protein ranged from median 7.86-9.21µg per wipe sample of the wood, granite and laminate table. The highest amount of peanut protein post application of 0.5ml of peanut was detected on one of the granite table samples (15.5µg peanut protein/wipe). Following a single wipe with water there was only a small reduction of peanut protein ranging from a median of 6.56-7.92 µg per wipe sample. Following vigorous detergent cleaning peanut protein was undetectable on granite table surfaces but still detectable on the laminate surface; median 0.47µg/wipe (range 0.42-0.55 µg per wipe) and wood surface; median 1.75µg/wipe (range 1.62-3.33 µg/wipe) (Figure 17). These results are also shown in Publication 1 (see Section 7.4). Peanut was undetectable before and after plain butter spikes. These results showed that peanut was resistant to usual methods of cleaning on laminate and wood tables, thus small amounts of peanut on table surfaces from other family members eating peanut could potentially come into contact with an infant's skin and sensitize them to peanut, even after the surfaces undergo usual cleaning processes.

Figure 17: Peanut protein on table surfaces after 0.5ml peanut butter spike then clean



5.5 Routes of transfer of peanut protein into the environment

5.5.1 Detection of peanut protein in blood

Whole dietary proteins have been shown to be transported directly into blood, [328] and there is a reported case of anaphylaxis suspected to be from passive transfer of peanut through a blood transfusion from the donor consuming peanut several hours before blood donation and administration to a peanut allergic individual.[329] One study involved measurement of histamine release from passively sensitized basophils, following IgE stripping, to investigate the absorption of peanut allergens from the buccal mucosa.[330] Basophils obtained from buffy coat bank were stripped of their IgE and passively sensitized with the IgE of patient with confirmed PA. Serum from patients who had eaten peanut was then mixed with these passively sensitized basophils and the histamine release from these basophils was used to quantify the allergenic activity of absorbed peanut in serum following peanut consumption. Histamine release from basophils were detected using serum collected 30 minutes after peanut consumption, reached a peak using serum collected 3 hours after peanut consumption and persisted up until 24 hours. There was no histamine release from basophils stripped and sensitized with serum from non peanut allergic (NA) individuals. ELISA was also used as a way of quantifying peanut in serum, though the authors reported that the ELISA was '*10 times less sensitive than basophil mediated histamine release*' (p. 1322).

5.5.2 Detection of peanut protein in hands and saliva

Ara h 1 has been measured in saliva in levels up to 40μg/ml (enough to cause an allergic reaction) immediately after peanut consumption; however, was undetectable in 87% of participants after 1 hour using a monoclonal ELISA against Ara h 1. [14] Using the same monoclonal ELISA Perry et al. (2004) placed peanut butter (5ml) onto the hands of participants which was then wiped/washed off with water, cleaners with bleach, sanitizing wipes, liquid soap or a bar of soap.[319] They used glass-fibre filter wipes moistened with extract solution (PBS

and 1% Tween) to sample the area before and after cleaning. They found Ara h 1 levels of 4.8-56.0µg/ml of extraction solution before cleaning which decreased to 0.2-8.3µg/ml on 25% of hands (after water clean) and 0.13-1.7µg/ml of hands (after hand sanitizer) in 50% of participants. All other participants had undetectable peanut levels after cleaning.

5.5.3 Evidence for airborne food protein on sensitization and allergy

Environmental exposure to peanut could lead to sensitization via both epicutaneous and inhalational routes of exposure. A classic example of sensitization to foods via inhalation is Baker's asthma which is one of the most common forms of occupational allergy;[331;332] however, there are also reports of occupational asthma to other foods such as cow's milk.[333] Occupational allergen analysis usually reveals high molecular weight type 1 sensitizers with molecular weights between 6-100kDa, which is the size of many inhalant and food allergens. In South-East Asia allergens such as buckwheat (often consumed as soba noodles) may induce sensitization through inhalational exposure from cooking or inhalational/epicutaneous exposure from pillows (buckwheat chaff-stuffed pillows). [334;335] Buckwheat allergy predominantly features as allergic rhinitis then asthma symptoms but can also lead to anaphylaxis. To date, there are no reports of occupational asthma caused by peanut exposure as a primary agent in the UK based SHIELD database (Surveillance Scheme for Occupational Asthma). [336] There was one case of a Turner (lathe wood worker) in 1997 who was reported to have developed occupational asthma due to oil mists (oil is rubbed into the wood after it is shaped), which is a common cause of occupational asthma. Peanut oil was stated as a secondary cause, however, it is not clear as to whether the oil mist that caused the occupational asthma was Arachis oil or another oil, such as pine oil, which is commonly used in this type of work.[337]

There are several reports of certain foods leading to IgE mediated hypersensitivity reactions via inhalation, including fish, egg and cow's milk. [338-340] Aerosolized food proteins have been measured using air sampling monitors in egg breaking plants[341] and fish markets.[342] There are anecdotal reports of allergic reactions following inhalation of peanut; however, when children with severe or reported inhalational reactions to peanut underwent blinded inhalational peanut challenges (peanut butter held 12 inches from the face for 10 minutes) these children had no allergic symptoms or signs.[343] An abstract reported peanut protein has been detected in the ventilation system filters of commercial airliners after 5000 flight hours using an inhibition assay with peanut extract; [344] however, this abstract was never published. Perry et al. (2003) evaluated airborne peanut using Personal Air Monitors attached to adult volunteers whilst eating peanut butter, shelling and eating roasted peanut, and opening small packets of dry roasted peanuts (to simulate the type of peanuts consumed in commercial flights). [319]Additionally, the room ventilation was switched off in some experiments to decrease air exchange so as to simulate the recirculation of air that occurs on commercial flights. Peanut was undetectable in all these experiment, even when the amount of peanut in the environment was increased artificially by stamping on peanuts on the ground. [319] However this study used a monoclonal ELISA against Ara h 1 which may not have been sensitive enough, given that a monoclonal ELISA will bind specifically to only one epitope of only one peanut allergen.

5.5.4 Threshold levels of environmental allergen exposure for sensitization

It is important to determine not just whether EPE is a risk factor for PA but also what threshold level of peanut protein would be sufficient to lead to PS. Allergen exposure threshold levels for sensitization have been described for inhalant allergens such as HDM (Der p: >2 μg/g), [345] cat (Fel d 1: 1-8 μg/g), [346;347] and cockroach (Bla g 1: >8 μg/g). [348;349] Witteman et al. [313] postulated that as 2 μg Der p 1 HDM allergen per gram of dust was enough to cause HDM sensitization and they had found ovomucoid levels greater than 2 μg/g

in dust, this might also be high enough to cause ovomucoid sensitization. No threshold studies for sensitization have been performed for food allergens in dust. However direct application of 100μg of peanut protein onto tape stripped skin [170] or via inhalation [350] is enough to induce a potent Th2 response in mice. For ethical reasons this has not been performed in humans.

5.5.5 Threshold levels of peanut exposure to elicit allergic reactions

It is important not only to consider whether the amount of peanut in the environment is sufficient to lead to PS, but also whether levels of peanut in the environment might be sufficient to elicit at allergic reaction in PA individuals. Simonte et al. (2003) showed that casual cutaneous contact with peanut was more likely to lead to local skin reactions rather than significant allergic reactions;[343] however, given that children may touch their mucosal surfaces with their hands after cutaneous contact and that young children may put toys and other items contaminated with peanut in their mouths, it is useful to consider what threshold level of peanut might lead to clinical reaction after oral exposure.

Studies have assessed the threshold levels of peanut protein required to elicit a clinical response in peanut allergic patients in DBPCFCs. In a review article of 12 studies which conducted low dose DBPCFCs to peanut, [351] the no observed adverse effect level (NOAEL), which is the highest dose observed not to produce any adverse effect, and the lowest observed adverse effect level (LOAEL), which is the lowest dose observed to produce any adverse effect, were used to create probability distribution models. The lowest doses predicted to elicit objective symptoms ranged from 11 mg whole peanut in threshold studies to 18.5 mg whole peanut in diagnostic challenges to 65.5 mg whole peanut in immunotherapy challenges; [351] some of the most sensitive peanut allergic individuals had objective reactions upon consumption of as little as 0.1-5 mg peanut protein (0.4-20 mg whole peanut). [352-354]

5.6 Indoor allergen measurement

Several of the issues surrounding the practicalities of collecting dust, quantifying, sieving, processing and extracting dust samples for analysis are discussed in a US Patent. [355] In this document recommendations are made regarding the apparatus, kit and method of dust collection such as using a vacuum adaptor to collect dust samples rather than collecting dust into a bag, collecting dust samples over a fixed area for a fixed period of time, sieving dust samples to accurately weigh fine dust samples and extracting them in proportional amounts of extraction buffer. The standard format to express exposure to environmental allergens in the literature is in μg/g of dust (allergen concentration).[356] However, some authors argue that using total recoverable allergen i.e. vacuuming the whole living room floor or bed may be a better marker of exposure [357] and recommend expressing environmental allergens in μg/metre^2.

5.7 ELISA kits for peanut protein quantitation

Peanut ELISA kits have been developed predominantly to assess contamination of foods with peanut protein. The US Food and Drug Administration Centre for Food Safety and Applied Nutrition and AOAC Research Institute conducted a Performance Tested Method Multiple Laboratory Validation Study of three peanut ELISA kits: [358] the Veratox polyclonal ELISA against 'total soluble peanut protein' (Neogen Corporation, Lansing, MI, USA); the polyclonal Ridascreen ELISA (R-Biopharm, UK) against 'total soluble peanut protein;' and the Biokits polyclonal ELISA against Ara h 1 (Tepnel Research Products and Services, Flintshire UK) (extrapolated to 'total soluble peanut protein'). All three ELISAs showed correct identification of test food samples which contained 5μg peanut/gram of food and showed no cross-reactivity to a panel of 32 foods including cereals, legumes, seeds and tree nuts.

Poms et al. (2005) performed an inter-laboratory validation study of these three commercial ELISA test kits (as well as two other peanut ELISAs). [359] All ELISA test kits performed better in the concentration range 5–10 mg

peanut per kg of food rather than in the low concentration range (2 or 2.5 mg/kg). The variation in the recoveries of peanut between the different test kits had a spread of 44–191% across all concentrations. Biokits and Veratox kits were the only two kits which did not give false negative results in dark chocolate but had a false negative rate of 1.9% and 2.1% respectively for biscuit. Recovery rate was 116-118% for the Biokits polyclonal ELISA against Ara h 1 and 151-188% for the Veratox polyclonal ELISA against peanut. On the basis of the sensitivity of the Biokits and Veratox kits I assessed these ELISAs as potential ELISAs for use in measuring peanut in the environment. I also assessed a monoclonal Ara h 1 ELISA (Indoor Biotechnologies, Warminster, UK) as this had been used previously in environmental sampling. [319] This work was performed as part of my MSc at the University of Southampton and is displayed in the online repository for publication 1 (see Section 7.4.1).[360]

5.8 Basophil activation test (BAT) to detect biological activity of peanut protein in dust

Although the Veratox polyclonal peanut ELISA assay was able to reliably quantify peanut protein levels in dust, it did not provide us with any information about the ability of peanut allergens in dust to bind to effector cells in peanut allergic patients. The BAT was therefore used to confirm biological activity of peanut protein in dust in collaboration with Dr Alexandra Santos, King's College London. The background to this assay is described below.

5.8.1 Basophils

Basophils are white blood cells which contain large granules inside their cytoplasm. Human basophils have high affinity IgE receptors which are antigen specific on their cell surface (FCεRI receptors). Once an antigen cross-linked antigen specific FCεRI receptors, the basophils degranulate and release proinflammatory allergic mediators such as histamine and secrete leukotrienes and cytokines. As such basophils have a similar pattern of reactivity to mast cells, however whereas mast cells reside in the tissues (such as the skin and gut), basophils

circulate in the peripheral blood and are therefore readily accessible for in vitro work. Although basophils are the least abundant white blood cell in the peripheral circulation, in sensitized individuals they represent the largest population of antigen specific cells in the circulation. [361] Basophils are therefore ideal cells to evaluate a functional biological response to an allergen rather than just an indirect marker such as sIgE.

5.8.2 Background of BAT

In previous studies the IgE-cross-linking capacity of Ara h 1, 2 and 3 was examined by measuring histamine release after incubating basophils from patients with PA as well as passively sensitized basophils with several dilutions of the allergens. [324] More recently, the BAT has evaluated expression of activation markers CD63 and CD203c on the surface of basophils of allergic patients by flow-cytometry (FACS) rather than just measuring degranulation products such as histamine or leukotrienes. [362] CD63 is a 53kDa tetraspanin which is translocated from the membrane of secretory granules to the cell surface during basophil activation from FCεRI cross-linking.[363] CD63 was originally thought to directly reflect histamine release; however, subsequently it has been shown to have differential expression to histamine release, although still being a good marker for basophil activation. [364] CD203c is a 150 kDa transmembrane glycoprotein in basophils and mast cells which is expressed in low levels on resting cells and is rapidly upregulated following activation using a distinct pathway from CD63.[364] The use of both cell surface activation markers has been argued to increase the sensitivity of this assay, as often CD63 upregulation only occurs in a subset of basophils that upregulate CD203c upon allergen specific IgE cross-linking.[361]

5.8.3 BAT applications

Although BAT has previously been used predominantly in the evaluation of drug and venom allergy,[365;366] BAT has more recently gained application in the diagnosis of food allergy, [367] including PA. [305;368;369] In the

most recent study by Santos et al. (2014), [369] using optimal cut-offs, BAT conferred an accuracy of 97%, PPV 95% and NPV 98% against clinically confirmed PA. PA was confirmed by OFC in 61/104 (58.7%) of children (5 had indeterminate challenges so were excluded). In the remaining children, PA was assumed due to a convincing history of reaction within the last year and peanut SPT or sIgE above 95% PPV and peanut tolerance was assumed if children were able to eat more than 4 grams twice a week (assessed by FFQ). [368] The BAT cut-offs and PPV were subsequently validated in an independent population of children in the LEAP study who were also undergoing OFCs to determine PA versus tolerance. BAT has also been shown to be a sensitive and specific tool to detect trace amounts of peanut in food matrices such as biscuits and chocolates.[370] Basophils from peanut allergic patients or peanut tolerant individuals were stimulated with peanut-spiked (0.1ppm, 0.01ppm, 0.001ppm) biscuits and chocolate extracts and highly significant activation of basophils with 0.1 and 0.01ppm samples was found.[370] Thus given the relevance of BAT in assessing clinical PA reactivity and its highly sensitive assay characteristics, BAT was used to determine the clinical reactivity of peanut protein in dust. The methodology employed for the BAT is described in Section 6.10.

5.9 Mass spectrometry (MS)

MS is a powerful analytical tool for protein and peptide analysis. Certain authors argue that MS analysis is important to confirm proteins detected by polyclonal ELISAs, as polyclonal ELISAs detect all peanut proteins which may or may not include the major allergens (although we confirmed that the Veratox peanut ELISA standards contained a protein band of equivalent size to Ara h 1: see Section 10.3). [371] I therefore wished to investigate whether peanut protein found in dust samples from the infants' bed-sheet and play-area contained the allergenic peanut proteins Ara h 1, 2, 3 and 6.

5.9.1 Background of MS

MS involves the ionisation of molecules and their subsequent separation and detection based on their mass (m) - to -charge (z) ratios (m/z). Ionisation is achieved by knocking one of more electrons off the sample so that the molecule has a positive charge. Ions of different m/z ratios are separated by their interaction with either an electric or magnetic field (depending on the type of instrumentation); lighter and more positively charged ions (e.g. 2+) are deflected more than heavier or less positively charged ions (e.g. 1+). The mass spectra is then produced with the m/z values plotted against signal intensity and this can be used to identify the protein of interest using peptide sequence tag analysis and database searching with PeptideSearch or Uniprot (which comprises Swissprot).

5.9.2 Sample clean-up requirements and MS analysis

When assessing complex mixtures of samples, such as food or dust it is necessary to use a sample clean-up method in order to isolate the peptides of interest. MS has been used to detect peanut in complex food matrices assisted by the use of reversed-phase liquid chromatography (RP-LC) using a gradient mode of separation of the complex mixture, [371] or through a combined ELISA immunoassay and MS assay.[372] In the study by Shefcheck et al. (2004), ice-cream was spiked with 0, 10 and 1000 µg/ml of Ara h 1.[371] Following extraction of protein from the ice-cream, they used a 50 kDa cut-off membranes to concentrate the Ara h 1 protein in the extract and to remove lower molecular weight proteins such as β-lactoglobulin which would interfere with ionization of Ara h 1 peptides. They performed sample digestion using Rapigest surfactant and a trypsin digest protocol

.

The peptide mixture obtained was separated out on a RP-LC column and detected with tandem mass spectrometry (MS/MS). In MS/MS collision-induced dissociation with energetic gas (hydrogen) molecules is used to generate a set of fragments from a specific peptide ion. The fragmentation occurs across the peptide backbone, cleaving the peptide (amide) bond and producing characteristic fragment ions that are indicative of the individual amino acids. Using this method Shefchek et al. (2004) were able to identify peptides with *m/z* 629.8, m/z 571.3, *m/z* 606.6 and *m/z* 869.9; using peptide sequence tag analysis and database searching with PeptideSearch, these peptides and their corresponding sequences were unique to Ara h 1. They were able to confirm the presence of Ara h 1 in levels as low as 10 ppm (10 µg/ml or 10,000 ng/ml); however, did not assess Ara h 1 concentrations lower than this. To date no study has assessed peanut levels in household dust using MS; however, I anticipated that using a similar experimental technique it would be possible to identify allergenic peanut proteins in dust.

5.10 Overall hypothesis and aims of study

The overriding hypothesis of this PhD is that early EPE, measured by peanut protein levels in household dust, is a risk factor for the development of PS and PA in children with an impaired skin barrier. The aims of this PhD were effectively to describe characteristics of environmental peanut exposure, the association between this and PS and PA in children with an impaired skin barrier and finally mechanistic work to support the concept that PS occurs through the skin using recall responses to peanut from memory Th2 cells. The methods employed to deliver these aims are describe below.

Chapter 6 Methods

6.1 Overview

There are effectively three themes to this PhD which each require a separate section on the methodology used The first is the section where I investigated peanut protein levels in the environment (dust, surfaces, and air), characteristics of this exposure (such as how peanut gets into the environment and persistence of peanut in the environment) and characteristics of the peanut protein (quantification, major allergen content and biological activity). The second is an epidemiological section where I addressed whether early EPE was associated with PS and PA (where available) in one atopic and two population-based cohorts and whether this exposure was modified by various measures of atopy or skin barrier impairment. In the third section I evaluated in-vitro responses to peanut from PA, PS and NA children, assessing memory T helper (Th) cell responses to peanut in skin- versus gut-homing Th cell subsets in order to assess whether epicutaneous sensitization was reflected in a predominant Th2 response to peanut from skin-homing Th cells.

Various sections to the methods are displayed below. Methods that are described in detail in the publications (which comprise the results section for this thesis incorporating publications) are not repeated in this Methods section. Where further background, validation or optimisation steps for the methods were taken (which are not described in the publications), these are included as Appendices. An overview of the methodology common to certain sections of the PhD is required, such as a table comparing the study design of the cohorts employed, assessment of potential confounding factors for the relationship between environmental peanut exposure (EPE) and peanut sensitization/allergy in the cohorts employed and prevalence of FLG loss-of-function mutations in the various populations from which the cohorts are derived. The methodology for sections which have not as yet been published (MS work, BAMSE cohort analysis) are included in full in this methods chapter.

6.2 Environmental peanut protein sampling methodology

Wipe and dust sample methodology for Publication 1, 2 and 4 (CoFAR cohort) are described in the methods sections of these publications. Justification for the methods employed and figures depicting environmental sampling are included in Section 10.1 (Appendix 1). The dust collection procedure for publication 3 (MAAS) is described in Section 10.11.2 (Appendix 11).

6.3 Dust sample processing

Methods for dust sample processing (sieving, quantification, weighing, extraction and storage) are described in Publication 1 and its online repository. Validation for the minimum weight of dust that could be used without affecting peanut protein quantification is describe in Section 10.2 (Appendix 2).

6.4 Peanut protein quantification by ELISA

The method and validation of the ELISA used in this PhD is described briefly in Publication 1 and its online repository. A more detailed description of ELISA methodology, the background, method and quality standards employed for the Veratox peanut ELISA as well as assessment of likely Ara 1 in the Veratox peanut standards is included in Section 10.3 (Appendix 3).

6.5 Semi-quantitative peanut FFQ

The peanut FFQ employed is described briefly in Publication 2. A full description of this FFQ's origins, validation, individual food peanut protein content and the actual FFQ is detailed in Section 10.4 (Appendix 4).

6.6 Participant questionnaire for Publication 2

A participant questionnaire was used to assess household, demographic and clinical factors which may affect levels of peanut protein in the home environment. This questionnaire is described briefly in Publication 2, therefore the full rationale and description is detailed in Section 10.5 (Appendix 5).

6.7 Recruitment of patients for environmental peanut sampling in Publication 1 and 2

Recruitment and study procedures are described briefly in Publication 1 and 2. Further details are provided on participant recruitment including the participant information sheet, participant flow-diagram and study procedures in Section 10.6 (Appendix 6).

6.8 Statistical powering and analysis for Publication 2

Statistical powering and analysis are described in publication 2. Considerations for depiction of correlation plots are explained in Section 10.7 (Appendix 7).

6.9 Persistence of peanut on human and environmental surfaces

Given that HPC was shown to positively correlate with peanut protein levels in dust and surface wipes, [373] it was important to determine how peanut might get into the environment, thus persistence of peanut on human hands and saliva after peanut consumption were assessed. Given that peanut protein is measureable in the infant's bed and play-area, and that this could be an important source of exposure leading to sensitization, it was important to assess whether a detergent wash was sufficient to remove peanut protein from these types of household items, for future use in interventional studies thus persistence of peanut on sofa-covers and pillows after peanut consumption were assessed. Methods for peanut sampling on human and environmental surfaces are described in Publication 1. Participant information sheet for peanut sampling on humans is shown in Section 10.8 (Appendix 8).

6.10 Whole blood basophil activation test (BAT)

This work was conducted in collaboration with Dr Alexandra Santos. Dust extracts with a known peanut protein level incubated with PBMCs of PA ad NA children was used to assess the biological activity of peanut protein. Patient selection and methodology of the BAT are described in Publication 2.

6.11 Mass Spectrometry (MS)

6.11.1 MS analysis at KCL

I aimed to confirm the presence of peanut using MS in collaboration with Dr. Mark Parkin, Lecturer in Analytical Science, Analytical and Environmental Research Division, Franklin-Wilkins Building, KCL and Prof James McDonnell, Professor of Molecular Immunology, Randall Division of Cell & Molecular Biophysics, KCL. Background of MS use as an analytical tool and previous studies using LC-MS/MS for the detection of Ara h 1 peptides in food is discussed in Section 5.9. Trypsin digestion was carried out to create peptides of Ara h 1 concentrate (Indoor Biotechnologies, USA) with the SOP described in Section 10.9.1 (Appendix 9).

6.11.1.1 MS validation work

Before analysing peanut peptides in dust (which contains many other proteins), an aqueous solution was spiked with Ara h 1 to determine the LLQ for Ara h 1 using LC-MS/MS. After tryptic digestion and solid-phase extraction for sample clean-up, the peptide mixture was separated out by reversed-phase liquid chromatography (RP-LC). Selected peptide ions were then bombarded with argon fracturing the peptide ions at the peptide bond into fragment ions. The peptide bond is the chemical bond formed between the carboxyl and amino-group of adjacent amino acids. As the peptide is fragmented at the peptide bond, the mass difference between two

fragment ions is the mass of an amino acid. A peptide sequence tag is determined using a 3 or 4 amino acid sequence (by calculating the mass difference between fragment ions), the total mass of the peptide and the mass of the fragmented peptide at the start and end of the amino acid sequence. These peptide sequence tags were then entered into SwissProt protein sequence database to identify the Ara h 1 protein. Peptides with MS/MS spectra corresponding to Ara h 1 were observed in the aqueous solutions comprising 1000 µg/ml and 500 ng/ml Ara h 1 with approximately 40% sequence coverage of the Ara h 1 protein.

Two of the more dominant peptide ions found in our aqueous solutions had peptide ions with *m/z* 607 (Figure 18) and *m/z* 630 (Figure 19, p149); these two peptide ions were also dominant in the study by Shefcheck et al. (2004) [(371)] (see Section 5.9), where food matrices were spiked with Ara h 1. Additionally, the *m/z* 607 peptide ion in our study had fragment ions with *m/z* 687.4, *m/z* 722.9, *m/z* 745.4, *m/z* 779.7, *m/z* 974.5, *m/z* 1073.5 (Figure 18); these closely resembled the *m/z* 607 peptide fragment ions (*m/z* 687.2, *m/z* 722.9, *m/z* 745.3, *m/z* 779.3, *m/z* 974.3, *m/z* 1073.3) obtained by Shefcheck et al. (2004). [(371)] Shefcheck et al. (2004) did not disclose the *m/z* 630 peptide fragment ions they obtained thus these could not be compared with our findings.

Figure 18: Selected ion chromatogram for peptide (*m/z* 607) (A), MS/MS spectrum of peptide *m/z* 607 (B)

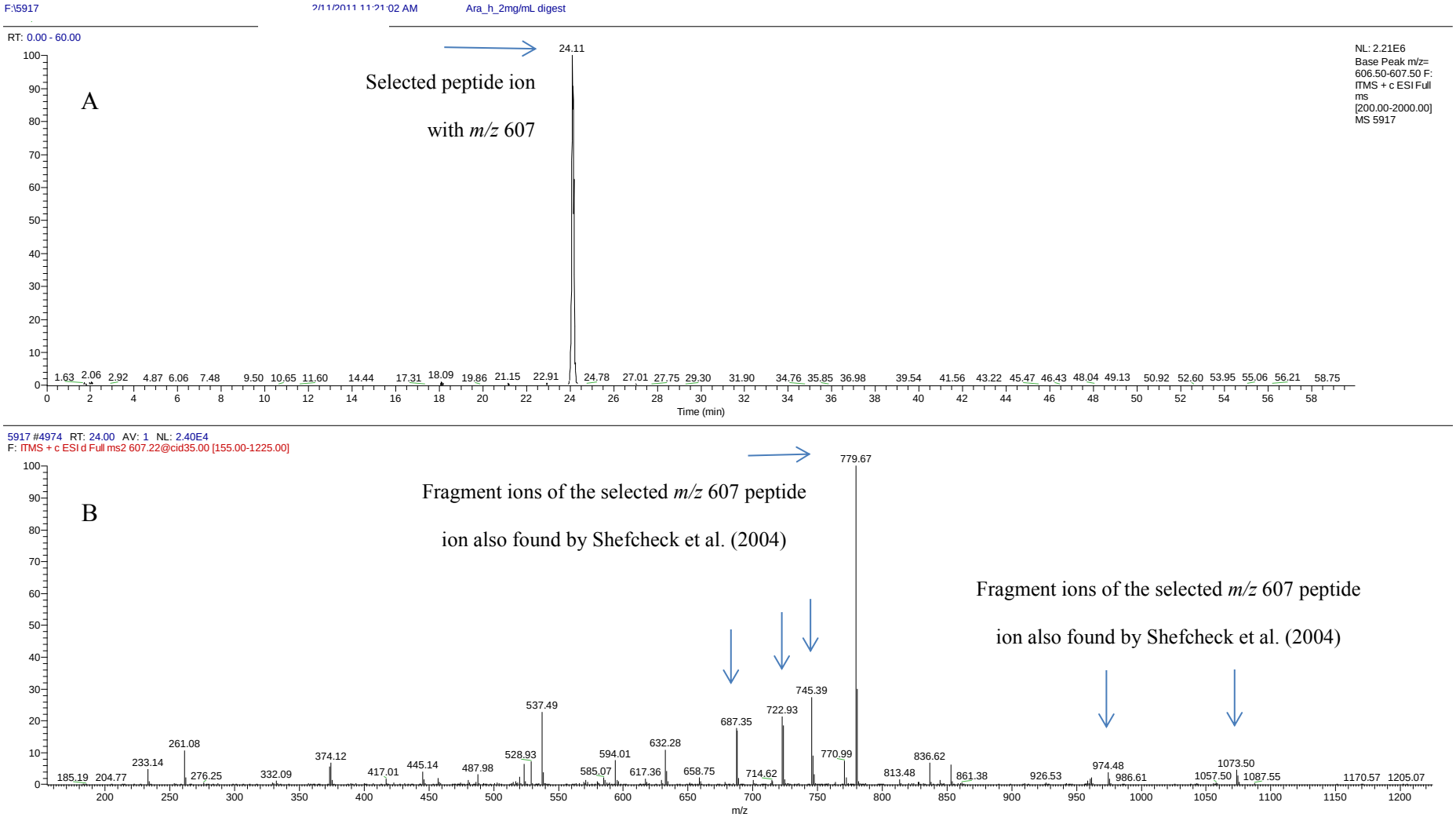
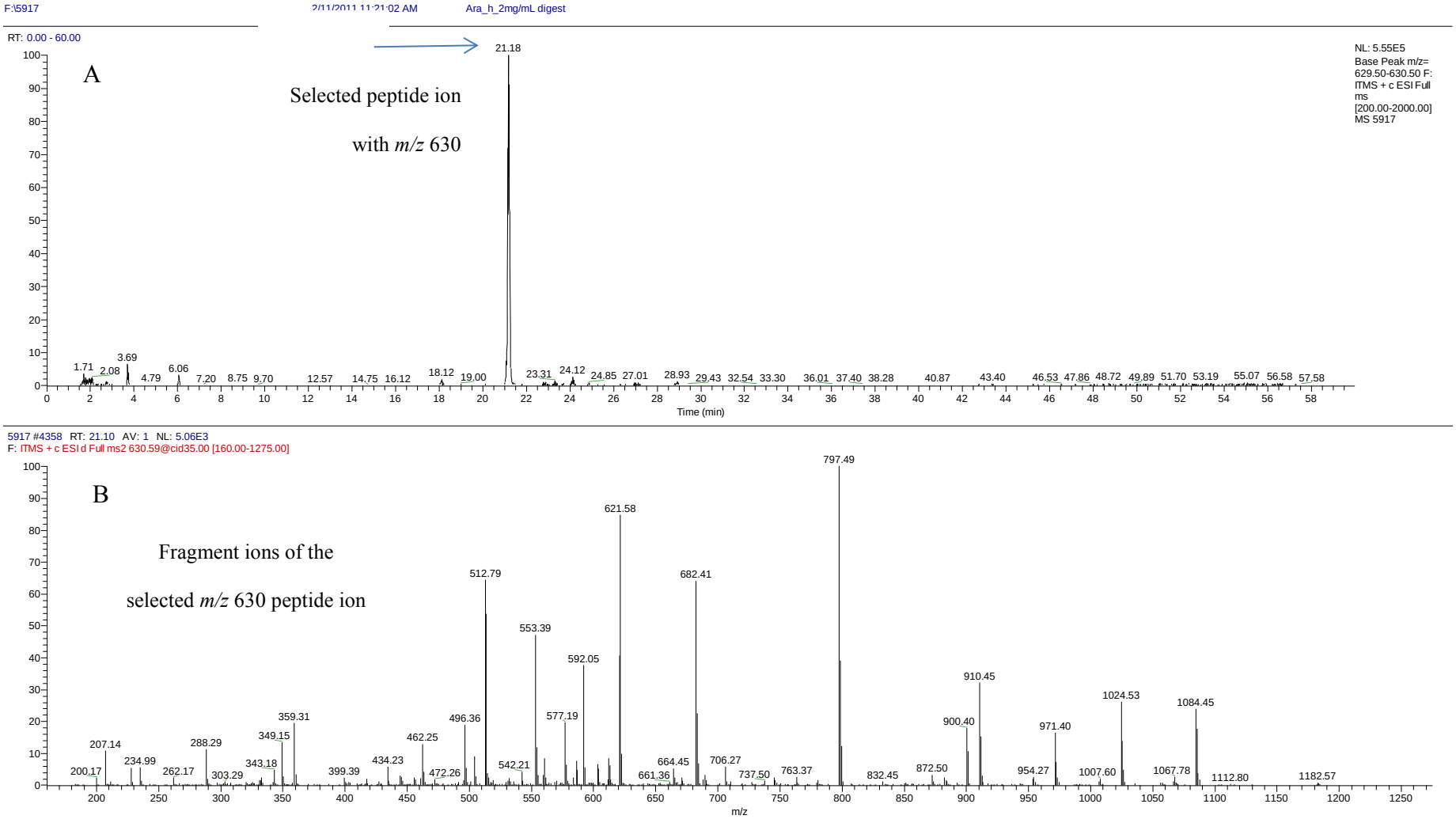


Figure 19: Selected ion chromatogram for peptide (*m/z* 630) (A), MS/MS spectrum of peptide *m/z* 630 (B)



6.11.1.2 Dust sample extract analysis

Subsequently, biological samples of dust were extracted as per the Veratox extraction protocol (see Section 6.4) with or without skim milk; these dust extracts had a peanut protein content below the Veratox ELISA LLQ (<25 ng/ml peanut protein) and were spiked with 500 ng/ml of Ara h 1. The total peanut content was calculated in the biological samples (as they contained other proteins apart from peanut) using a Nanodrop Spectrophotometer and proportional amounts of trypsin were added to the sample for the trypsin digest. A 500 ng/ml Ara h 1 aqueous sample was used as a positive control. Samples were run on the LC-MS/MS (Linear trap quadrupole (LTQ), Thermo Scientific). Although peptides were seen in the positive controls of aqueous samples spike with 500 ng/ml Ara h 1, no Ara h 1 peptides were detected in the dust extracts; however, many keratins were found which are known to interfere with ion detection in LC-MS/MS. [374]

6.11.1.3 Sample clean-up

Therefore, a strong cation ion exchange method was used for sample clean-up prior to LC-MS/MS. The isoelectric point for Ara h 1 is 4.55, which is the point at which positive and negative charges are equal so there is no net charge. Dust sample extracts containing no peanut were spiked with 500ng/ml and 1000ng/ml Ara h 1. Aqueous solution was also spiked with 500ng/ml and 1000ng/ml Ara h 1 as a positive control. The spiked aqueous solution and dust extracts were run through a solid phase extraction cartridge (C18 cartridges, Agilent Technologies) on a vacuum manifold is separate experiments. By adding ammonium hydroxide to increase the pH of these samples to 11.5 it was anticipated that Ara h 1 would bind to the cartridge given the isoelectric point for Ara h 1 of 4.55. Ara h 1 was then eluted off the cartridge using three washes at pH 5.5, pH 4.5 (the isoelectric point where 50% of Ara h 1 would be bound and 50% would be unbound) and pH 2.5. A sensitive

triple quadrupole MS analyser (TQS – Thermo Scientific, UK) was then used to analyse these samples. Following the strong cation ion exchange method clean-up, no peptides were detected in either the dust extracts or the positive controls. Therefore further MS analysis was performed in collaboration with Professor Clare Mills and Dr Phil Johnson at the University of Manchester.

6.11.2 MS analysis at the University of Manchester

Three dust samples collected from the homes of families participating in the correlation study (see Section 6.7) were sent to the Manchester Institute of Biotechnology for analysis. Two dust samples from an infant's bed-sheet (Y175) and play-area (Y231) contained high levels of peanut protein (797.5µg/g and 870 µg/g respectively). One dust sample from a maternal mattress (P77) contained peanut protein levels below the LLQ of the Veratox peanut ELISA.

6.11.2.1 Dust sample preparation and clean-up

Dust samples (5mg) were extracted with 50 mM Tris Cl^- (pH 8.8), 50 mM DTT, 0.04 % Rapigest^{TM} (1 in 20 w/v) for 15 min with heating at 60^oC with continuous sonication and vortex mixing every 5 min. Samples were sequentially reduced, alkylated and digested using an in solution protocol designed to reduce volumes of reagents and therefore maximise protein concentration in the final sample (see Section 10.9.2). Sample clean-up was performed using Pierce C18 spin columns (Thermo Scientific, UK) according to manufacturer's instruction. Samples were stored frozen at -20^oC prior to analysis.

6.11.2.2 MS analysis

Dust sample extracts were subjected to reverse phase HPLC electrospray ionisation mass spectrometry (RP-HPLC-ESI-MS) using a NanoAcquity LC (Waters, Manchester, UK) coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific, Waltham, USA). Samples (20µl) were applied to the analytical column (1.7µm ACQUITY™ C18 (Waters, Manchester, UK)) and eluted with a 2% (v/v) acetonitrile : water gradient for 10min, followed by a linear gradient to 60% (v/v) from 10 to 45min, finishing with a linear gradient to 90% (v/v) acetonitrile at 50 min. Formic acid (0.1% (v/v)) (final concentration) was included throughout. Mass spectra were obtained in positive ion electrospray mode. The mass range for the survey scans was *m/z* 300 – 2000, resolution 60,000, with *m/z* values determined by the Orbitrap fourier transform mass spectrometry (FTMS) stage. The FTMS fill target was 700,000 ions with a maximum fill time of 1000ms. The resultant monoisotopic masses were accurate to better than 10ppm. MS/MS spectra were obtained using collision induced dissociation with collision voltage 35V with *m/z* values determined by the Linear Ion Trap stage. The MS/MS was triggered by a minimal signal of 5000 ions with a fill target of 30,000 ions and 150ms maximum fill time. A maximum of 6 MS/MS spectra per survey scan were obtained by defaulting to the most abundant ions, with *m/z* values determined to better than ~ 0.5 Daltons (Da).

6.11.2.3 Data analysis

MS data were analysed using PEAKS Client 6.0 build 2012620 (Bioinformatic Solutions Inc, Waterloo, Canada). Analytical variables were set as follows: parent ion mass tolerance 5ppm, MS2 ion tolerance 0.5 Da, 2+ and 3+ charge states only. Up to 1 missed cleavage (trypsin) was allowed at either end of the peptide. The database used was a FASTA file of all peanut proteins (TAXID 3818: *A. hypogaea*) current as of 11/4/13.

Subsequent analysis consisted of identification of sequences corresponding to those present in a collated, non-redundant allergen sequence database (personal communication Dr P Johnson, University of Manchester, September 2014). Additional extracted ion chromatograms (EICs) and parent ion spectra were generated using the QualBrowser software package (Thermo Scientific) using an m/z range of the theoretical m/z +/- 0.01 Da.

6.12 Airborne peanut (publication 1)

To determine whether peanut could be aerosolised, experiments were conducted to try to capture airborne peanut was captured by glass-fibre filters inserted into the inhalable occupational medicine (IOM) sampling head of a personal air sampling monitor (Casella Tuff, Bedford, UK). This method is described briefly in Publication 1. A full description with figures of the experiments performed is in Section 10.10 (Appendix 10).

6.13 Influence of early EPE on PS and PA in cohort studies

6.13.1 Study design

Cohort studies from the UK, USA and Europe were employed to assess the hypothesis that early EPE, measured by peanut protein levels in household dust, is a risk factor for the development of PS and PA in children with an impaired skin barrier. A summary of the three cohorts employed are described in Table 3. The MAAS and CoFAR study are described in the methods sections of Publication 3 and 4 respectively. Further details on the definition of PA (OFC criteria) for MAAS as well as the differences in dust collection procedure for MAAS are provided in Section 10.11 (Appendix 11). The third cohort employed (BAMSE) is not yet published thus I include full details of this cohort's methodology in the Methods Section 0.

Table 3: Descriptive of three cohort studies included in this PhD

	MAAS	BAMSE	CoFAR
Full name of study	Manchester Allergy and Asthma Study	Barn Allergy Milieu Stockholm Epidemiology	Consortium of Food Allergy Research
Country	UK	Sweden	US
Study format	Observational study but with randomised controlled trial within high-risk sub-cohort.	Observational study	Observational study
Commenced	1995	1994	2005
Recruitment	Antenatal	Antenatal	3-15 months
Atopy status	Unselected population based study	Unselected population based study	Egg / milk allergy or moderate-severe eczema with positive SPT for egg/ milk
Intervention arm	HDM reduction measures	Nil	Nil
Sample size recruited	1184	4089	512
Final sample size used in PhD	623	1878	359 (living room), 173 (bed-sheet)
Dust site	Upholstered furniture	Maternal mattress	Bed-sheet of infant or living room floor
Dust collection	Antenatally till 12 months	Two months after birth	3-15 months
PS outcomes	8 and 11 years	4 and 8 years	3-15 month
Definition PS: SPT	SPT≥3mm	N/A	SPT≥3mm
Definition PS: sIgE/ CRD	>0.35kU/L (CRD)	≥0.35kU/L (sIgE/CRD)	≥0.35kU/L (sIgE)
Clinical allergy	OFC or 95% PPV	Questionnaire based only	Not yet available (due OFC results)

6.14 Confounding factors for all cohorts

The following demographics, clinical outcomes and potential sources of peanut exposure were requested from the databases of the cohorts described above (where available) so that the association between these variables and PS or PA could be assessed.

1. Parental atopy: PA is more common in children with atopic parents, as atopy is inherited, and PA is higher in first degree family members of peanut allergic children (see Section 1.7). [63] Parental atopy also increases the risk of other atopic diseases (eczema, egg sensitization/allergy, allergic rhinitis) and may influence behaviour towards peanut consumption during pregnancy, breastfeeding and early childhood given previous recommendations, [100] as well as duration of breastfeeding. [119]

2. HPC: HPC is a known risk factor for PA; however, these data were only available by proxy (peanut butter in the home during breastfeeding) in CoFAR and was not available in MAAS or BAMSE. [104]

3. Infant peanut consumption was not available in all three cohort studies, which is unfortunate given that this might impact both on PS and PA (potentially via oral tolerance induction) and on environmental peanut levels (potentially via epicutaneous sensitization).

4. Sex: Lifetime prevalence rate of PA is higher in male children than female children both in the US [13] and the UK. [132] In the US self-reported allergy to peanut and tree nuts was 1.7% in male versus 0.7% in female children aged 0-18 years ($P = 0.02$).[13] In England, using GP recorded diagnosis of PA, the crude lifetime prevalence rate of PA was 1.77/1000 (95% CI: 1.63-1.91) in males versus 1.39/1000 (95% CI 1.27-1.52) in females between the ages of 0-19 years which was significantly different ($P<0.001$). [132]

5. Ethnicity was included for the following reasons:

a) Non-Caucasian participants were excluded when analysis of *FLG* loss-of-function mutations (R501X, 2282del4, S3247X, R2447X, 3673delC and 3702delG) were entered into the LR model; these *FLG* mutations have been associated with eczema and other atopic conditions only in Caucasian individuals. [258]

b) Race (Black ethnicity) has been associated with raised peanut sIgE in several studies; [9;375;376] however, in the LEAP study screening participants Black ethnicity was negatively associated with peanut SPT. [9]

6. Number of older siblings: The number of older siblings in the home could reduce the likelihood of PA on the basis of increased microbial exposure preventing atopy (as per the Hygiene hypothesis); [377] however, conversely it is possible that a large number of children in the home could lead to more peanut being consumed in the home and thus more EPE during infancy (prior to the infant consuming peanut).[104]

7. Maternal age: Food allergy in the child was associated with a higher maternal age in a Massachusetts case control study; the offspring of mothers aged over 30 years of age at the time of their child's delivery, had a 3.7- fold (95% CI: 1.67-8.20) increased risk of food allergy defined as a history of an acute allergic reaction and positive SPT of sIgE to that allergen. This risk was present after adjusting for socioeconomic status (using private versus no private medical insurance as a proxy) and birth order of the child. [378]

8. Infantile eczema (presence and severity): Presence and severity of eczema are well known risk factors for PS and PA.[49;143] Additionally, it is important to consider including eczema in the statistical model when assessing *FLG* loss-of-function mutations as a predictor of PS and PA as *FLG* is also a strong predictor of eczema. [259] Brown et al. (2011) found that the presence of a *FLG* loss-of-function mutation was a risk factor

for PA even after adjusting for eczema thus showing an independent effect of *FLG* mutations increasing the risk of PA.[286]

9. Egg SPT or sIgE sensitization: Children with egg allergy are highly atopic and at risk of developing PA, with approximately 20% to 30% demonstrating sensitization to peanut on SPT (data from the ALSPAC study).[49;104] More recently the LEAP study found that infants with egg allergy were 2.3 times [95% CI, 1.39-3.86] more likely to be peanut SPT sensitized than children without egg allergy recruited to the LEAP study (with moderate-severe eczema).

10. Allergic rhinitis: Tree and grass pollen allergy can lead to cross-reactive antibodies that lead to positive in vitro (sIgE) and in vivo (SPT) peanut cross-reactivity, but are not indicative of primary PA. Ara h 8 may be raised due to cross-reactivity with birch pollen (Bet v 1).[379] Ara h 5, a profilin (panallergen) may be raised due to cross-reactivity with grass or tree pollen (Bet v 2).[380] CCD (carbohydrate cross-reactive determinants) may be raised due to cross-reactivity with grass pollen.[381]

11. HDM intervention (MAAS cohort only): Impermeable mattresses and acaricide treatments of soft furnishing have the potential to reduce levels of HDM allergen exposure,[301] and affect exposure to other allergens such as peanut antigen or endotoxin.[382]

12. Peanut consumption during pregnancy and breastfeeding: The literature surrounding this as a possible risk factor for PA is discussed in Section 2.3.

13. Duration of maternal breastfeeding: Many studies have investigated the role of breastfeeding on the risk of eczema and food allergy. The literature surrounding this is discussed in Section 2.3.4.

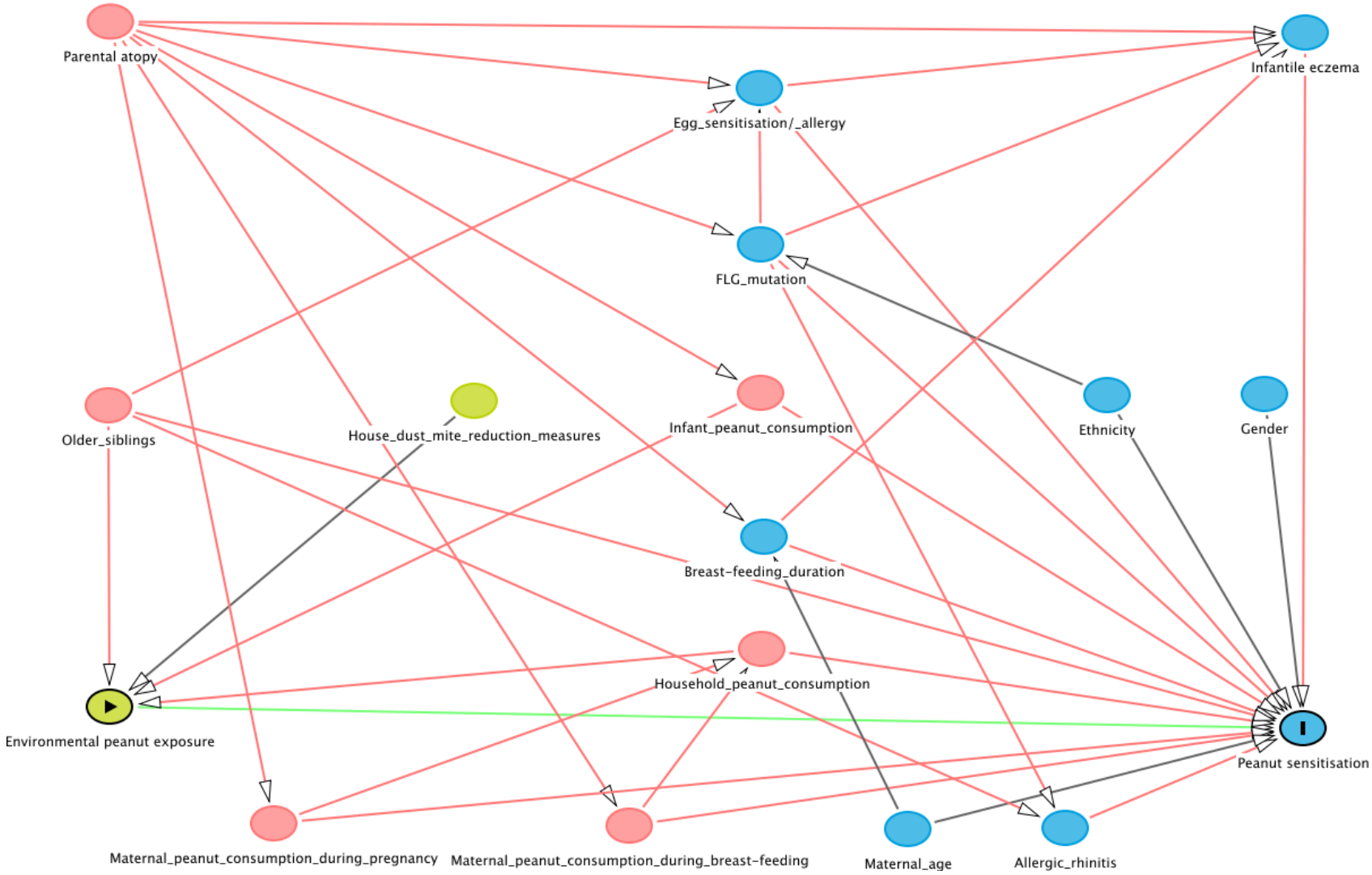
6.14.1 Relationship between variables and potential confounding factors

To assess potential confounding factors should be accounted for in the statistical analysis I used software for Direct Acyclic Graphs (http://www.dagitty.net/dags.html). This method assesses causal networks between variables under study to determine potential confounding variables, and has been recommended and used by various research groups.[383;384] The conventional method to determine covariates that need to be adjusted for is by performing a univariate followed by multivariate LR analysis with the outcome of interest. Figure 20 depicts possible associations between predictor variables (demographic, environmental, and behavioural) and the outcome variable PS. Figure 20 additionally depicts possible associations between demographic, environmental, and behavioural variables on the predictor variable of interest (EPE). An explanation of the hubs and pathways is given below:

1) Green hubs: Exposure variable and ancestor of exposure variables
2) Blue hubs: Outcome variable and ancestor of outcome variable
3) Pink hubs: Ancestors of exposure and outcome variables
4) Green lines: Causal pathways
5) Pink lines: Biasing pathways

Variables related to both the main predictor (EPE) and outcome variable (PS) were parental atopy, older siblings, infant peanut consumption, HPC, maternal peanut consumption during pregnancy and breastfeeding. Factors related only to the outcome variable (PS) were infantile eczema, *FLG* mutations, egg sensitization, ethnicity, sex, breastfeeding duration, maternal age and allergic rhinitis. HDM reduction measures were only related to EPE as there is currently no evidence at present to suggest that these measures reduce the risk of PS.

Figure 20: Possible associations between EPE and PS and other factors



6.15 *FLG* genotyping

FLG genotyping was performed using genomic DNA extracted from blood. Although DNA can also be extracted from buccal swabs this method is less reliable so only blood for genotyping was used.[385] *FLG* loss-of-function mutation prevalence, association with eczema and penetrance in studies from countries similar to the cohorts employed in this PhD are described in

Table **4**. *FLG* genotyping on the following six *FLG* loss-of-function mutations was performed in MAAS: R501X, 2282del4, S3247X, R2447X, 3673delC and 3702delG. The reason for selecting these *FLG* mutations was that in previous literature each of these had been shown to be associated with eczema in the UK or Ireland. R501X and 2282del *FLG* mutations were the most prevalent *FLG* loss-of-function mutations in the UK and Ireland and were more highly associated with eczema than S3247X or R2447X *FLG* mutations. [386;387] S3247X and R2447X *FLG* loss-of-function mutations were not significantly associated with eczema in the UK case control study (Cumbria); however, in the Irish case control study *FLG* genotypes S3247X, R2447X and 3702delG were significantly associated with childhood eczema.[268] These discrepant findings may have been because in the Irish study controls were compared against children with moderate to severe eczema, [251] whereas the UK study compared controls against flexural eczema without selecting the more severe eczema group. [387]

Details of *FLG* genotyping for MAAS and CoFAR are described briefly in Publication 3 and 4. *FLG* genotyping was performed by each respective cohort (except CoFAR where genomic DNA was sent to Professor Irwin McLean's Team at the College of Life Sciences, University of Dundee). Although I did not perform *FLG* genotyping I discussed the protocol and observed the methodology when I visited the Genomics

Team at The University of Manchester and University of Dundee. Further description of *FLG* genotypes, RT-qPCR background and methods employed in MAAS and CoFAR are described in Section 10.12 (Appendix 12).

Table 4: Review of *FLG* loss-of-function mutations in studies from countries from respective cohorts (MAAS: UK, CoFAR: USA and BAMSE: Sweden)

Country + *FLG* mutations assessed	**Study population**	**Prevalence of at least one *FLG* mutation**	**Prevalence of eczema in *FLG* mutations**	**Association with eczema OR (95% CI)**
Sweden (Stockholm) [388] R501X 2282del4	Eczema families recruited from dermatology clinic with at least 2 siblings affected (n=921)	**All patients:** R501X: 3.2% 2282del4: 7% Combined:9.6% **Severe eczema:** Combined genotype: 43.4%	Severe eczema: Wild-type *FLG*:16% Heterozygous *FLG* mutation: 42% Homozygous *FLG* mutation: 67%	R501X: OR2.68 (1.34-5.33) 2282del4: OR1.85 (1.24-2.76) Combined: OR 2.21 (1.5-3.25) Severe eczema: OR 4.73 (2-11.29)
US (North America) [389] R501X 2282del4	Childhood Asthma management program (children with asthma) n=646 children	**All children:** R501X: 3.7% 2282del4: 5.6% Combined: 8.7% **No eczema:** R501X: 2.5% 2282del4: 4.9% Combined: 7.6% **Eczema last 2 yrs:** R501X:7.3% 2282del4: 8.3% Combined 14.3%	Wild-type *FLG*: 27% Heterozygous *FLG* mutation: 37% Homozygous *FLG* mutation: 100%	R501X: OR3.1 (1.4-6.8) 2282del4: OR 2.0(1.1-3.7) Combined: OR 2.4 (1.5-3.9)
Cumbria, UK [386;387] R501X 2282del4 R2447X S3247X 3702delG	Population birth cohort school aged children (n=792)	**Control popn:** R501X: 5.98% 2282del4: 4.83% R2447X: 1.33% S3247X: 0.66% 3702delG: 0.0% **Eczema popn:** R501X: 8.76%	Wild-type *FLG*: 14.3% Heterozygous *FLG* mutation: 16.3% Homozygous *FLG* mutation: 55.6%	Using allelic model R501X: OR 1.6 (0.9-2.8) 2282del4: OR 2.0 (1.1-3.7) R2447X: OR 1.2 (0.3-4.5) S3247X: OR 2.3 (0.5-10.5)

		2282del4: 9.28% R2447X: 1.56% S3247X: 1.54% 3702delG: 0.52%		3702delG: N/A Combined OR 1.8 (1.2-2.6)

6.16 Statistical powering

Hypothesis: Early EPE in children with *FLG* loss-of-function mutations is a risk factor for PS

Simulations were carried out by Professor Andy Grieve (Professor of Statistics, KCL) to determine the power of the study to detect a range of values for the slope of the interaction of *FLG* loss-of-function mutation and environmental exposure in the logistic model. The estimated prevalence of PS and PA (where available), *FLG* mutation rate and mean and standard deviation of log transformed peanut protein levels in 20 random dust samples from each cohort were used for this simulation (see Table 5).

Table 5: Data used to determine power of each cohort study (using estimated data)

Study	Estimated prevalence of PS	Estimated *FLG* mutation rate	Mean log transformed peanut protein in dust	Standard deviation of peanut protein in dust
MAAS	4.2%*	6.6%	2.62	1.17
BAMSE	3.3%*	6.6%	3.11	1.07
CoFAR	17.5%	15%	3.75	1.15

*A correction factor of 0.6 in BAMSE and MAAS was included to account for pollen cross-reactivity.

It was subsequently possible to predict the detectable interaction *FLG* genotype by environmental peanut protein exposure odds ratio for each cohort given an estimated sample size based on communication with

principle investigators from these cohorts on how much dust was available and how many children had had *FLG* genotyping. On the basis of the sample sizes below each study had ≥80% power to detect an interaction odds ratio of 2.5 (Table 6).

Table 6: Detectable interaction OR for *FLG* genotype by EPE on PS

Study	Estimated sample size	Detectable interaction OR
MAAS	750	2.3
BAMSE	2132	1.9
CoFAR	140 (bed-sheet)	2.5

6.17 Statistical analysis for MAAS and CoFAR

Statistical analysis for results obtained for MAAS and CoFAR are described briefly in Publication 3 and 4 respectively. A detailed description of the statistical methods and the background to these methods is discussed in Section 10.13 (Appendix 13).

6.18 BAMSE study

The BAMSE study is an unselected Swedish population birth cohort which recruited 4089 children antenatally from 1994-1996.[390] The BAMSE study was an observational study to evaluate health conditions over time. No intervention was performed. The BAMSE study obtained ethical approval for environmental sampling of dust.

6.18.1 BAMSE data sources and definition of variables

Serum sIgE to peanut (ImmunoCAP system, Thermo Scientific, Sweden) was measured at 4 and 8 years and children were defined as 'peanut sIgE sensitized' if peanut sIgE was ≥0.35 kU/L. Component allergens (Ara h 1, 2, 3, 8 and 9) were assessed at 8 years (ImmunoCAP system, Thermo Scientific, Sweden) and children were considered to be 'peanut CRD sensitized' if Ara h 1, 2 or 3 was ≥ 0.35kU/L and non CRD sensitized if Ara h 1, 2 and 3 were <0.35kU/L or peanut sIgE was <0.35kU/L at 8 years. Children with positive peanut sIgE at 8 years of age but no component allergen analysis were excluded from the peanut CRD analysis. Peanut SPT was only available in 49 participants. Peanut SPT sensitization was defined as a mean wheal size ≥3mm. Egg sensitization was defined as egg sIgE ≥ 0.35KU/L at 4 years of age. Parental atopy was defined as a doctor's diagnosis of asthma and prescription of asthma medication and/or a doctor's diagnosis of hay fever in combination with furred pets- and/or pollen allergy at the time of questionnaire.

Genotyping was performed for *FLG* mutations common in Scandinavia by using TaqMan allelic discrimination assays for R501X and R2447X and matrix-assisted laser desorption/ionization-time-of-flight MS for 2282del4. Children with a mutation in any of these positions were classified as having a *FLG* loss-of-function mutation. A reported history of infantile eczema was assessed by questionnaire and was defined as dry skin, itchy rashes for 2 weeks or more and specific localization (face or arms/legs extension surfaces or arms/legs flexures or

wrists/ankles flexures) and/or doctor's diagnosis of eczema during the first year of life. Ethnicity was based on where the parents were born. Allergic rhinitis at 4 and 8 years was assessed using ISAAC validated questionnaires.[391] Clinical symptoms of reactivity to peanut were reported in 3.8% of children at 4 years and 5% at 8 years in this cohort but no food challenges were performed.

6.18.2 BAMSE dust samples collection procedure

Dust samples were collected at a median of 2 months of age from the mother's mattress. The mother's mattress was vacuumed for 2.5 minutes with a small disposable filter bag (Allergy Control Products Inc. Ridgefield CT, USA) inserted in the front hose of the vacuum. The dust containing filter bags were sealed in plastic bags and stored at -20ºC. Dust samples were sieved and fine dust was weighed and extracted in proportional volumes of extraction solution as previously described.[360] Peanut protein in dust was determined using the Veratox polyclonal ELISA against whole peanut protein (Neogen Corporation, Lansing, MI, USA). The lower limit of quantitation (LLQ) of the assay was defined as 100ng/ml and samples below this value were defined as 50ng/ml (LLQ/2).[392] Details of dust processing and extraction are described in Section 6.2. Peanut protein in dust was determined using the Veratox polyclonal ELISA as described in Section 6.4. Results were converted from ng/ml into μg peanut protein/gram dust. Due to batch to batch variability of the Veratox ELISA results were batch corrected prior to being entered into the final statistical analysis.

6.18.3 Batch correction

Due to the large number of dust samples in the BAMSE study it was not possible to analyze all dust samples using the same batch of Veratox peanut ELISAs. Different batches were therefore used and a correction factor

was employed to adjust for this when converting from ng/ml to µg/gram peanut protein. This batch correction was feasible because independent positive controls were run along the whole range of the standard curve at 0ng/ml, 250ng/ml, 500ng/ml and 1000ng/ml with each new ELISA kit. There were four batches which had similar results for the independent standards assessed; however, three batches had significantly higher results along the trajectory of the standard curve. The results obtained from ELISA batches which had similar results for the independent peanut standards were combined into a 'low batch' trend line to define the equation for this group, which was y = 1.0298x + 30.196. The three batches which ran higher than the independent peanut standard controls had trend equations as described below and in Figure 21:

1) Batch 26099: y = 1.2901x + 25.592
2) Batch 26100: y = 1.3411x + 13.036
3) Batch 26101: y = 1.5258x + 12.762

To correct for the higher results I used the following equations for batch correction:

1) All samples for Batch 26099:

   a. Subtract 25.59, divide value by 1.2901, multiply by 1.0298 and add 30
   b. INTO EXCEL FUNCTION =((value-25.59)/ 1.2901)*1.0298+30.196

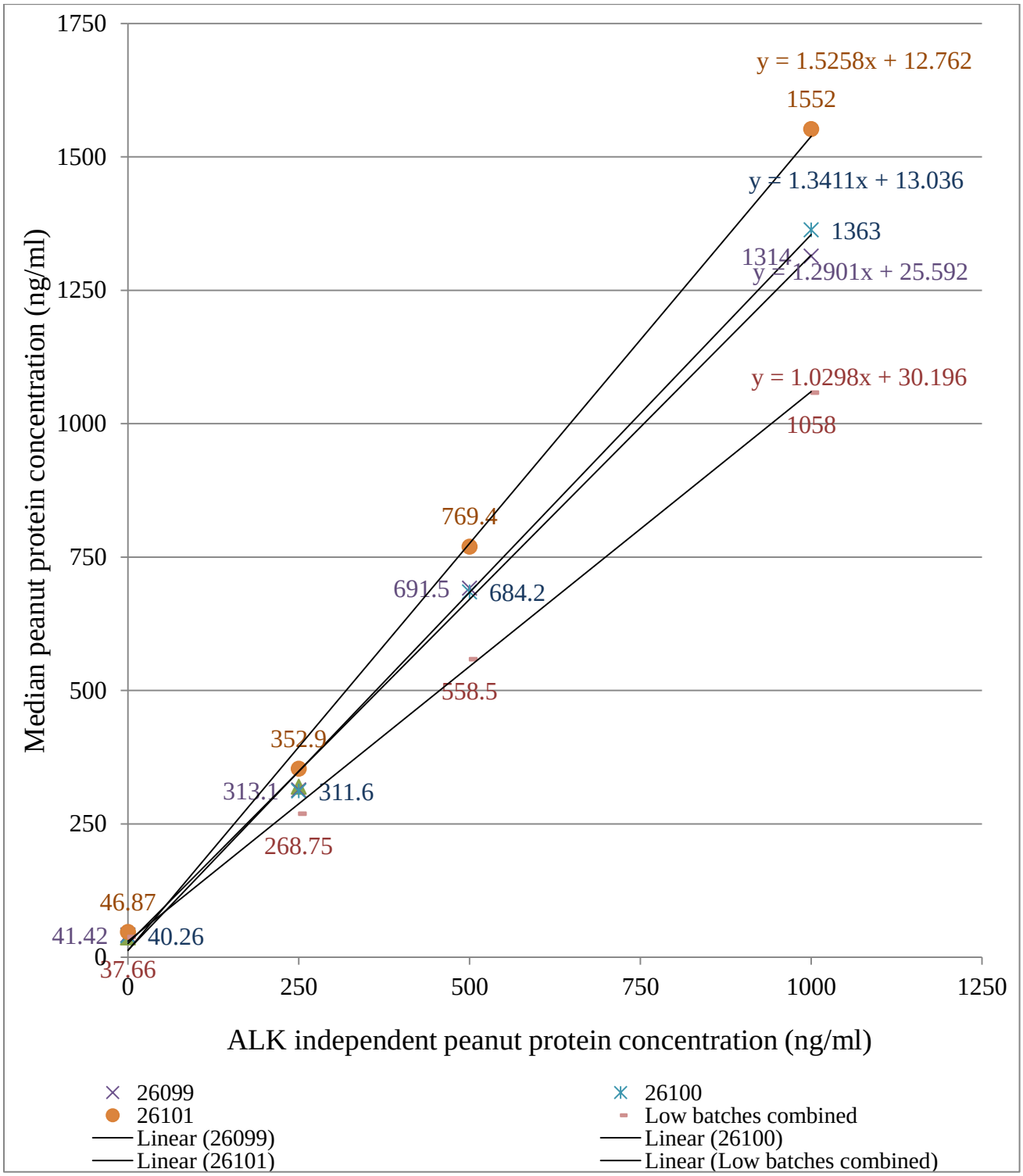
2) All samples for Batch 26100:

   a. Subtract 13.04, divide value by 1.3411, multiply by 1.0298 and add 30
   b. INTO EXCEL FUNCTION =((value-13.04)/ 1.3411)*1.0298+30.196

3) All samples for Batch 26101:

   a. Subtract 12.76, divide value by 1.5258, multiply by 1.0298, then add 30
   b. INTO EXCEL FUNCTION=((value-12.76)/1.5258)*1.0298+30.196

Figure 21: Results obtained for independent peanut protein along standard curve of Veratox ELISA



6.18.4 Statistical analysis for BAMSE study

Data were entered into an SPSS (SPSS 19.0; SPSS Inc, Chicago, IL, USA) and STATA spreadsheet (Timberlake Consultants Ltd London, UK). Peanut protein levels in dust (µg/gram) underwent natural log transformation. Initial nested case control analysis was performed with peanut sIgE sensitized cases at 4 years matched for sex and parental atopy with a 2:1 control (n=274) to case (n=137) matching. Children with peanut CRD sensitization defined by Ara h 1, 2 or 3 ≥0.35kU/L at 8 years of age were also matched against controls at age 8 years matched for sex and parental atopy with a 2:1 ratio of controls (n=130) to cases (n=65). Conditional LR (incorporating matching) was performed for the case control analysis using robust standard error for peanut sIgE sensitization at 4 years and peanut CRD sensitization at 8 years.

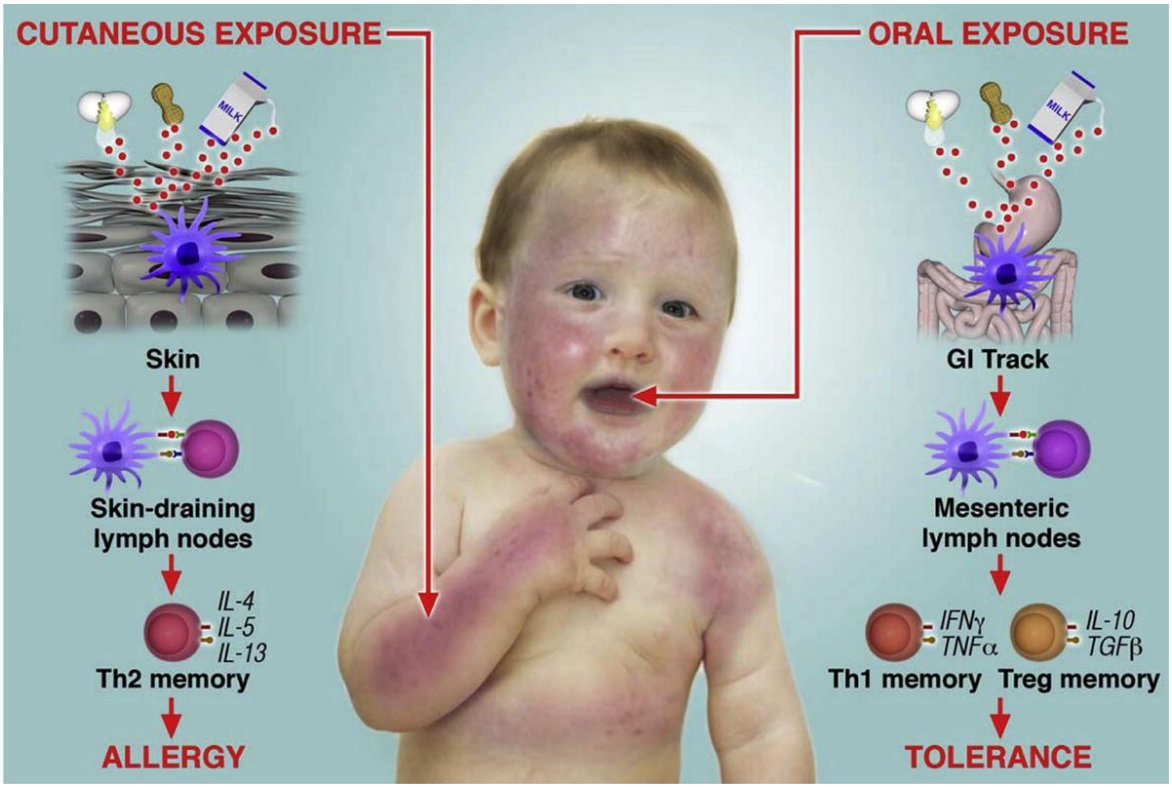
Subsequently I assessed the relationship between early EPE and PS in children from the whole BAMSE cohort with available postnatal maternal bed-dust and *FLG* genotyping (n=1878). LR analysis was performed for peanut CRD sensitization at 8 years of age. Factors associated with peanut sIgE sensitization were assessed using Generalized Estimating Equations (GEE) with an exchangeable working correlation matrix to account for repeated measures within individuals at 4 and 8 years. Univariate followed by multivariate regression analysis was performed including EPE, *FLG* mutations, infantile eczema and egg sensitization at 4 years plus other covariates significantly associated with PS ($P \leq .05$) that improved the quality of fit of the multivariate model using the Akaike information criterion (AIC) for LR and the Quasi Likelihood Independence models criterion (QIC) for GEE. Where the *FLG* mutation covariate was entered into the statistical model, participants with missing ethnicity data (35/1878=1.9%) and non-Caucasians (165/1842=8.8%) were excluded as distinct *FLG* loss-of-function mutations are present in different populations and the *FLG* loss-of-function mutations assessed in this study have only been associated with eczema in Caucasian European populations.[257;277] Peanut levels in dust (µg/g) were compared between groups using the Mann-Whitney U test. Proportions between groups (e.g. infantile eczema and *FLG* mutations) were compared using Pearson chi-squared. Statistical significance was assessed at $P<05$.

6.19 Differential gene expression in skin versus gut Th cell recall responses to peanut

6.19.1 Relevance to PhD hypothesis

The overriding hypothesis of this PhD is that early EPE, measured by peanut protein levels in household dust, is a risk factor for the development of PS and PA in children with an impaired skin barrier. I sought to use mechanistic work to support the concept that PS occurs through the epicutaneous exposure using recall responses to peanut from memory Th2 that are derived from the skin. The hypothesis for this mechanistic section was that memory peanut specific Th2 from the skin would upregulate genes within the Th2 cytokine network when cultured in peanut. Conversely memory peanut specific Th2 from the gut would upregulate genes within the Th1 or Treg network. This finding would also support the Dual Allergen Exposure Hypothesis, [393] which hypothesises that allergen exposure via the skin leads to a Th2 phenotype whereas allergen exposure through the gut leads to T regulatory function and oral tolerance induction (Figure 22).[205]

Figure 22: Dual Allergen Exposure Hypothesis
Reprint from Lack et al. (2012)[394] with permission from Elsevier provided by Copyright Clearance Center.



6.19.2 Patient selection and methodology

Children with PA, PS and atopic NA children were recruited from St. Thomas Hospital Children's Allergy Unit to assess differential gene expression in recall responses to peanut from skin versus gut-homing Th cells. The methods for PBMC isolation, culture of PBMCs in peanut protein versus medium alone, cell staining and FACS, RNA extraction and quantification, cDNA conversion, amplification, quantification, fragmentation and biotinylation for hybridization onto the Affymetrix microarray, microarray data normalisation and quality check, Partek analysis and automatic classification approach, and confirmatory RT-qPCR and intracellular cytokine staining (ICCS) are described in the online repository of Publication 5 (Section 7.10.1). Various optimisation steps which are not described in the online repository of Publication are detailed in Section 10.14 (Appendix 14), including the choice of medium for peanut culture, concentration of peanut protein for culture and measures to improve and confirm the quantity and quality of RNA extracted from PBMCs. Confirmatory RT-qPCR results are described only briefly in the online repository, therefore a more detailed description and justification of the methods employed are displayed in Section 10.15 (Appendix 15).

Chapter 7 Results

This PhD incorporates publications as results chapters, thus these publications include a brief introduction, method, results section (with associated tables and figures) and discussion. Not all of the work performed has been published to date (MS work and BAMSE cohort analysis) thus these results are included as separate subsection of the results section. The publications can be grouped into the three themes of this PhD:

(1) Characterisation of EPE

(2) Influence of EPE on PS and PA in children with an impaired skin barrier

(3) In-vitro responses to peanut from PA, PS and NA children, assessing memory T helper (Th) cell responses to peanut in skin- versus gut-homing Th cell subsets.

The publications and sections are listed below according to which theme they belong to:

7.1 Characterisation of EPE

1) Publication 1: Brough H.A., Makinson K, Penagos M, Maleki S, Cheng H, Stephens AC, Turcanu V, Lack G. Distribution of peanut protein in the home environment. J Allergy Clin Immunol 2013; 132(3):623-9

2) Publication 2: Brough H.A., Santos A, Makinson K, Penagos M, Stephens AC, Turcanu V, Lack G. Peanut protein in household dust is related to household consumption and is biologically active. J Allergy Clin Immunol 2013; 132(3):630-8

3) MS findings: This section is as yet unpublished and therefore the results are detailed in this PhD as a separate section.

7.2 Influence of EPE on PS and PA

1) Publication 3: Brough H.A., Simpson A., Makinson K., Hankinson J., Brown S., Douiri A et al. Peanut allergy: Impact of EPE in children with a filaggrin loss-of-function mutation. J Allergy Clin Immunol. 2014;134:867-75

2) Publication 4: Brough H.A., Sicherer S., Liu A., Makinson K., Douiri A., Wood R. et al. Atopic dermatitis increases the impact of exposure to peanut antigen in dust on peanut sensitization and peanut allergy. J Allergy Clin Immunol. 2015;135(1):164-170

3) BAMSE cohort study findings: This section is as yet unpublished and therefore the results are detailed in this PhD as a separate section.

7.3 Recall responses to peanut in children with PA, PS and NA in skin versus gut-homing Th cells

1) Publication 5: Brough H.A., Cousins D.J., Muntaenu A., Wong Y.F., Sudra A, Stephens A. et al. IL9 is a key component of memory Th cell peanut-specific responses from peanut allergic children. J Allergy Clin Immunol. 2014;134(6):1329-1338

7.4 Publication 1: Distribution of peanut protein in the home environment

Brough H.A., Makinson K, Penagos M, Maleki S, Cheng H, Stephens AC, Turcanu V, Lack G. Distribution of peanut protein in the home environment. J Allergy Clin Immunol 2013; 132(3):623-9

Reprinted from Brough et al. (2013) with permission from Elsevier.[360]

Distribution of peanut protein in the home environment

Helen A. Brough, MSc, MRCPCH,[a,b] Kerry Makinson, MSc,[a] Martin Penagos, MSc, MD,[a] Soheila J. Maleki, PhD,[c] Hsiaopo Cheng, MS,[c] Abdel Douiri, PhD,[d] Alick C. Stephens, PhD,[a] Victor Turcanu, PhD,[a]* and Gideon Lack, MD, FRCPCH[a]* *London and Southampton, United Kingdom, and New Orleans, La*

Background: To halt the increase in peanut allergy, we must determine how children become sensitized to peanut. High household peanut consumption used as an indirect marker of environmental peanut exposure is associated with the development of peanut allergy.
Objective: We sought to validate a method to quantify environmental peanut exposure, to determine how peanut is transferred into the environment after peanut consumption, and to determine whether environmental peanut persists despite cleaning.
Methods: After initial comparative studies among 3 ELISA kits, we validated and used the Veratox polyclonal peanut ELISA to assess peanut protein concentrations in dust and air and on household surfaces, bedding, furnishings, hand wipes, and saliva.
Results: The Veratox polyclonal peanut ELISA had the best rate of recovery of an independent peanut standard. We demonstrated 100% sensitivity and specificity and a less than 15% coefficient of variation for intra-assay, interassay, and interoperator variability. There was high within-home correlation for peanut protein levels in dust and household surface wipes. Airborne peanut levels were lower than the limit of quantitation for the Veratox polyclonal peanut ELISA in a number of simulated scenarios, except for a brief period directly above peanuts being deshelled. Peanut protein persisted on hands and in saliva 3 hours after peanut consumption. Peanut protein was completely removed from granite tables after cleaning with detergent, and levels were reduced but still present after detergent cleaning of laminate and wooden table surfaces, pillows, and sofa covers.
Conclusions: Peanut spread easily around the home and might be resistant to usual cleaning methods. Peanut protein can be transferred into the environment by means of hand transfer and saliva but is unlikely to be aerosolized. (J Allergy Clin Immunol 2013;132:623-9.)

Key words: *Peanut, sensitization, allergy, environment, dust, aerosolized, airborne, saliva, hand, ELISA, validation*

Peanut allergy is an important public health concern.[1] Ongoing studies on oral tolerance induction to peanut aim to address these issues (www.leapstudy.co.uk).[2] To halt the increase in peanut allergy,[3,4] we must first understand the mechanism of peanut sensitization. Household peanut consumption is 10 times higher in infants with peanut allergy versus high-risk (with egg allergy) control subjects.[5] In this study household peanut consumption was considered an indirect marker of environmental peanut exposure; however, peanut protein levels in the home were not directly quantified.

Few studies have assessed the distribution of peanut in the environment. Surface wipes from desks, cafeteria tables, and water fountains of 6 schools found little evidence of peanut using a monoclonal ELISA against Ara h 1 (INDOOR Biotechnologies, Warminster, United Kingdom).[6] Most cleaning agents (plain water, dishwashing liquid, sanitizing wipes, and bleach cleaner) were able to remove Ara h 1 from tables and hands spiked with 5 mL of peanut butter. Dish soap left residual Ara h 1 on 33% of tables (40-140 ng/mL), and Ara h 1 remained on 25% and 50% of hands after use of water and hand sanitizer, respectively.[6] Previous studies have quantified egg (ovomucoid), milk (β-lactoglobulin), and fish levels in household settled dust.[7,8] More recently, Ara h 2 has been quantified in bedroom dust of 18 (23.4%) of 77 children with asthma.[9] We have shown that peanut levels increase on bed sheets (on which participants have slept) the day after a single peanut-containing meal.[10]

As well as quantifying environmental peanut exposure, it is important to determine how peanut can be transferred into the environment from persons eating peanut. Aircraft often impose restrictions on peanut consumption because of concerns that persons with peanut allergy might inhale airborne peanut from other passengers eating peanuts on board.[11] There are anecdotal reports of allergic reactions after inhalation of peanut; however, when children with severe or reported inhalational reactions to peanut

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*These authors contributed equally to this work.

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Abbreviations used
IOM: Inhalable occupational medicine
LLQ: Lower limit of quantitation
VPPE: Veratox polyclonal peanut ELISA

underwent blind inhalational peanut challenges (peanut butter held 12 inches from the face for 10 minutes), these children had no allergic symptoms or signs.[12] Peanut protein has been detected in the ventilation system filters of commercial airliners after 5000 flight hours by using an inhibition assay with peanut extract[13]; however, the results of this abstract have not been replicated. Peanut protein might be transferred into the environment after peanut consumption through hand transmission[5] or saliva.[14] Ara h 1 has been measured in saliva in levels up to 40 μg/mL (enough to cause an allergic reaction) immediately after peanut consumption; however, it was undetectable in 87% of participants after 1 hour using a monoclonal ELISA against Ara h 1.[14]

This study was designed to validate a method to quantify environmental peanut protein levels in household dust, surfaces, bedding, furnishings, and air to quantify environmental peanut exposure and its potential role in peanut sensitization and allergy. We also wished to assess potential routes of peanut transfer into the environment and the effect of usual detergent cleaning on reducing environmental peanut levels.

METHODS

The study was approved by the Brent Medical Research Ethics Committee. Informed consent was obtained before environmental sampling and from participants who provided saliva and hand-wipe samples before and after peanut consumption.

Validation of method to quantify peanut protein in dust and wipes

The Veratox polyclonal peanut ELISA (VPPE) used in this study was validated according to the International Conference on Harmonization guidelines for validation of analytic procedures.[15] We also assessed aspects of dust processing related to peanut protein. Details of the methods used are included in the Methods section in this article's Online Repository at www.jacionline.org, including the following:

1. Details of samples used
2. Rate of recovery of an independent peanut standard comparing 3 validated commercial ELISA kits:
   A. VPPE (Neogen, Lansing, Mich)
   B. Biokits polyclonal Ara h 1 ELISA (Tepnel Research Products and Services, Flintshire, United Kingdom)
   C. Monoclonal ELISA against Ara h 1 (INDOOR Biotechnologies)
3. Performance characteristics of VPPE:
   A. Sensitivity and specificity
   B. Lower limit of quantitation (LLQ)
   C. Assay precision
4. Dust processing:
   A. Peanut protein in sieved fine dust versus residual fluff
   B. Extraction assays
   C. Effect of freezing and thawing extracted dust samples.

Peanut protein in household dust and surfaces

Forty-five families with infants were recruited from pediatric allergy clinics. Dust samples were obtained from the bed sheets of all household members and from the infant's play area; participants were asked not to wash or vacuum these for 5 days before the home visit. Dust samples were taken from each side of the parent's bed. The infant's play area was the place where the infant spent most of his or her day (eg, play mat/quilt and living room carpet).

A Philips cylinder vacuum FC8262 (1600 W) was connected to a Dustream adaptor and collector with a disposable nylon collection filter (pore size, 40 μm; INDOOR Biotechnologies). Bed sheets and the infant's play area were vacuumed for 2 minutes within a 1-m^2 surface area; the infant's bed sheet was vacuumed for 1 minute within a 0.5-m^2 area. Dust samples were sieved with a 300-μm copper sieve (Endecotts, London, United Kingdom), and fine dust was weighed to express results in micrograms of peanut protein per gram of dust. Dust was extracted in proportional volumes of the VPPE extraction solution and heated for 15 minutes at 60°C (see the Methods section in this article's Online Repository for further details). Dust samples of less than 5 mg were excluded.

Wipe samples made from Benchkote filter paper (Whatman, Maidstone, United Kingdom) cut to 4 × 4 cm and moistened with 0.5 mL of PBS were obtained from the parent's table, infant's highchair table, tap, dishwasher handle, refrigerator handle, and infant's crib rail. Table-surface wipes were collected within A4 paper–sized templates. Wipes were weighed before and after sampling to calculate results in micrograms of peanut protein per gram. Wipe samples were extracted in 2 mL of VPPE extraction solution in a sealed syringe. We used the VPPE to quantify peanut protein levels in dust and wipes. All samples collected were blinded from the researcher performing the ELISAs.

Airborne peanut

Airborne peanut was captured with glass-fiber filters (pore size, 0.7 μm) inserted into the inhalable occupational medicine (IOM) sampling head of a personal air-sampling monitor (TUFF; Casella Measurement, Bedford, United Kingdom). The pump was run at 2 L/min, as recommended by the manufacturer, which is equivalent to an infant's minute volume (tidal volume [5 mL/kg] × respiratory rate [40 breaths/min]), using an estimated weight of 10 kg. Glass-fiber filters were processed in the same way as wipes and analyzed with the VPPE. The VPPE LLQ was 100 ng/mL (equivalent to 2.5 μg/m^3). The following experiments were performed to detect airborne peanut:

1. The sampling head was held 1 cm (n = 3) and 1 m (n = 3) above a peanut butter jar/dry-roasted peanut bag for 22 hours and above a simmering pan of satay sauce (10.8 g of peanut; Amoy, Hayes, United Kingdom) for 10 minutes.
2. While eating peanut butter or dry-roasted peanuts, the sampling head was pinned to the researcher's clothes, placed on the dining room table, breathed on for 10 minutes, or placed overnight on the bedside table (n = 3).
3. The IOM was run for 22 hours in homes with high peanut protein levels in dust (n = 5; median peanut protein, 163.8 μg/g; range, 51.2-365.2 μg/g).
4. The sampling head was held 1 cm and 1 m above peanuts being deshelled. New glass-fiber filters were run in the IOM for 10 minutes before, during, immediately after, and 30 minutes and 1 hour after deshelling peanuts (n = 6).

Peanut protein on hands and saliva after peanut consumption

Hand-wipe and saliva samples were taken before and 3 hours after consuming 50 g of salted peanuts (n = 6; KP Nuts, Hayes, United Kingdom). Participants were asked not to eat peanut for 24 hours before and 3 hours after this peanut meal. Hand samples were taken with Benchkote wipes of the right palm (all subjects were right handed) and processed as described above. Saliva samples were collected into Eppendorf tubes and analyzed directly for peanut protein by using the VPPE without extraction.

Persistence of peanut despite cleaning

Table surfaces. Three table surfaces (wood [unpainted], granite, and laminate) were cleaned with water and allowed to air dry. A5 paper templates were sellotaped to the tables (n = 3). Smooth peanut butter (0.5 mL; Sun-Pat; Premier Foods Group, Manchester, United Kingdom) was spread evenly onto

TABLE I. Correlation grid of peanut protein levels (in micrograms per gram) in household dust and surface wipes

Maternal bed dust (n = 41)	Paternal bed dust (n = 38)	Infant's bed dust (n = 38)	Infant's play area dust (n = 38)	Sibling's bed dust (n = 17)	Parent's table wipe (n = 27)	Dishwasher wipe (n = 27)	Refrigerator handle wipe (n = 35)	Tap wipe (n = 36)	Infant's crib rail (n = 24)
4.19 (0.54 to 24.89)	*5.11 (0.92 to 25.63)*	*4.79 (0.76 to 33.27)*	*6.88 (3.25 to 48.95)*	*2.83 (0.39 to 50.4)*	*0 (0 to 0.68)*	*0 (0 to 1.28)*	*0 (0 to 1.52)*	*0 (0 to 0.49)*	*0 (0 to 0)*
Maternal bed dust	0.864 (0.753 to 0.927)*	0.844 (0.708 to 0.919)*	0.635 (0.383 to 0.799)*	0.700 (0.313 to 0.887)†	0.540 (0.224 to 0.753)†	0.619 (0.288 to 0.818)†	0.790 (0.610 to 0.892)*	0.516 (0.210 to 0.729)†	0.595 (0.253-0.805)†
	Paternal bed dust	0.760 (0.564-0.875)*	0.720 (0.501 to 0.852)*	0.609 (0.163 to 0.848)†	0.656 (0.382 to 0.824)*	0.773 (0.530 to 0.898)*	0.833 (0.676 to 0.917)*	0.668 (0.411 to 0.826)*	0.512 (0.137 to 0.758)†
		Infant's bed dust	0.862 (0.734 to 0.930)*	0.757 (0.241 to 0.914)*	0.645 (0.344 to 0.825)*	0.691 (0.381 to 0.861)*	0.728 (0.488 to 0.865)*	0.490 (0.151 to 0.726)†	0.692 (0.360 to 0.868)*
			Infant's play area dust	0.725 (0.339 to 0.902)†	0.639 (0.335 to 0.822)*	0.731 (0.473 to 0.873)*	0.694 (0.440 to 0.845)*	0.419 (0.07 to 0.677)†	0.693 (0.362 to 0.896)*
				Sibling's bed dust	0.532 (−0.026 to 0.837)‡	0.131 (−0.543 to 0.702)‡	0.595 (0.033 to 0.871)†	0.501 (−0.069 to 0.824)‡	0.247 (−0554 to 0.810)‡
					Parent's table wipe	0.571 (0.228 to 0.788)†	0.728 (0.499 to 0.862)*	0.759 (0.554 to 0.877)*	0.574 (0.212 to 0.797)†
						Dishwasher wipe	0.743 (0.500 to 0.877)*	0.770 (0.546 to 0.891)*	0.570 (0.141 to 0.818)†
							Refrigerator handle wipe	0.756 (0.566 to 0.870)*	0.633 (0.299 to 0.828)*
								Tap wipe	0.559 (0.201 to 0.785)†
									Infant's crib rail

Median peanut protein levels and interquartile ranges are displayed in italics. Spearman rho correlation coefficients (r_s) and 95% CIs are displayed for each combination. Statistical significance is shown as follows: *$P \leq .001$, †$P = .002$-$.049$, and ‡$P \geq .05$.

an index card, placed onto the template area with the peanut side down, held for 5 seconds, and removed. Three table surfaces were also exposed to plain butter as negative controls (n = 3). Benchkote wipes were used to obtain samples before the peanut spike, immediately after, after the water wipe, and after detergent cleaning (n = 3). The water-wipe technique was a single wipe with a clean paper kitchen towel (Bounty; Proctor & Gamble, Weybridge, United Kingdom) moistened with 1 mL of water. In the detergent clean the template was lifted, and the area was vigorously cleaned with 6 circular motions with a kitchen towel and washing up liquid (Fairy Liquid, Proctor & Gamble) and 1 mL of water followed by a water wipe with a new kitchen towel. New templates were placed over the original area, and wipe samples were taken again.

Pillows and sofa covers. Dust samples were vacuumed from 5 sofa covers (70 cm × 70 cm removed from sofa cushions) and 5 standard pillows (65 cm × 45 cm with pillow cases removed) to determine the effect of a detergent wash on household bedding and furnishings. The sofa covers and pillows were washed separately in a Hotpoint washer/dryer (model BHWD129; GE Appliances, Fairfield, Conn) on a 60°C cotton cycle with a 40-minute tumble dry using 1 cap of washing detergent (Lenor, Procter & Gamble) and fabric softener (Fairy). Peanut butter was consumed while sitting on the sofa cover (n = 5), and the pillow was slept on overnight (n = 5). Repeat dust samples were collected from the sofa covers and pillows; these were then washed separately, and when completely dry, further dust samples were collected (n = 5). In between each detergent wash, the washing machine was cleaned by using a 90°C cotton cycle and 1 cap of soda crystals.

Statistical analysis

Data were entered into an SPSS spreadsheet (SPSS 17.0; SPSS, Chicago, Ill) for the purposes of analysis. Spearman rank correlation coefficients (r_s) were used for all correlations, and 95% CIs were calculated by using Fisher r-to-z transformation. Paired differences between peanut protein levels in dust versus fluff, and in hand wipes and saliva before and after peanut consumption were assessed by using the Wilcoxon signed-rank test. Paired analysis between peanut protein levels in extracted dust processed immediately or frozen and then thawed over 2 or 24 hours was performed with the Friedman test. Statistical significance was assessed at a P value of less than .05.

RESULTS

Validation of method to quantify peanut protein in dust and wipes

Full details are described in the Results section in this article's Online Repository at www.jacionline.org. To summarize, the VPPE kit showed the best rate of recovery (66.3% to 119.8%) of the ALK-Abelló (Hørsholm, Denmark) independent peanut standard compared with the polyclonal and monoclonal Ara h 1 ELISA kits. Sensitivity and specificity were 100% with receiver operating characteristic curve analysis comparing 20 wooden tables spiked with peanut (and cleaned with detergent) versus tables that had not been spiked with peanut. There was no cross-reactivity with potentially cross-reactive proteins, including soya milk; crushed almond, cashew, or pistachio nuts; house dust mite; and human skin cells (given dust composite). We determined an LLQ of 62.5 ng/mL using the signal-to-noise approach (see Fig E1 in this article's Online Repository at www.jacionline.org). The coefficient of variation was less than 15% for intra-assay, interassay, and interoperator variability (see Fig E2 in this article's Online Repository at www.jacionline.org) for the VPPE standard curve, independent peanut standard, and peanut in dust. There was no significant difference in peanut protein levels in sieved dust versus residual fluff (see Fig E3 in this article's Online Repository at www.jacionline.org). The efficacy of extraction was greater than 99% (see Fig E4 in this article's Online Repository at www.jacionline.org). No peanut remained on the disposable nylon collection filter (see Fig E5 in this article's

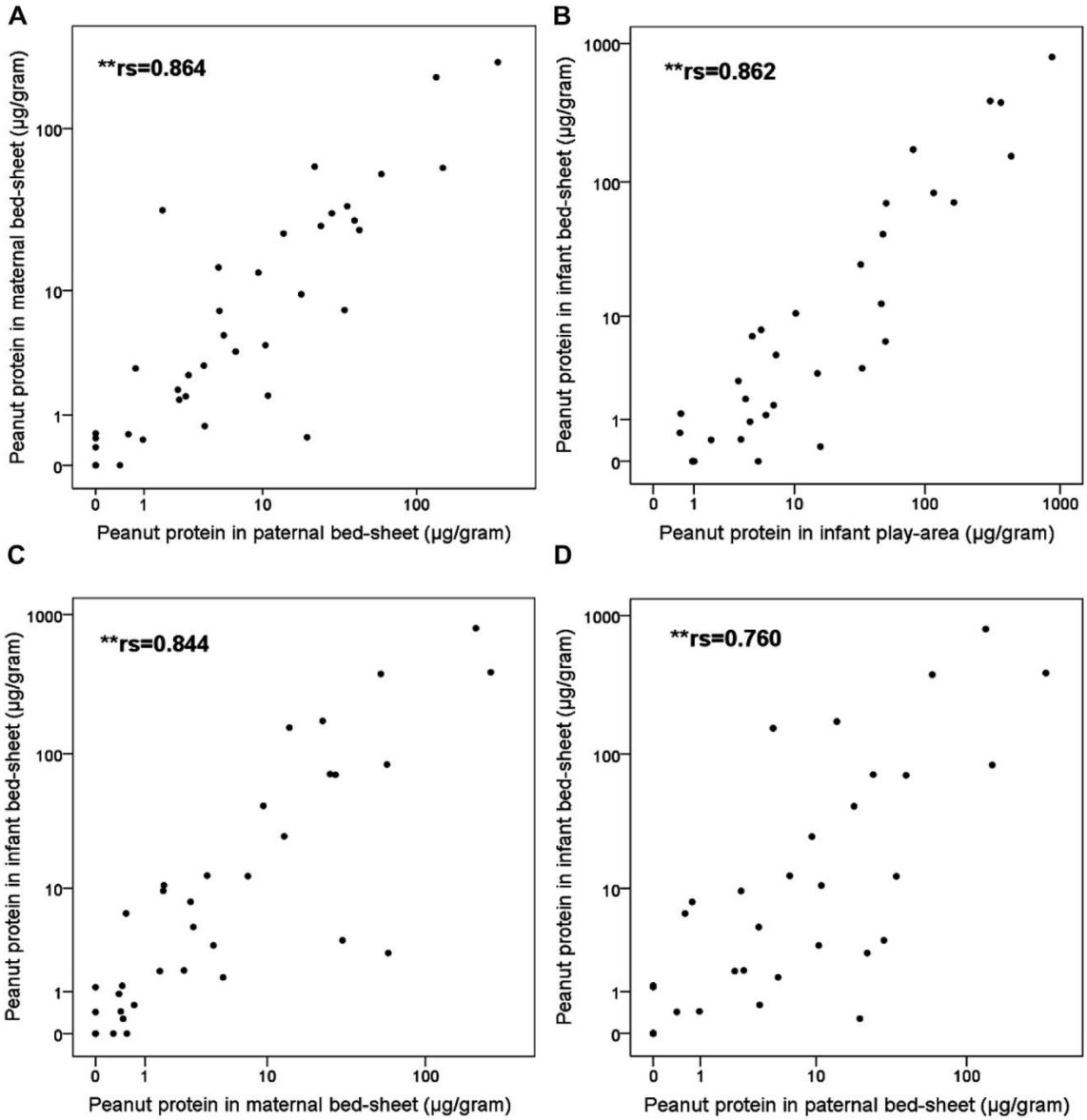

FIG 1. Within-home environmental peanut protein correlation. Correlations between peanut protein levels (in micrograms per gram) in maternal and paternal bed sheets (**A**; n = 38, r_s = 0.864; 95% CI, 0.753-0.927; $P < .001$), infant's bed sheets and play areas (**B**; n = 32; r_s = 0.862; 95% CI, 0.734-0.930; $P < .001$), maternal and infant's bed sheets (**C**; n = 34; r_s = 0.844; 95% CI, 0.708-0.919; $P < .001$), and paternal and infant's bed sheets (**D**; n = 33; r_s = 0.760; 95% CI, 0.564-0.875; $P < .001$) are shown. Axes are displayed in log scale.

Online Repository at www.jacionline.org). Freezing and thawing extracted dust samples did not affect peanut protein levels (see Fig E6 in this article's Online Repository at www.jacionline.org).

Peanut protein in household dust and wipes

Details of peanut protein levels in dust and wipes, as well as correlations between peanut protein levels in dust and wipes, are shown in Table I. Dust from the infant's play area had the highest peanut protein concentration, followed by dust from the paternal bed, infant's bed, maternal bed, and then sibling's bed. Peanut protein levels were lower in wipe samples than dust samples. Median results for peanut protein were less than the LLQ in wipe samples; however, the 75th percentile was highest for the refrigerator handle, followed by the dishwasher handle, parent's table, tap, and then infant's crib rail and table. There was high within-home correlation between peanut protein levels in dust, particularly between the maternal and paternal bed, the infant's bed and play area, and the maternal and infant's bed (all $r_s > 0.840$, $P < .001$, Fig 1). Peanut protein levels also correlated between dust samples and surface wipes (Table I). There were only 3 infant's table-surface wipe samples with peanut levels greater than the LLQ, and thus we did not include this in the correlation analysis.

Peanut protein levels measured with air-sampling monitors

Median peanut protein levels were less than the LLQ (2.5 $\mu g/m^3$) at all time points, except while peanuts were being deshelled. The median peanut protein level extracted from glass-fiber filters 1 cm above peanuts being deshelled (n = 6) was 330.90 $\mu g/m^3$ (range, 292.64-692.08 $\mu g/m^3$) and was still detectable 1 m above peanut deshelling (median, 4.76 $\mu g/m^3$;

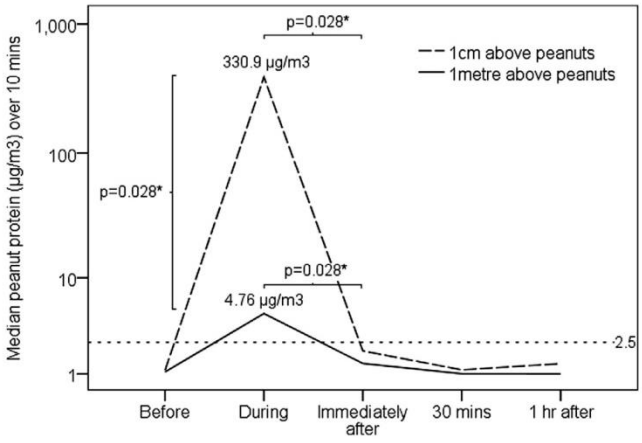

FIG 2. Time course of airborne peanut during peanut deshelling. The air sampling monitor was run for 10 minutes before, during, immediately after, and 30 and 60 minutes after deshelling peanuts at 1 cm and 1 m above the peanuts (n = 6). The *y-axis* is displayed in logarithmic scale. The LLQ (2.5 µg/m^3) of the VPPE is depicted by a *horizontal dotted line*. Median peanut protein levels (in micrograms per cubic meter) are depicted during peanut deshelling at 1 cm versus 1 m above peanuts ($P = .028$*), with different peanut protein levels during peanut deshelling versus immediately after deshelling both at 1 cm and 1 m ($P = .028$*).

range, 2.50-7.19 µg/m^3; Fig 2). Median peanut protein levels decreased to less than the LLQ immediately after peanuts stopped being deshelled, even at 1 cm above the peanut shells.

Peanut protein on hands and saliva after peanut consumption

Peanut protein levels increased from less than the LLQ (0.025 µg/ml) to a median of 3.34 µg/mL (range, 0.03-12.19 µg/mL) for saliva (n = 6) and 0.39 µg per wipe (0.03-1.18 µg per wipe) for the right hand (n = 6) 3 hours after peanut consumption (Fig 3).

Persistence of peanut protein despite cleaning

Table surfaces. After spiking wooden, granite, and laminate tables with peanut butter, median peanut protein levels increased from less than the LLQ (0.2 µg per wipe) to a median of 7.86 to 9.21 µg of peanut protein per wipe sample (Fig 4). The highest peanut protein samples obtained after spiking were from granite tables. After a single water wipe, there was only a small reduction in peanut protein levels to a median of 6.56 to 7.92 µg per wipe. After vigorous detergent cleaning, peanut protein levels were less than the LLQ on granite table surfaces but still present on the laminate (median, 0.47 µg per wipe; range, 0.42-0.55 µg per wipe; n = 3) and wooden (median, 1.75 µg per wipe; range, 1.62-3.33 µg per wipe; n = 3) tables. Peanut protein levels were less than the LLQ after plain butter spikes.

Sofa covers and pillows. The median peanut protein level in sofa cover dust (n = 5) at baseline was 38.74 µg/g (range, 10.05-485.43 µg/g), which decreased to 0.73 µg/g (range, 0.25-30.73 µg/g) after the first machine wash. After peanut consumption, peanut protein levels in the sofa cover dust increased to 6217.74 µg/g (range, 1293.02-6460.84 µg/g) and then decreased to 6.06 µg/g (range, 0.25-829.50 µg/g) after the second machine wash, constituting a median 1000-fold reduction in peanut protein (Fig 5, *A*). In a similar experiment peanut protein levels in pillow dust (n = 5) decreased from a median of 2.40 µg/g (range, 1.77-2.78 µg/g) to 1.14 µg/g (range, 0.85-1.76 µg/g) after the first machine wash and from 76.75 µg/g (range, 24.92-183.92 µg/g) after peanut consumption to 1.80 µg/g (range, 0.66-15.19 µg/g; Fig 5, *B*) after the second machine wash, constituting an approximate 40-fold reduction. The LLQ was 0.5 µg/g.

DISCUSSION

In this study we validated a sensitive, specific, and reliable method to quantify peanut protein levels in the home environment. Given that household peanut consumption is a risk factor for the development of peanut allergy,[5] this assay now allows direct quantitation of environmental peanut exposure. Using this commercial peanut ELISA kit, we detected high within-home correlation of peanut protein in household dust and surfaces, which suggests that peanut spreads easily around the home. We showed that hands and saliva are potential routes of peanut transfer into the environment after peanut consumption because peanut is present on both hands and saliva 3 hours after peanut consumption. Furthermore, we have shown that certain household surfaces, bedding, and furnishings retain peanut protein, even after cleaning with detergent; thus environmental peanut exposure in the home might remain after the usual cleaning methods.

Peanut is unlikely to be transferred into the environment by means of aerosolization because peanut could not be detected in a variety of aerosolization experiments, only temporarily while being propelled into the air by peanut deshelling. Previous authors were also unable to detect aerosolized peanut using air monitors strapped to the participants' heads while they ate and stamped on peanuts.[6] One could argue that the air monitor flow rate in our study was not strong enough (2 L/min); however, a previous study detected aerosolized egg at a flow rate of 1.7 to 2 L/min.[16] Additionally, we did not wish to artificially increase the flow rate to greater than a small child's minute volume because this would not have biological plausibility with regard to environmental peanut exposure and possible sensitization through the inhalational route. We ran the air samplers for as long as possible (22 hours) so as to be comparable with other studies detecting aerosolized egg (8 hours)[16] and fish (4-37 hours).[17] The fact that we could measure peanut protein at 1 cm and 1 m above deshelling peanuts proves that the Casella TUFF Air Monitor was able to capture aerosolized peanut protein. Similar findings have been reported by another group while deshelling peanuts using a SpinCon 3000 air collector (InnovaPrep, Drexel, Mo).[18] In our study median aerosolized peanut protein levels at 1 cm above peanut deshelling (330.9 µg/m^3) were similar to those found for aerosolized egg protein in egg factory transfer (644 µg/m^3) and egg-breaking rooms (255 µg/m^3)[16] but much higher than aerosolized fish protein in fish markets (2-25 ng/m^3).[17] Our study importantly showed that aerosolized peanut protein disappeared immediately after peanut deshelling stopped; thus we believe that the physical action of deshelling peanuts propelled peanut particles into the air, but these rapidly settled and did not remain airborne. Formation of large peanut protein aggregates after roasting could render the peanut particles less airborne, which might explain this observation.[19,20] Thus peanut protein is unlikely to cause either peanut sensitization or allergic manifestations in patients with peanut allergy through inhalation unless the peanuts are deshelled in close proximity to them. However, it is possible that a different cooking or handling method that was not tested here could lead to detectable airborne peanut levels. Additionally, these findings might not be applicable to pressurized and recirculated air systems, such as those found in commercial airliners; thus further studies are warranted in this field.

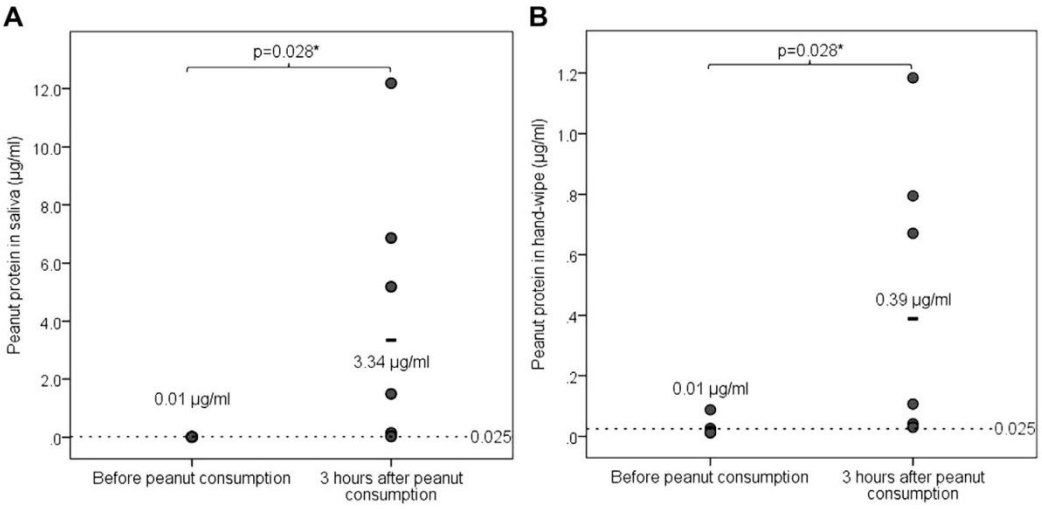

FIG 3. Peanut protein levels (in micrograms per milliliter) in saliva **(A**; n = 6**)** and on hands **(B**; n = 6**)** before and 3 hours after consumption of 50 g of peanut. Median peanut protein levels (in micrograms per milliliter) are displayed before and after peanut consumption (P = .028*). The LLQ (0.025 µg/mL) is displayed as a *horizontal dotted line*.

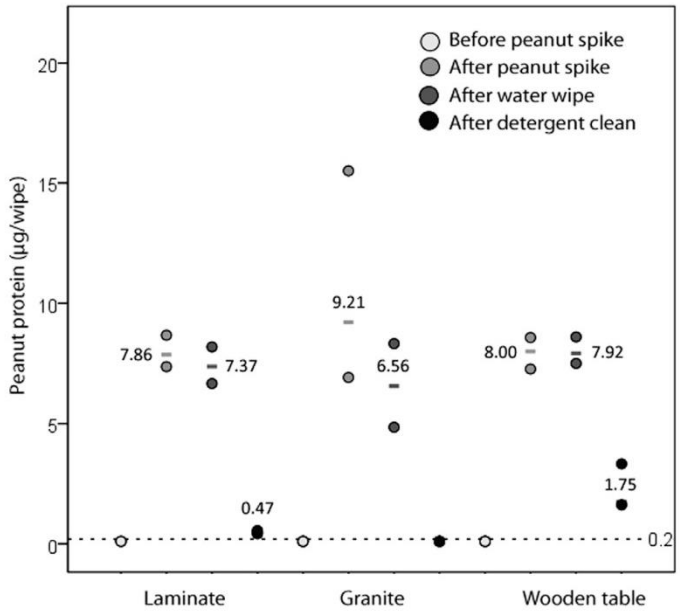

FIG 4. Peanut protein levels (in micrograms per wipe) on 3 different table surfaces (granite, laminate, and wood) before and after a 0.5-mL peanut butter spike, water wipe, and vigorous detergent cleaning (n = 3). Median peanut protein levels (in micrograms per wipe) are displayed as a *short horizontal line*. The LLQ (0.2 µg per wipe) is displayed as a *dotted line*.

Fox et al[5] postulated that environmental peanut exposure might occur through hand transmission because high household consumption of peanut butter (which is sticky and easily transferred on hands) was associated with a higher risk of peanut allergy than household consumption of covered peanuts (eg, chocolate). In this study we found peanut protein on hands and in saliva for a longer time period and at higher concentrations than found in other studies using a monoclonal ELISA against Ara h 1.[6,14] Peanut protein levels in saliva were almost 10 times higher than those in hand-wipe samples after peanut consumption. This might be because participants washed their hands during the 3-hour period but did not brush their teeth.[14] Vigorous cleaning with detergent did not remove peanut protein from laminate and wooden table surfaces, which is in contrast to previous literature.[6] This is likely due to the sensitivity of the VPPE used in this article. However, peanut levels were less than the LLQ on granite tables after detergent cleaning, presumably because it was easier to remove peanut from a smooth uncorrugated surface, such as granite, as shown by the high initial peanut result found on granite after peanut spiking.

We assessed the efficacy of detergent on removing peanut from the dust of sofa covers and pillows as a reflection of an infant's play and sleeping environment, respectively. Although machine washing significantly reduced peanut protein levels, there remained microgram quantities of peanut protein per gram of dust. Similar machine wash experiments have reduced house dust mite allergen levels but have had less success with cat allergen.[21] Persistence of environmental peanut protein after cleaning was still 1000 times lower than that required to elicit a peanut-induced allergic reaction (11.9-65.5 mg)[22]; however, this could be high enough to sensitize young children.

The VPPE had a more sensitive recovery rate of an independent peanut standard than the monoclonal or polyclonal Ara h 1 ELISA. This would be expected because a polyclonal anti-peanut ELISA has a greater diversity of antibodies than a monoclonal ELISA, and Ara h 1 is only one of the peanut proteins detected by using the VPPE.[23] We found similar peanut protein levels in sieved dust versus residual "fluff," which has also been found for Der p 1.[24] However, our experience was that the fluff was more difficult to centrifuge down, and thus we continued to use sieved dust for extraction and analysis. The nylon collection filter did not retain peanut protein, and thus dust could be tipped out of the filter without losing peanut protein. Freezing at −80°C and different durations of thawing did not affect peanut protein levels in extracted dust, which would be expected given that Ara h 1 and 2 are stable allergens resistant to other forms of environmental stress, such as heating and gastric digestion.[25,26]

The shortcomings of this study included the lack of an internationally recognized peanut reference standard to compare the peanut ELISA kits. However, steps are being taken toward having a suitable peanut reference standard to use as quality control material.[27] We addressed this by quantifying the peanut protein concentration and Ara h 1, 2, and 3 levels in the ALK-Abelló independent peanut standard.

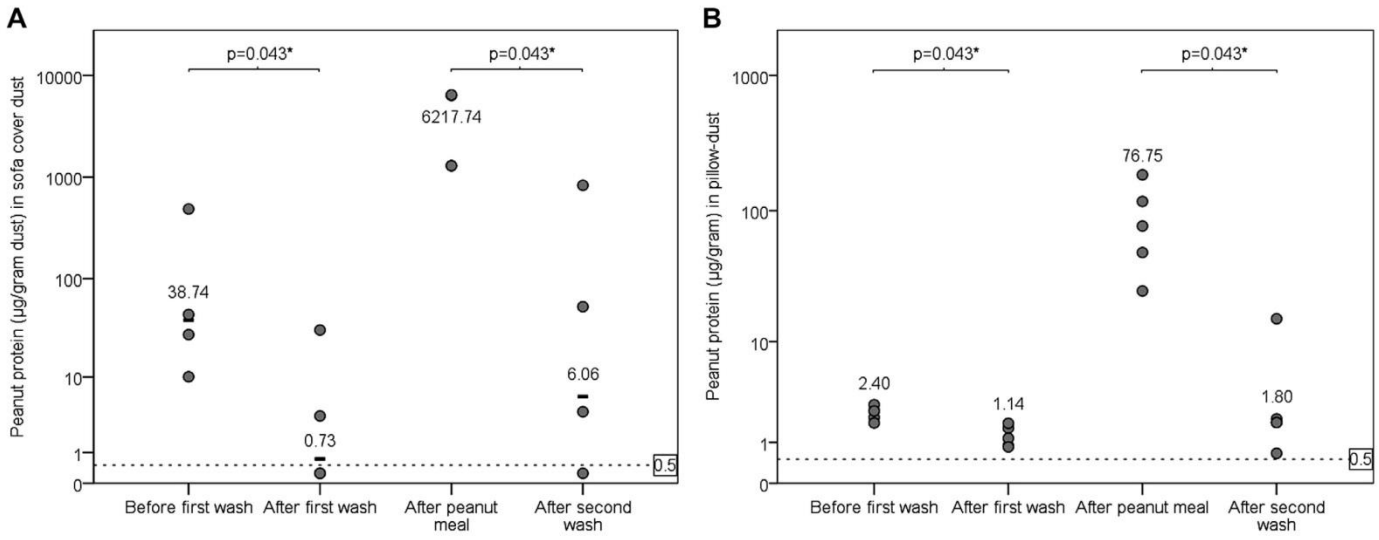

FIG 5. Peanut protein levels (in micrograms per gram of dust) in sofa covers **(A)** and pillows **(B**; n = 5 each**)** at baseline and after a machine detergent wash and then after peanut consumption and repeat machine detergent wash. Median peanut protein levels (in micrograms per gram of dust) are shown for each time point. The LLQ (0.5 µg/g dust) is displayed as a *horizontal dotted line*.

In conclusion, we have shown that peanut in the environment is measurable and transferrable and might persist despite usual detergent cleaning methods. Peanut protein can be transferred into the environment through hands or saliva but is unlikely to become airborne. Further research into the significance of environmental peanut exposure is required. We plan to further evaluate this in multicenter cohort studies.

We thank Dr Mohammed H. Shamji for advice on validation of assays, Ms Anjeli Chadha for collecting saliva and hand-wipe samples, and ALK-Abelló for providing the independent peanut standard.

Clinical implications: Environmental peanut protein exposure might be an important route of peanut sensitization. This study validates quantification of environmental peanut exposure for use in future studies.

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7.4.1 Online repository Publication 1: Distribution of peanut protein in the home environment

Online Repository for Distribution of peanut protein in the home environment

1) Samples used:

i. Independent peanut protein standard (provided by ALK-Abello, Hørsholm, Denmark)

ii. Benchkote wipes (Whatman, Maidstone, UK): negative control laboratory surfaces, cross-reactive food experiment, ROC curve analyzis for 'negative' and 'positive' table surfaces, table peanut spiking then cleaning experiment,

iii. Dust samples: Negative control new mattress, family bed-sheets and infant play-areas.

iv. 25mm circular glass microfibre filters (Whatman, GE Healthcare UK Ltd, Little Chalfont, UK) for insertion into Air Monitor pump.

2) Rate of recovery of independent peanut standard comparing three ELISA kits

We reviewed available validated commercial ELISA kits for the detection of peanut in food or food processing equipment to determine which would be most suitable for quantifying environmental peanut. [1;2] The Veratox polyclonal peanut ELISA (Neogen Corporation, Lansing, MI, USA) and Biokits polyclonal Ara h 1 ELISA (Tepnel Research Products and Services, Flintshire UK) correctly identified 99% of 240 test food samples spiked with 5µg peanut/gram or no peanut, displayed no cross-reactivity to 32 foods and showed the lowest false negative rate. [1] We also assessed a monoclonal Ara h 1 ELISA (Indoor Biotechnologies, Warminster, UK) as this had been used previously in environmental sampling. [3]

We compared these three ELISA kits for rate of recovery of an independent peanut standard (ALK Abello, Hørsholm, Denmark). To create this peanut standard raw peanut was extracted in cold isotonic phosphate buffer pH 6.5 at 100 mg/ml and magnetically stirring for 90 ± 15 minutes at 2-8°C. The extract was centrifuged at 20,000G for 30±5 minutes at 2-8°C then clarified by filtration through glass-fibre filters or 0.5 µm membrane filters. The pH was checked and adjusted to 6.5 ± 0.5. The extract was sterilized by filtering it twice through 0.2µm filters then

filled at 1ml per vial into sterilized glass vials and freeze dried. We confirmed peanut protein in the peanut standard using a number of methods repeated twice: O.D. 280, Biorad protein assay (14±1.1 µg/µl), SDS-Page and densitometry (15±3 µg/µl) and Biorad Experion chip (17 µg/µl) which we averaged out to 15.3 µg/µl. SDS-PAGE and densitometry, Experion capillary electrophoresis and ELISA determined the peanut allergen percentage: Ara h 1 mean 36±10%, Ara h 2 mean 10.7±5%, Ara h 3 mean 26.3±12%.

The Veratox peanut ELISA, Biokits polyclonal Ara h 1 ELISA and monoclonal Ara h 1 ELISA were carried out according to manufacturer specifications. Serial dilutions of the independent peanut standard were prepared using the relevant extraction solution for each ELISA kit. The polyclonal peanut ELISA and polyclonal Ara h 1 ELISA both gave results in ng/ml and ppm (parts per million) peanut. In order to convert values obtained from peanut to peanut protein we divided results obtained by 4 according to manufacturer specifications. The monoclonal ELISA expressed results in ng/ml of Ara h 1. We used a correction factor of 36% to convert Ara h 1 into peanut protein as we had already determined that there was 36±10% Ara h 1 in the independent peanut standard. These conversions were used to compare the rate of recovery of samples spiked with serial dilutions of the independent peanut standard for all three ELISAs.

3) Performance characteristics of Veratox peanut ELISA kit

Peanut standards provided by the Veratox peanut ELISA kit (100 ng/ml, 200 ng/ml, 400 ng/ml, 1000 ng/ml) were used to form the standard curve. Sample extraction was performed in a water bath at 60°C for 15 minutes. For each ELISA run we used an independent positive control from the batch of aliquoted peanut standards (ALK-Abello) (250 ng/ml) and a negative control (plain extraction solution). Samples were performed in duplicate. Only 24 wells were performed at once as recommended by the manufacturer. Samples were analyzed at 650nm wavelength on a Precision EMax microplate reader (Molecular Devices Inc. USA).

i) Sensitivity and Specificity

We obtained 20 'negative' wipe samples from a clean wooden table within A5 templates. We subsequently applied 0.5 ml peanut butter onto the table and cleaned this four times with detergent. In the detergent clean the template was lifted and area was vigorously cleaned with 6 circular motions with kitchen towel and Fairy Liquid washing up liquid (Proctor & Gamble, Weybridge, Surrey, UK) and 1 ml water followed by a water wipe with a new kitchen towel. We then took repeat 'positive' wipe samples from the same area. The 20 'positive' and 'negative' samples were used to define a (ROC: receiver operator characteristic) curve. We used the lower limit of quantitation of 100 ng/ml specified by the manufacturer. We vacuumed dust from a new spring mattress, took wipe samples from laboratory surfaces and table surfaces before and after spiking with possible cross-reactive proteins: soya milk (Alprosoy, Alpro UK limited, Burton Latimer, UK), crushed whole almond, cashew nut and pistachio. We assessed cross-reactivity to house dust mite and human skin cells (as dust contains house dust mite and skin cells) using Soluprick SQ House Dust Mite 10 HEP (ALK-Abello, Hørsholm, Denmark) (n=6) and human heel skin scrapings (n=3).

ii) Lower limit of quantitation

The Veratox polyclonal peanut ELISA stipulates a 100 ng/ml lower limit of quantitation. A 'signal to noise' approach was used to reassess this. We compared the optical density of serial dilutions of the lowest peanut standard provided by the Veratox kit (100 ng/ml down to 3.125ng/ml) in plain extraction solution and dust samples with undetectable peanut. We thus established the minimum concentration at which peanut could be reliably quantified with a signal-to-noise ratio of 10:1.

iii) Assay precision

We prepared aliquots of serial dilutions of the ALK-Abello peanut standard at 1000 ng/ml, 500 ng/ml and 250 ng/ml to cover the range of the Veratox peanut ELISA standard curve. Intra-

assay variability was assessed by running 13 wells (maximum available within one ELISA) for each peanut standard. Inter-assay variability was assessed for peanut levels in 13 dust samples and aliquots of three ALK-Abello peanut standards 20 times in separate ELISA run by the same operator. Inter-operator variability was assessed for three different operators for the Veratox ELISA standard curve and the independent peanut standard.

4) Processing dust samples

We assessed whether to sieve or not sieve dust, the efficacy of our extraction method and the impact of freezing and thawing extracted dust samples on peanut levels.

i) Peanut protein in sieved fine dust versus residual fluff

To assess whether peanut protein is found in the fine dust that is obtained after sieving or in the residual 'fluff' remaining (consisting of hair and other larger structures), we used a 300µm copper sieving device. Samples were extracted as follows: Eight level scoops (each approx. 4.38 grams) of extraction reagent (skim milk + additive) were added to 1 litre of PBS and warmed to 60°C in a pre-heated water bath. Two ml of extraction solution was added to 100mg of fine dust or fluff and this was placed in a 60 °C water bath shaker (50 rev/minute) for 15 minutes. After cooling, samples were centrifuged at 1467G (2500 rpm) for 20 minutes at 21 °C. The supernatant was removed, placed in a new Eppendorf tube and re-centrifuged as above. The supernatant was then aspirated and placed into a new Eppendorf to ensure no residual dust and frozen at -80°. Extracted samples were thawed over 2 hours and vortexed prior to analyzis.

ii) Extraction assays

We wanted to ensure that our method of extraction would get all peanut protein available for analyzis out of the collecting system. Our first concern was that the extraction technique recommended by the Veratox peanut ELISA kit would not remove all the peanut protein from

vacuumed dust. Our second concern was that a significant amount of dust and peanut protein would be stuck to the sides of the disposable nylon collection filter. We addressed these concerns with two different experiments:

a) Efficacy of extraction from dust

Vacuumed dust was collected using a 'Dustream' vacuum adaptor, collector and disposable nylon filter (Indoor Biotechnologies, Warminster, UK) from bed-sheets 18-24 hours after peanut butter consumption. We collected and extracted 7 dust samples in 16 mls of extraction solution (to cover the dust and filter) at 60°C for 15 minutes. Samples were centrifuged at 1467G (2500 rpm) for 20 minutes at 21 °C, the supernatant was tipped out and the dust was retained in the filter for a subsequent round of extraction. In 4 of the 7 samples the filter and dust were re-extracted without a water rinse. In the remaining 3 dust samples the filter and dust were re-extracted after the filters were rinsed with water to remove residual extraction solution.

b) Efficacy of extraction from collection apparatus

To assess whether peanut was stuck to the sides of the collection filter we vacuumed dust from bed-sheets after peanut butter consumption and analyzed the filter and dust by tipping the dust out of the filter and extracting the dust and filter separately in 16 mls of extraction solution.

iii) Impact of freezing and thawing

We assessed whether freezing and different methods of thawing extracted dust samples impacted on the level of measurable peanut in six dust samples. Dust extract was divided into three aliquots and was either processed immediately, or after being frozen at -80 °C for a week and then thawed either over 2 hours or 24 hours at room temperature.

Results

1) Comparison of three peanut ELISA kits

The rate of recovery for the monoclonal Ara h 1 ELISA ranged from 42.9- 93.0%; the polyclonal Ara h 1 ELISA ranged from 37.9-46.5% and the polyclonal peanut ELISA ranged from 66.3-119.8%. Thus polyclonal peanut ELISA underestimated peanut the least of all three ELISA kits; it slightly underestimated at values near the top of the standard curve and overestimated at values nearer the lower limit of quantitation (100 ng/ml).

2) Performance characteristics of the Veratox peanut ELISA kit

i) Sensitivity and Specificity

Analyzis of 20 table surface wipes before and after peanut spiking then cleaning with detergent was used to define the ROC curve. The area under the ROC curve was 1.00, 95% CI:1.0-1.0, $p<0.001$. This gave the test sensitivity and specificity of 100%. Putative negative surfaces from laboratory surface and a new mattress were also below the lower limit of quantitation. Wipes from table surfaces spiked with crushed almond, cashew, pistachio and soya had undetectable peanut. Human skin cells and house dust mite extract showed no detectable peanut signal.

ii) Lower limit of quantitation

Serial dilutions of the Veratox peanut standard (100 ng/ml down to 3.125 ng/ml) were added to either plain extraction solution or dust samples with undetectable peanut. The minimum concentration at which peanut could be reliably detected was 6.25 ng/ml above the plain extraction solution (Fig E1a) and 3.125ng/ml above the negative dust sample (Fig E1b). Thus using a conservative estimate the lower limit of quantitation was 10 x 6.25 = 62.5ng/ml. We found a lower limit of quantitation (62.5ng/ml) than that specified by the Veratox ELISA kit (100ng/ml), however given that the Veratox peanut ELISA slightly overestimated at lower values we adhered to the 100 ng/ml lower limit of quantitation in all samples tested.

3) Assay precision

i) Intra-assay variability: Mean results for the ALK-Abello peanut standards 1000 ng/ml, 500 ng/ml and 250 ng/ml were 972.6 ng/ml (SD 73.9), 488.6 ng/ml (SD 32.7) and 267.7 ng/ml (SD 32.42) respectively. Overall coefficient of variation (CV) was 8.73%.

ii) Inter-assay variability: ALK-Abello peanut standards (1000 ng/ml, 500 ng/ml and 250 ng/ml) analyzed in 20 separate ELISAs gave a mean of 1064 ng/ml (SD 84.2), 563 ng/ml (SD 35.2) and 285 ng/ml (SD 19.5) respectively (CV 8.46%). Inter-assay variability for 13 dust samples in three ELISAs was CV 13.7%.

iii) Inter-operator variability: Correlation coefficient for the ELISA standard curve was $r = 0.982$, $p<0.001$ (CV 2.13%) (Fig E2a) and $r = 0.95$ $p<0.001$ (CV 7.95%) for the aliquoted independent peanut standards (Fig E2b).

4) Collecting and measuring peanut protein in dust

i) Peanut protein in sieved fine dust versus residual fluff

There was no significant difference between paired levels of peanut protein in fine sieved dust versus the residual fluff left over after sieving ($p=0.95$) (Fig E3).

ii) No peanut remains in the dust or collection apparatus following single round of extraction

One of the four dust samples had detectable levels of peanut (330 ng/ml) when the disposable nylon filter and dust sample were re-extracted without a water rinse; this was 14.5% of the peanut measured on first extraction (1939 ng/ml) (Fig E4a). However all dust samples that were re-extracted after rinsing with water ($n=3$) had undetectable levels of peanut. Notably one of these dust samples had a very high peanut concentration following the first extraction (2513.5ng/ml) (Fig E4b). The three disposable nylon filters (with dust tipped out) had undetectable peanut despite the dust containing significant peanut levels (Fig E5).

iii) Freezing and different thawing methods do not impact on peanut levels in extracted dust

Freezing at -80°C and thawing dust samples for 2 versus 24 hours at room temperature did not significantly impact on the level of peanut detected in the extracted dust sample (p=0.311) (Fig E6).

References

(E1) Poms RE, Agazzi ME, Bau A, Brohee M, Capelletti C, Norgaard JV et al. Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. Food Additives & Contaminants 2005; 22(2):104-12.

(E2) Park LP, Coates S, Brewer VA, Garber AE, Abouzied M, Johnson K et al. Performance tested method multiple laboratory validation study of ELISA-based assays for the detection of peanuts in food. J AOAC Int 2005; 88(1):156-60.

(E3) Maloney JM, Chapman MD, Sicherer SH. Peanut allergen exposure through saliva: Assessment and interventions to reduce exposure. J Allergy Clin Immunol 2006;(3):719-24.

Legends for online repository of distribution of peanut protein in the home environment

FIG E1: Signal to noise approach to determine the lower limit of quantitation for the Veratox peanut ELISA using plain extraction solution (1a) and negative peanut dust samples (1b).

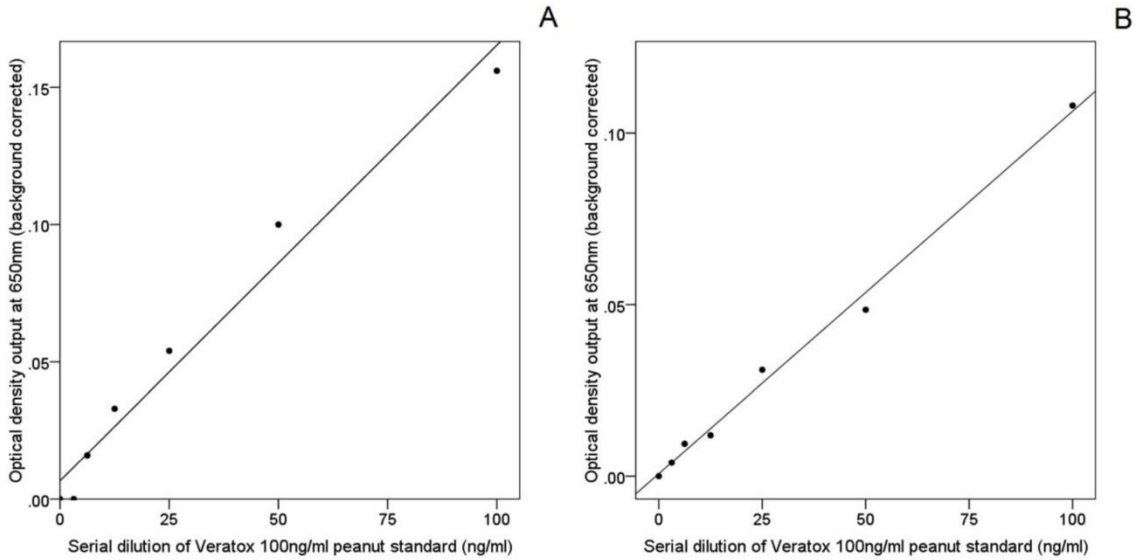


FIG E2: Inter-operator variability (n=3) for serial dilutions of the Veratox peanut ELISA standard curve (a) and ALK-Abello peanut standards (b). Spearman correlation coefficients shown were highly statistically significant with p<0.001**.

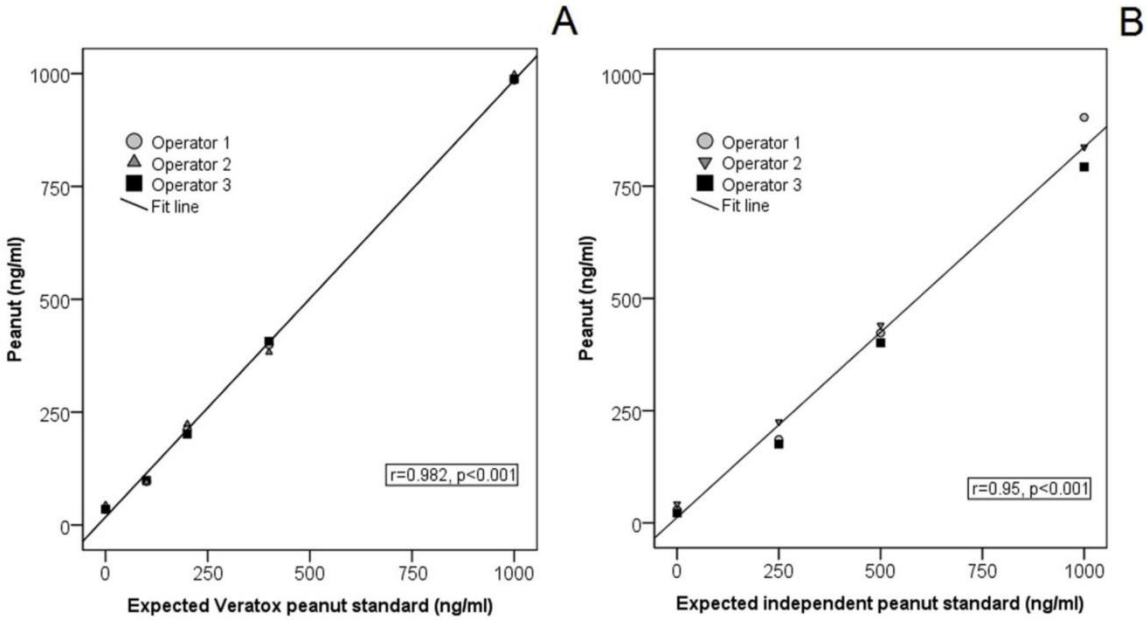


FIG E3: Peanut protein concentration (µg/g) in fine dust versus residual 'fluff' after sieving (n=15).

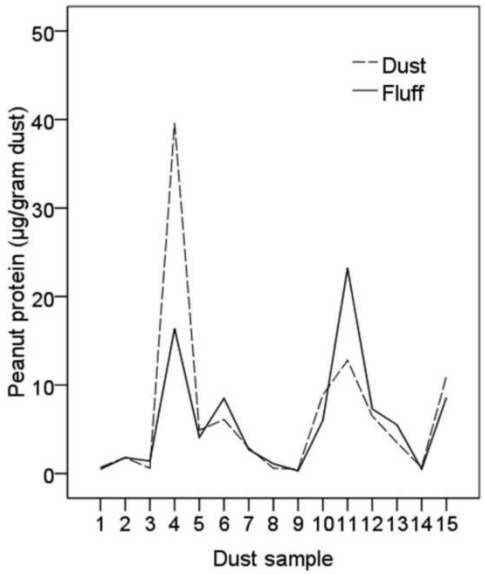


FIG E4: Efficacy of extraction procedure. Dust extracted inside nylon dust collection filter and then re-extracted with new extraction solution (n=4) (A). Dust extracted inside nylon dust collection filter then re-extracted with new extraction solution after rinsing the nylon filter (n=3) (B). Dotted line represents the lower limit of quantitation (100ng/ml).

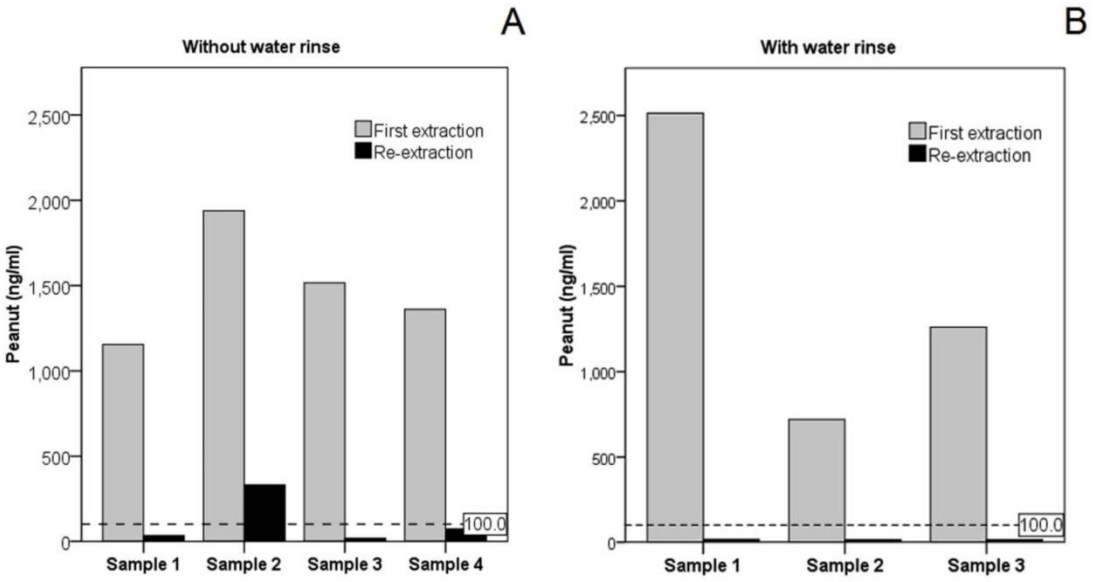


FIG E5: Efficacy of extraction from collection apparatus. Dust tipped out from nylon collection filter, extracted and analyzed for peanut. The nylon collection filter was then extracted and analyzed for peanut. The dotted line represents the lower limit of quantitation (100ng/ml).

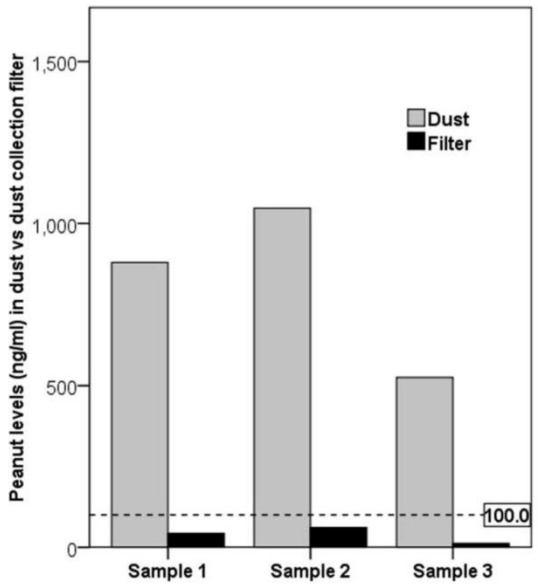
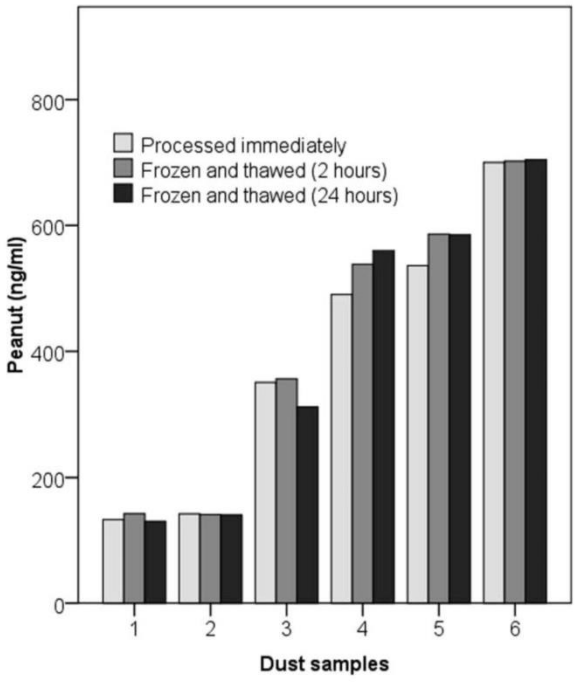


FIG E6: Peanut (ng/ml) in dust samples extracted and analyzed immediately, or frozen at -80°C for a week and then thawed at room temperature for either 2 or 24 hours (n=6).



7.5 Publication 2: Peanut protein in household dust is related to HPC and is biologically active

Brough H.A., Santos A, Makinson K, Penagos M, Stephens AC, Turcanu V, Lack G. Peanut protein in household dust is related to household consumption and is biologically active. J Allergy Clin Immunol 2013; 132(3):630-8

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Peanut protein in household dust is related to household peanut consumption and is biologically active

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Background: Peanut allergy is an important public health concern. To understand the pathogenesis of peanut allergy, we need to determine the route by which children become sensitized. A dose-response between household peanut consumption (HPC; used as an indirect marker of environmental peanut exposure) and the development of peanut allergy has been observed; however, environmental peanut exposure was not directly quantified.

Objective: We sought to explore the relationship between reported HPC and peanut protein levels in an infant's home environment and to determine the biological activity of environmental peanut.

Methods: Peanut protein was quantified in wipe and dust samples collected from 45 homes with infants by using a polyclonal peanut ELISA. Environmental peanut protein levels were compared with peanut consumption assessed by using a validated peanut food frequency questionnaire and other clinical and household factors. Biological activity of peanut protein in dust was assessed with a basophil activation assay.

Results: There was a positive correlation between peanut protein levels in the infant's bed, crib rail, and play area and reported HPC over 1 and 6 months. On multivariate regression analysis, HPC was the most important variable associated with peanut protein levels in the infant's bed sheet and play area. Dust samples containing high peanut protein levels induced dose-dependent activation of basophils in children with peanut allergy.

Conclusions: We have shown that an infant's environmental exposure to peanut is most likely to be due to HPC. Peanut protein in dust is biologically active and should be assessed as a route of possible early peanut sensitization in infants. (J Allergy Clin Immunol 2013;132:630-8.)

Key words: *Peanut, sensitization, allergy, environment, dust, ELISA, biological activity, basophil activation test*

Peanut allergy is increasing,[1,2] is a leading cause of anaphylaxis in food allergy,[3] and has a significant effect on quality of life for the child and his or her family.[4,5] Most children react on first known oral exposure to peanut, thus sensitization must be occurring earlier.[6-9] Understanding the way children become sensitized to peanut is therefore imperative to prevent this condition. Observational[9] and animal[10] work suggest that epicutaneous peanut exposure might play an important role in peanut sensitization; both early-onset severe eczema and the application of Arachis (peanut) oil onto eczematous skin are risk factors for the development of peanut allergy.[9] A dose-response relationship has been demonstrated between household peanut consumption (HPC; used as an indirect marker for environmental peanut exposure) and the risk of peanut allergy in young children.[11] Environmental peanut levels were not directly measured in the study by Fox et al[11]; however, previous studies have demonstrated quantifiable levels of egg, milk, fish, and peanut (Ara h 2) in vacuumed household settled dust.[12-14] In one study egg and milk protein levels in dust were high enough to elicit a positive RAST result with the sera of patients with egg and milk allergy, respectively.[12]

In addition to objectively quantifying environmental peanut protein, we wished to determine whether peanut allergens in dust maintain their biological activity. Functional *in vitro* assays that closely resemble *in vivo* allergic reactions have been used in previous studies to verify the allergenic potential of peanut proteins (ie, their ability to trigger mast cell and basophil activation and degranulation), both pure and in complex mixtures.[15,16] Levels of mediators, such as β-hexosaminidase and histamine, released by rat basophilic leukemia cells and stripped human basophils passively sensitized with plasma from allergic patients have

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*These authors contributed equally to this work.

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Abbreviations used
BAT: Basophil activation test
FFQ: Food frequency questionnaire
HPC: Household peanut consumption

been measured after stimulation with allergen.[17] Recently, assays evaluating the expression of activation markers on the surfaces of basophils of allergic patients by using flow cytometry (basophil activation test [BAT]) have gained application in the diagnosis of IgE-mediated food allergy,[18] including peanut allergy.[19,20] The BAT has also been used to assess the allergenicity of foods[21] and has been shown to be a sensitive and specific tool to detect trace amounts of peanut in food matrices.[22]

Given that household peanut exposure is a risk factor for the development of peanut allergy,[11] direct quantification of environmental peanut exposure might be important in future studies to determine threshold levels of peanut exposure associated with peanut sensitization, allergy, or both. Therefore in this study we aimed to relate peanut levels in an infant's home environment to peanut consumption by other household members and other potentially contributing factors. Additionally, we anticipated that the BAT would be useful in verifying the biological activity of peanut proteins in household dust samples.

METHODS

The study was approved by the Brent Medical Research Ethics Committee and South East London Research Ethics Committee 2. Forty-six families with infants were recruited from pediatric allergy clinics. One family did not complete their dietary questionnaire and thus was not included in the study. Three peanut-monoallergic and 3 nonallergic children (not allergic to peanut or to common food or airborne allergens) were recruited for the basophil activation assays. Written informed consent was obtained from parents of all children before any study procedures.

Dust and wipe samples

Families were asked to avoid washing their bed sheets and vacuuming their infant's play areas for 5 days before our home visit. Wipe samples were obtained from the parent's table, infant's highchair table, tap, dishwasher handle, refrigerator handle, and infant's crib rail with 4 × 4 cm Benchkote filter paper (Whatman, Maidstone, United Kingdom) and moistened with PBS. Wipes were weighed before and after sampling to calculate results in micrograms of peanut protein per gram of filter. Dust samples were obtained from the beds of all household members and from the infant's play area. Parents usually slept in the same bed, and thus dust samples were taken from each side of the bed. The infant's play area was the place where the infant spent most of his or her day (eg, Moses basket, play mat/quilt, and living room carpet). A Philips cylinder vacuum FC8262 (1600 Watts) was connected to a Dustream adaptor and collector (INDOOR Biotechnologies, Warminster, United Kingdom) with a nylon collection filter (pore size, 40 μm). The infant's bed sheet was vacuumed for 1 minute within a 0.5-m^2 surface area. Other bed sheets and the infant's play area were vacuumed for 2 minutes within a 1-m^2 surface area. Further details of the dust processing and analysis are described in a separate article.[23] We used the Veratox polyclonal peanut ELISA (Neogen, Lansing, Mich) to quantify peanut protein in dust and wipes. This ELISA has been validated for sensitivity, specificity, reliability, and efficacy of extraction for determining peanut protein contamination in food.[24-26] We have also performed our own validation of this ELISA to measure peanut protein levels in dust and wipe samples.[23] Results were converted from nanograms per milliliter into micrograms of peanut protein per gram of dust. All wipe and dust samples collected were blinded from the researcher performing the ELISA analysis. The main caregiver was asked to complete a questionnaire detailing factors that could affect environmental peanut levels.

Semiquantitative peanut food frequency questionnaire

The food frequency questionnaire (FFQ) used in this study has been validated for recall against the same FFQ over 2 years.[27] It has also been validated against a 7-day food diary over 6 months for maternal and infant peanut consumption.[28] The FFQ combined peanut-containing foods and other foods so as to prevent too much emphasis on peanut and potential recall bias. Standard portion sizes were obtained by using the Ministry of Agriculture's food portion sizes.[29] Frequency of peanut consumption was assessed over 1 week, 1 month, and 6 months. Moderate to high peanut protein consumption was defined as 10 g/wk or greater, as previously described.[28] HPC was the sum of peanut protein consumed by all household members.

Whole-blood BAT

Six extracted dust samples containing high (19.3-43.5 μg/mL, n = 3) or low (0.01-0.015 μg/mL, n = 3) levels of peanut protein were used to stimulate basophils of 3 peanut-monoallergic and 3 nonallergic children, resulting in 5 pairs of dust sample–patient experiments. The extracted dust samples with high peanut content were dilution-adjusted to a maximum concentration of 10 μg/mL and then underwent serial dilutions for comparability of dose-response. The low peanut dust samples were prepared by using the same dilution factor to make comparable other potential components of the dust extract that could activate basophils independently of peanut protein. Peanut allergy was diagnosed based on a combination of a recent history of an immediate allergic reaction to peanut and peanut skin prick test responses of 8 mm or greater (Stallergenes, Antony, France) and serum peanut-specific IgE levels of 15 KU/L or greater (ImmunoCAP; ThermoFisher Scientific, Uppsala, Sweden).[30] Nonallergic patients were eating peanuts regularly and had negative skin prick test results and negative serum specific IgE levels to peanut and common food and airborne allergens.

Heparinized venous blood was incubated for 30 minutes at 37°C, with equal volumes of extracted dust samples or a peanut protein standard (ALK-Abelló, Hørsholm, Denmark) and diluted in RPMI medium at serial 10-fold dilutions from 10,000 to 0.1 ng of peanut protein per milliliter. Anti-IgE (Sigma-Aldrich, Dorset, United Kingdom) and N-formyl-methionyl-leucyl-phenylalanine (Sigma-Aldrich) were used as positive controls. RPMI alone was used as a negative control. Cells were stained for CD123–fluorescein isothiocyanate, HLA-DR–peridinin-chlorophyll-protein, and CD63–allophycocyanin. Surface expression of these markers was evaluated by using flow cytometry in a FACSCanto II with FACSDiva software (BD Biosciences, San Jose, Calif). Data were analyzed with FlowJo software (version 7.6.1; TreeStar, Ashland, Ore).

Sample size calculation

To determine the sample size for this study, we used data from our previous study of peanut protein in dust.[31] Peanut levels in dust from beds on which participants had slept before and 18 to 24 hours after eating a peanut butter sandwich (n = 10) were used as an estimate for peanut levels in moderate to high (≥10 g/wk) and low (<10 g/wk) peanut-consuming families.[28] The mean difference in peanut levels before and after peanut consumption was 2.9 μg/m^2, with an SD of 2.9. Using a paired *t* test, we would need 17 moderate to high and 17 low peanut-consuming families (n = 34); however, adding 15% for unknown distribution/nonparametric test, this resulted in a sample size of 40 with a power of 80% and an α value of .05. We accounted for 15% dropout because of withdrawal or dust levels of less than 5 mg and thus recruited 46 families.

Statistical analysis

Data were entered into an SPSS spreadsheet (SPSS 17.0; SPSS, Chicago, Ill) for the purposes of analysis. Spearman rank correlation was used

TABLE I. Correlation grid (Spearman rank: r_s, 95% CI, and *P* value) between peanut protein consumption over 6 months (median [IQR] grams per week) and environmental peanut protein levels (median [IQR] μg/g) in household dust and surface wipes

Median (IQR)	Maternal peanut consumption, 2.40 g/wk (0.11-5.90 g/wk)	Paternal peanut consumption, 2.97 g/wk (0.00-10.80 g/wk)	Combined parental consumption, 5.40 g/wk (1.03-19.50 g/wk)	Infant peanut consumption 0.00 g/wk (0.0-0.00 g/wk)	HPC 7.38 g/wk (1.84-19.50 g/wk)
Peanut protein in maternal bed dust, 4.19 μg/g (0.54-24.89 μg/g)	r_s = 0.477 (0.199 to 0.684)†	r_s = 0.588 (0.335 to 0.762)*	r_s = 0.698 (0.498 to 0.827)*	r_s = 0.364 (0.064 to 0.604)†	r_s = 0.689 (0.484 to 0.852)*
Peanut protein in paternal bed dust, 5.11 μg/g (0.92-25.63 μg/g)	r_s = 0.390 (0.081 to 0.631)†	r_s = 0.591 (0.331 to 0.807)*	r_s = 0.672 (0.449 to 0.816)*	r_s = 0.346 (0.030 to 0.599)†	r_s = 0.692 (0.479 to 0.828)*
Peanut protein in infant bed sheet dust, 4.79 μg/g (0.76-33.27 μg/g)	r_s = 0.417 (0.108 to 0.652)†	r_s = 0.579 (0.305 to 0.764)*	r_s = 0.699 (0.489 to 0.832)*	r_s = 0.540 (0.267 to 0.733)*	r_s = 0.713 (0.510 to 0.840)*
Peanut protein in infant play area dust, 6.88 μg/g (3.25-48.95 μg/g)	r_s = 0.370 (0.058 to 0.616)†	r_s = 0.563 (0.293 to 0.750)*	r_s = 0.674 (0.452 to 0.817)*	r_s = 0.510 (0.228 to 0.713)*	r_s = 0.718 (0.517 to 0.843)*
Peanut protein on infant crib rail wipe, 0.0 μg/g (0.0-0.0 μg/g)	r_s = 0.150 (−0.260 to 0.514)‡	r_s = 0.606 (0.269 to 0.852)†	r_s = 0.612 (0.287 to 0.811)*	r_s = 0.782 (0.560 to 0.899)*	r_s = 0.585 (0.247 to 0.796)†
Peanut protein on dishwasher wipe, 0.0 μg/g (0.0-1.28 μg/g)	r_s = 0.311 (−0.078 to 0.617)‡	r_s = 0.454 (0.090 to 0.711)†	r_s = 0.601 (0.287 to 0.798)*	r_s = 0.594 (0.277 to 0.794)*	r_s = 0.685 (0.413 to 0.845)*
Peanut protein tap wipe, 0.0 μg/g (0.0-0.49 μg/g)	r_s = 0.256 (−0.079 to 0.539)‡	r_s = 0.300 (−0.042 to 0.579)‡	r_s = 0.431 (0.120 to 0.665)†	r_s = 0.355 (0.030 to 0.612)†	r_s = 0.468 (0.165 to 0.690)†
Peanut protein on refrigerator handle wipe, 0.0 μg/g (0.0-1.52 μg/g)	r_s = 0.508 (0.211 to 0.719)†	r_s = 0.559 (0.267 to 0.757)*	r_s = 0.653 (0.409 to 0.809)*	r_s = 0.520 (0.226 to 0.727)*	r_s = 0.632 (0.379 to 0.797)*
Peanut protein on parent table wipe, 0.0 μg/g (0.0-0.68 μg/g)	r_s = 0.462 (0.136 to 0.698)†	r_s = 0.336 (−0.020 to 0.616)‡	r_s = 0.455 (0.127 to 0.693)†	r_s = 0.173 (−0.294 to 0.573)‡	r_s = 0.509 (0.217 to 0.717)†

Statistical significance is shown as follows: *$P \leq .001$, †$P = .002$-$.049$, and ‡$P \geq .05$.
IQR, Interquartile range.

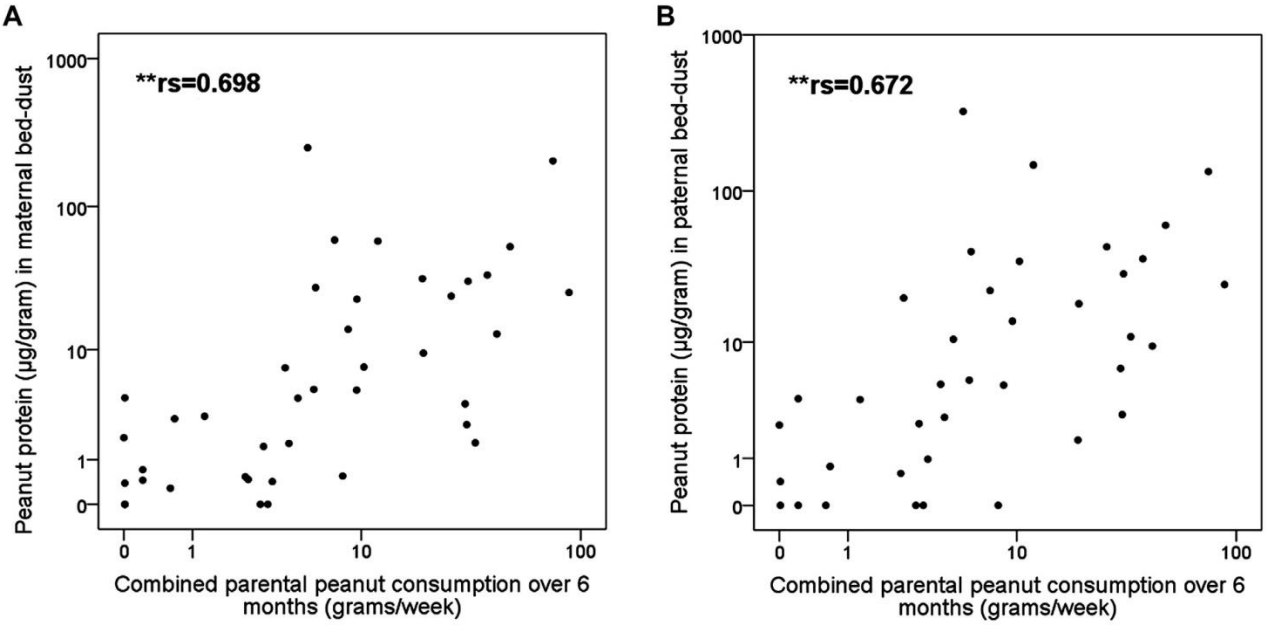

FIG 1. Correlation between combined parental peanut consumption (grams per week) over 6 months and peanut protein levels in the maternal bed (**A**; n = 41; r_s = 0.698; 95% CI, 0.460-0.811; $P < .001$) and paternal bed (**B**; n = 37; r_s = 0.672; 95% CI, 0.475-0.829; $P < .001$). Axes are displayed in log scale.

to correlate environmental peanut levels and average weekly peanut consumption; 95% CIs were calculated by using Fisher r-to-z transformation. Kendall tau correlation was used to confirm Spearman rank correlations between variables with multiple low values (infant peanut consumption and surface wipes). Peanut protein levels in the infant's environment were compared in moderate to high versus low peanut-consuming households by using the Mann-Whitney *U* test. Clinical, household, and behavioral factors affecting peanut protein levels in the home analysis. Peanut protein levels in dust and HPC underwent natural log transformation to meet the assumptions of a regression analysis. Univariate factors associated with peanut protein levels in the infant's bed dust and play area ($P \leq .1$) were included in the multivariate regression model. Basophil activation (as measured by the percentage of $CD63^+$ basophils) induced by serial dilutions of high or low peanut extracted dust samples, independent peanut standard, and controls in patients with peanut allergy and nonallergic children was compared by using the Mann-Whitney *U*

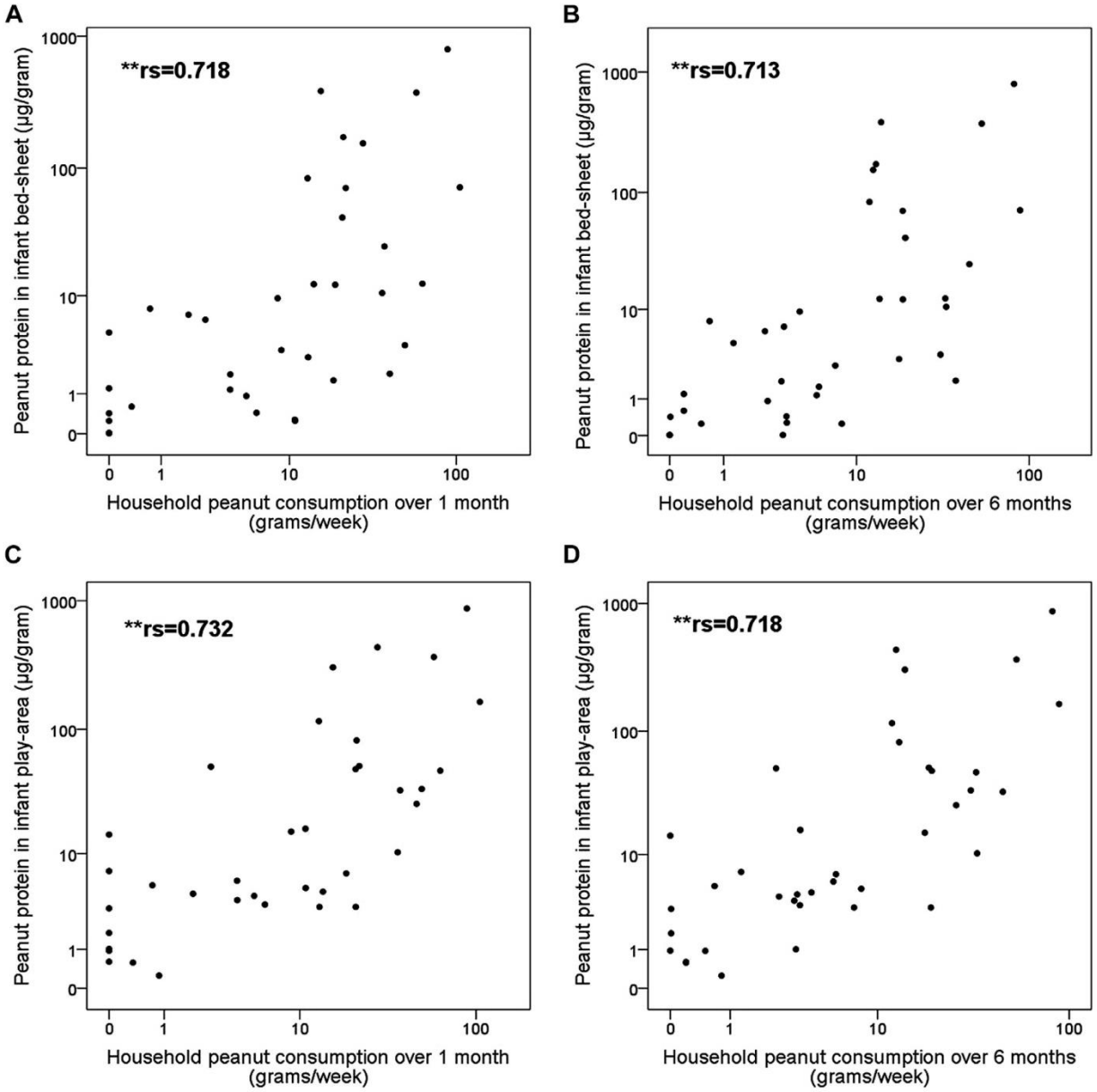

FIG 2. Correlation between peanut protein (micrograms per gram of dust) in the infant's bed sheet and HPC (grams per week) over 1 month (**A**; n = 38; r_s = 0.718; 95% CI, 0.517-0.843; $P < .001$) and 6 months (**B**; n = 38; r_s = 0.713; 95% CI, 0.510-0.840; $P < .001$). Correlation between peanut protein in the infant's play area and HPC over 1 month (**C**; n = 38; r_s = 0.732; 95% CI, 0.539-0.852; $P < .001$) and 6 months (**D**; n = 38; r_s = 0.718; 95% CI, 0.517-0.843; $P < .001$). Axes are displayed in log scale.

RESULTS

Peanut consumption is positively correlated with peanut protein levels in the environment

Results and correlations between environmental peanut levels in dust and surface wipes and peanut consumption in the home are displayed in Table I. Individual paternal and maternal peanut consumption was associated with peanut in the respective person's bed dust. However, combined parental peanut consumption showed higher correlation coefficients for peanut in the maternal and paternal sides of the bed (Fig 1). Infant peanut consumption was related to peanut levels in the infant's home environment (bed, crib rail, and play area). All 6 infants consuming peanut had high (≥10 µg/g dust) peanut protein levels in their play areas and bed dust.

We then went on to test whether parental peanut consumption and HPC affected peanut protein levels in the infant's environment. Paternal peanut consumption was more highly correlated with peanut levels in the infant's bed and play area than maternal peanut consumption. HPC was most highly correlated with peanut protein in the infant's bed and play area dust, with an r_s value of greater than 0.700 ($P < .001$) both at 1 and 6 months (Fig 2). There was also a positive correlation between HPC and peanut protein levels on the infant's crib rail and kitchen surfaces, particularly dishwasher and fridge handles. Spearman rank correlations were similar to Kendall tau correlations between surface wipes and infant peanut consumption (data not shown). Infant highchair table wipes were not included because only 3 wipes had detectable peanut levels. Peanut protein levels were

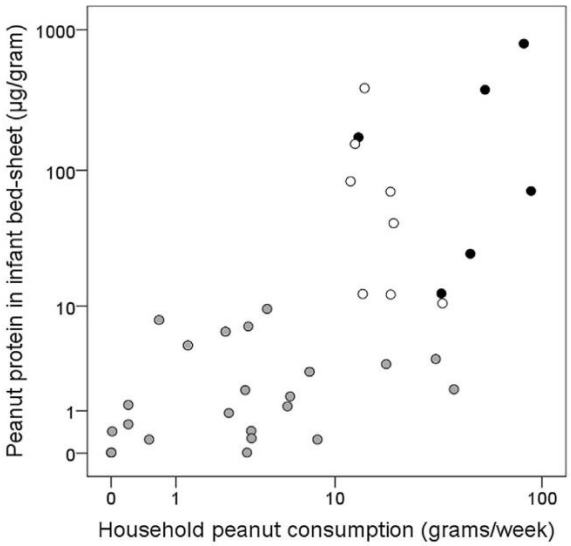

FIG 3. Average HPC over 6 months (grams per week) versus peanut protein levels in the infant's bed dust. High (>10 µg/g) peanut protein levels in the infant's bed dust are present when both the infant and parents eat peanut *(black dots)*. If the infant is not eating peanut, high peanut protein levels (>10 µg/g) in their bed dust is associated with moderate to high parental peanut consumption (≥10 g/week, *white dots*). Axes are displayed in log scale.

significantly higher ($P < .001$) in the infant's bed and play area in moderate to high (≥10 g/wk) versus low (<10 g/wk) peanut-consuming households.

High peanut protein levels in the infant's bed dust were found if both infants and parents were eating peanuts (Fig 3, black dots). If the infant was not eating peanut, high peanut protein levels (>10 µg/g) in their bed dust was associated with moderate to high parental peanut consumption (≥10 g/wk; Fig 3, white dots). Importantly, when we excluded families with infants consuming peanut from the analysis (thereby excluding high peanut-consuming parents and households), there was still a significant positive correlation between HPC and peanut protein in the infant's bed dust (r_s = 0.620; 95% CI, 0.341-0.798; $P < .001$) and play area (r_s = 0.629; 95% CI, 0.360-0.801; $P < .001$).

Linear regression analysis of factors affecting peanut protein levels in the environment

Associations between clinical, environmental, and behavioral characteristics and environmental peanut protein levels in the infant's bed dust are displayed in Table II. There was a positive linear relationship between HPC (*ln* grams per week over 6 months) and peanut protein in the infant's bed dust (*ln* micrograms per gram; coefficient [B] = 0.733; 95% CI, 0.399-1.068; $P < .001$) and play area (B = 0.602; 95% CI, 0.346-0.859; $P < .001$). The unstandardized coefficient (B) shows that for a natural log unit of peanut consumption (2.72 g/wk), peanut protein in the infant's bed dust increases by *ln* 0.733 units (2.08 µg/g). Maternal, paternal, combined parental, and infant peanut consumption were also associated with peanut protein in the infant's bed and play area dust but were not included in the multivariate analysis because HPC comprises each of these variables.

A reported family member with peanut allergy was associated with lower environmental peanut protein levels in the infant's bed and play area. On subgroup analysis, peanut protein levels were highest in the infant's bed in homes with no peanut allergy, followed by households with infants with peanut allergy, followed by households with "other" members with peanut allergy (excluding those with infants with peanut allergy). This pattern was mirrored by HPC (Fig 4). Age at assessment, sex, siblings, peanut allergy in the infant, size of the home, antiallergenic covers in the infant's crib, and age of the infant's mattress were not associated with peanut levels in the infant's bed (Table II).

In the multivariate regression model HPC was the only variable that remained significantly associated with peanut protein levels in the infant's bed dust (B = 0.492; 95% CI, 0.084-0.900; $P = .020$), adjusting for a household member with peanut allergy, ethnicity, where the infant slept, and dust weight. HPC was also the determining factor for peanut protein levels in the infant's play area (B = 0.539; 95% CI, 0.275-0.804; $P < .001$), adjusting for a household member with peanut allergy. Other clinical and household factors were not associated with peanut protein levels in the infant's play area. "Old" mattresses (>10 years) were associated with higher environmental peanut protein levels in the paternal (B = 1.945; 95% CI, 0.455-3.434; $P = .012$) and maternal (B = 1.434; 95% CI, −0.031-2.899; $P = .055$ [trend]) sides of the bed.

Biological activity of peanut protein in house dust

Three children with peanut allergy and 3 nonallergic children were recruited for the basophil activation assay on dust samples. Two patients with peanut allergy were sensitized to the 3 major peanut allergens (Ara h 1, Ara h 2, and Ara h 3), and 1 patient was sensitized to Ara h 1 and Ara h 2. None of these patients were sensitized to any other food or airborne allergens. Extracted dust samples with high or low peanut protein content were tested by using blood from children with peanut allergy and nonallergic children, forming 5 pairs of dust sample–patient experiments. Dust samples containing peanut protein levels of less than 0.375 ng/mL did not induce significant basophil activation either in children with peanut allergy (Fig 5, *A*) or in nonallergic children (Fig 5, *C*). However, basophils of children with peanut allergy showed clear dose-dependent upregulation of the activation marker CD63 with serial dilutions of peanut protein levels in dust (Fig 5, *B*), as opposed to basophils from nonallergic children, which did not respond to peanut protein in dust (Fig 5, *D*). The proportion of $CD63^+$ basophils was significantly higher in children with peanut allergy at peanut concentrations ranging between 1 and 10,000 ng/mL ($P < .01$). The threshold for basophil activation was 1 ng/mL using basophils from the 3 selected donors with peanut allergy (Fig 5, *B* [with peanut allergy], vs 5, *D* [nonallergic]). Basophils of allergic and nonallergic patients showed comparable spontaneous basophil activation and CD63 expression induced by anti-IgE and N-formyl-methionyl-leucyl-phenylalanine. In children with peanut allergy, the dose response of basophil activation by dust samples was comparable with the dose response to similar concentrations of the independent peanut standard. Basophils of nonallergic patients were not activated by the peanut standard up to 10 µg/mL peanut protein.

DISCUSSION

This is the first study to demonstrate that peanut protein levels in an infant's home environment are positively correlated with HPC and that peanut protein in household dust is biologically active. Previous work showed that HPC was a risk factor for the development of peanut allergy.[11] One criticism of this study was

TABLE II. Clinical, behavioral, and household factors associated with peanut protein levels in the infant's bed dust (*ln* micrograms per gram)

Clinical, environmental, and behavioral factors	Median (IQR) or frequency (%)	Univariate analysis for peanut protein in the infant's bed dust (*ln* μg/g)			Multivariate analysis for peanut protein in the infant's bed dust (*ln* μg/g)		
		Coefficient	95% CI	*P* value	Coefficient	95% CI	*P* value
Age at assessment	9 mo (6-10.5 mo)	−0.150	−0.396 to 0.095	.222		NA	
Female sex	Male: 29/45 (64.4%) Female: 16/45 (35.6%)	1.243	−0.344 to 2.830	.121		NA	
White ethnicity	Nonwhite: 14/45 (31.1%) White: 31/45 (68.9%)	1.748	0.180 to 3.316	.030*	0.533	−0.969 to 2.034	.474
Household siblings	Yes: 30/45 (66.7%) No: 15/45 (33.3%)	0.598	−1.004 to 2.200	.454		NA	
Questionnaire: infant with peanut allergy	No: 37/45 (82.2%) Yes: 8/45 (17.7%)	−0.967	−2.950 to 1.015	.329		NA	
Questionnaire: family member with peanut allergy	No: 32/45 (71.1%) Yes: 13/45 (28.9%)	−2.179	−3.684 to 0.674	.006*	−0.794	−2.428 to 0.840	.328
Size of home	≤1 bed: 3/45 (6.7%) 2-3 beds: 28/45 (62.2%) ≥4 beds: 14/45 (31.1%)	0.124	−1.299 to 1.548	.860		NA	
Age of infant's mattress	<5 y: 31/37 (83.8%) ≥5 y: 6/37 (16.2%)	−0.714	−2.275 to 2.004	.785		NA	
Infant anti-allergenic covers	Yes: 9/45 (20%) No: 36/45 (80%)	−0.768	−2.582 to 1.045	.396		NA	
Infant's sleep location	Parent bed: 8/45 (17.8%) Crib: 37/45 (82.2%)	1.706	−0.351 to 3.763	.101*	0.534	−1.503 to 2.572	.596
Infant's bed dust weight (*ln* mg)	19.00 mg (7.25-76.80 mg [not *ln* transformed])	0.828	0.060 to 1.596	.035*	0.602	−0.061 to 1.266	.074
Maternal peanut consumption (*ln* g/wk)	2.40 g/wk (0.11-5.90 g/wk [not *ln* transformed])	0.329	0.044 to 0.614	.025*		NA	
Paternal peanut consumption (*ln* g/wk)	2.97 g/wk (0.0-10.80 g/wk [not *ln* transformed])	0.301	0.420 to 0.561	.025*		NA	
Combined parental peanut consumption (*ln* g/wk)	5.40 g/wk (1.03-19.50 g/wk [not *ln* transformed])	0.610	0.333 to 0.887	<.001*		NA	
Infant's peanut consumption (*ln* g/wk)	0.00 g/wk (0.0-0.00 g/wk [not *ln* transformed])	0.636	0.293 to 0.980	.001*		NA	
HPC (*ln* g/wk)	7.38 g/wk [1.84-19.50 g/wk [not *ln* transformed])	0.733	0.399 to 1.068	<.001*	0.492	0.084 to 0.900	.020*

Initial univariate linear regression analysis was performed, followed by multivariate regression analysis.
IQR, Interquartile range; *NA*, not applicable.
*Factors with *P* values of .1 or less on univariate analysis were included in the multivariate model. Maternal, paternal, combined parental and infant peanut consumption were not entered into the multivariate model as HPC comprises each of these variables.

that peanut levels in the environment were not directly measured. Our study has now confirmed the relationship between HPC and peanut protein levels in the infant's home environment. A univariate followed by multivariate regression analysis was carried out to determine the factor that best explained peanut levels in the infant's home environment. HPC was the most important factor for peanut protein in the infant's bed and play area dust. A household member with peanut allergy was associated with lower peanut protein levels in the infant's bed and play area; this was a result of lower HPC because in the multivariate model only HPC remained significant. Interestingly, lower peanut consumption was most pronounced when a household member other than the infant was allergic to peanut; thus it might take time for households to change their peanut eating habits after a new household member (the infant) is given a diagnosis of peanut allergy. Alternatively, cessation of peanut consumption might not be reflected in peanut dust levels for several months.

The presence of detectable biologically active peanut protein in dust lends credence to the notion that high environmental peanut exposure can lead to peanut allergic sensitization. To give more weight to the notion that peanut in dust can sensitize young children, we wanted to confirm that we were measuring peanut levels that were biologically significant in patients with peanut allergy. Therefore we demonstrated the ability of peanut protein in dust to cause allergen-specific activation of basophils from children with peanut allergy. Peanut protein in household dust induced a dose-dependent activation of basophils from children with peanut allergy that was comparable with the dose-response to similar concentrations of an independent peanut standard. These observations are highly suggestive of a peanut-specific basophil response. Given that these children were solely allergic to peanut and neither sensitized nor allergic to any other food or airborne allergen, basophil activation is very unlikely to have been caused by allergens other than peanut present in the dust samples (eg, house dust mite or grass and tree pollen allergens). The allergen-specific response is further confirmed by the fact that activation was not observed when basophils from nonallergic children were stimulated with the high peanut level–containing dust samples. Furthermore, the inability of dust samples with negligible levels of peanut to cause basophil activation in both children with peanut allergy and nonallergic children excludes non–allergen-specific basophil activation. Taken together, these findings confirm that

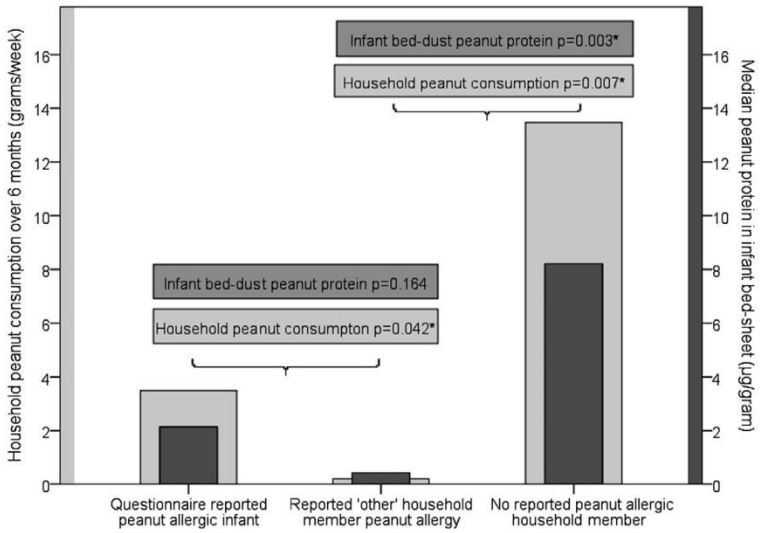

FIG 4. Peanut protein (micrograms per gram of dust) in the infant's bed *(dark gray)* and HPC (grams per week over 6 months, *light gray*) comparing households with reported peanut allergy in the infant (n = 8) versus reported peanut allergy in another household member (n = 5) versus no reported household member with peanut allergy (n = 32). *$P < .05$.

peanut allergens in house dust are able to interact with immune cells, such as basophils, and we hypothesize that, being biologically active, peanut allergens in house dust are not only able to activate mast cells and basophils but also to be captured by dendritic cells and presented to T cells in the context of a T_H2-driven immune response leading to allergic sensitization.

Allergen exposure threshold levels for sensitization have not been described for food allergens but have been described for inhalant allergens, such as house dust mite (*Dermatophagoides pteronyssinus*, >2 μg/g),[32] cat (Fel d 1, 1-8 μg/g),[33,34] and cockroach (Bla g 1, >8 μg/g).[35,36] Aeroallergens might behave differently to food allergens with respect to their mechanism of sensitization, and thus these allergen threshold levels might not be directly comparable. We found higher peanut protein concentrations in dust (median, 4.95 μg/g; interquartile range, 1.12-29.34; range undetectable to 870 μg/g) than previously reported for peanut (Ara h 2; median, 1.13 μg/g; range, 0.40-9.79 μg/g),[14] which is likely to be because the Veratox polyclonal peanut ELISA is directed against whole peanut protein. Additionally, peanut proteins are very stable allergens and thus might build up in reservoirs, such as mattresses; this is supported by peanut protein levels being higher in older mattresses. We do not believe environmental peanut protein levels are likely to cause clinical reactions to peanut but might be sufficient to induce sensitization. This is particularly pertinent in the context of infantile eczema in which skin is inflamed and abraded and immune cells are potentially directly exposed to peanut proteins in dust.

Peanut protein levels in bed dust were also related to individual peanut consumption; combined parental peanut consumption was positively correlated with peanut levels in the maternal and paternal sides of the bed, and infant peanut consumption was positively correlated with peanut protein in the infant's bed (Table I). These findings suggest that peanut protein in bed dust can be used as an objective marker of peanut consumption. This might be useful in intervention studies in which peanut consumption versus avoidance needs to be assessed, such as in peanut allergy prevention or peanut oral immunotherapy randomized controlled trials. Although 39 (86.7%) of 45 of the infants consumed no peanut, there were high (>10 μg/g dust) peanut protein levels in the infant's bed dust of all 6 infants consuming peanut. This supports the hypothesis that if an infant eats peanut, then there will be peanut protein in the infant's bed dust; however, larger studies with infants eating peanut are required to confirm this.

Environmental peanut exposure is only one consideration in the development of peanut sensitization and allergy. There are many other factors to consider, such as genetic factors,[37] the hygiene hypothesis,[38] gut flora,[39] and numerous other factors.[40] There is ongoing work on the induction of oral tolerance through high-dose early peanut consumption (www.leapstudy.co.uk)[41] on the basis of previous observational work showing a protective effect of early peanut consumption in infants.[42] In the study by Fox et al,[11] children who had consumed peanut before 1 year of age were less affected by high HPC. In animal studies high-dose repeated peanut consumption leads to oral tolerance induction; however, this might be prevented by preceding epicutaneous sensitization.[43] Recent *in vitro* work has shown differential proliferation of peanut-specific skin-homing versus gut-homing memory T cells in the presence of peanut in children with peanut allergy versus peanut-tolerant children, respectively. In children with peanut allergy, skin-homing T cells (cutaneous lymphocyte antigen positive) proliferated and produced a predominantly T_H2 cytokine profile. In peanut-tolerant children gut-homing T cells ($\alpha4\beta7$ positive) proliferated and produced a predominantly T_H1 cytokine profile. This suggests that in children with peanut allergy, allergic sensitization occurs through the skin, whereas in peanut-tolerant children they become tolerant through the gut.[44] Thus it might be that environmental peanut exposure poses a risk for the development of peanut sensitization through epicutaneous exposure only in the absence of the protective effect of oral peanut consumption (the dual-allergen exposure hypothesis).[40]

In conclusion, this study confirms the relationship between HPC and environmental peanut exposure in young children and demonstrates that peanut measured in dust is biologically active. Further prospective environmental population studies are needed to address whether there is a threshold level of environmental peanut exposure that can lead to peanut sensitization.

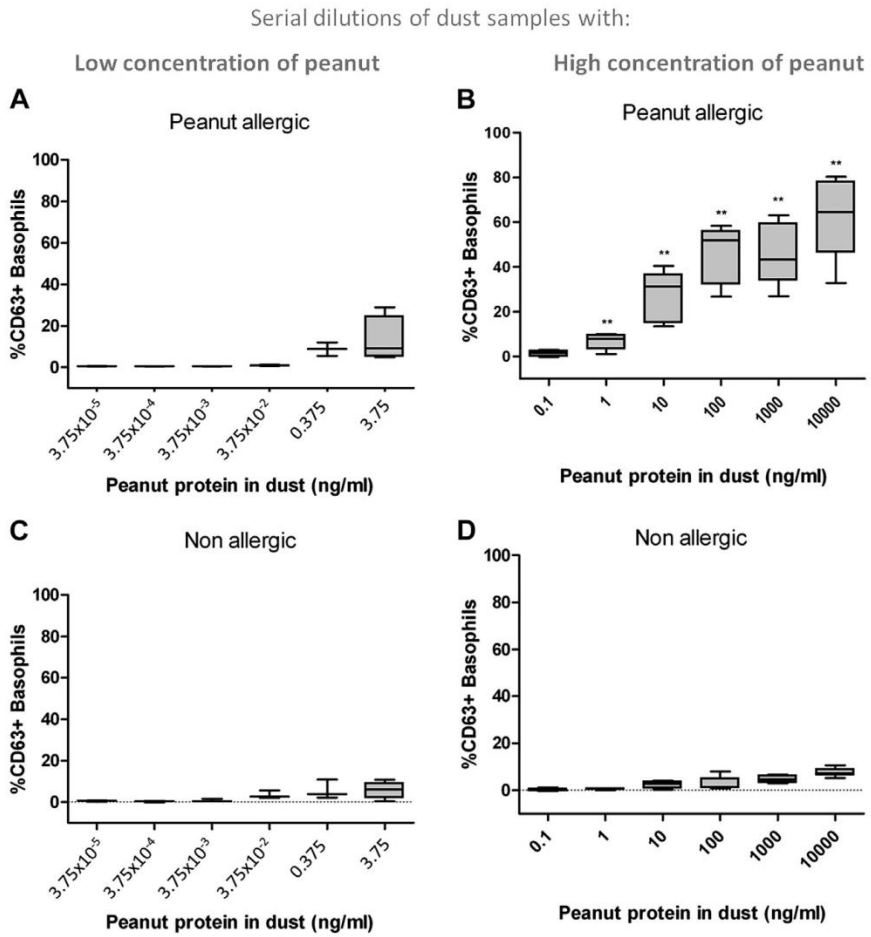

FIG 5. Basophil activation by serial dilutions of dust samples containing low (**A** and **C**) or high (**B** and **D**) peanut protein in peanut-monoallergic (Fig 5, *A* and *B*) or nonallergic (Fig 5, *C* and *D*) patients (n = 5 pairs of sample-patient experiments). Medians, 25th and 75th percentiles, and minimum and maximum percentages of $CD63^+$ basophils are displayed. High peanut dust extracts were dilution-adjusted to the maximum same concentration of peanut protein (10 μg/mL) and then underwent serial dilutions for comparability of dose-response, and low peanut dust samples were prepared by using the same dilution factor. Basophil activation for each concentration of peanut was compared between allergic and nonallergic children (ie, Fig 5, *A*, vs Fig 5, *C*, and Fig 5, *B*, vs Fig 5, *D*). $^{**}P < .01$.

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Clinical implications: Peanut dust levels in the infant's environment correlate with household consumption of peanut. The peanut in dust is biologically active. Environmental peanut exposure should be studied as a possible risk factor for peanut sensitization.

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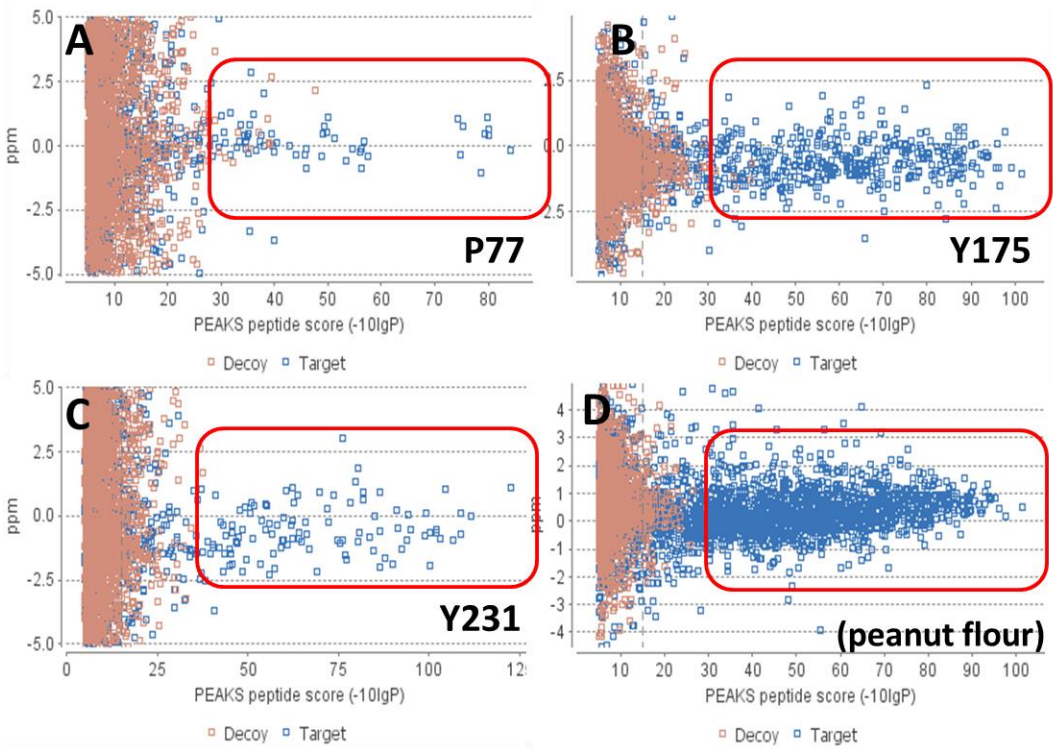
7.6 MS findings

MS was used to determine more detailed analysis of the peanut peptides in dust. The MS methodology and validation work used is detailed in Section 6.11.1 (KCL analyses) and Section 6.11.2 (University of Manchester analyses). Dust samples from an infant's play-area (Y175) and bed-sheet (Y231) contained (870 µg/g and 797.5 µg/g peanut protein respectively). Dust from a maternal mattress (P77) had levels below the LLQ of the Veratox peanut ELISA. Initial analysis of the MS/MS data obtained from these extracted dust samples and a peanut flour comparator/control is shown in Figure 23. Searches of MS/MS data against a decoy database (obtained automatically using the PEAKS software package) was performed to indicate the chance of peanut peptide detection through chance alone. In the case of the maternal bed-sheet (P77) (suggested by ELISA analysis to contain little or no peanut protein) there was an indication of some peptides attributable to peanut with quality scores of 50-100 indicating authentic detection. However, analysis of infant bed-dust (Y231) and play-area (Y175) samples indicated a far higher number of detected peanut peptides at a higher quality score, indicating an increased presence of peanut proteins in these samples. As expected, the peanut flour comparator contained abundant significant peanut peptide hits in greater numbers than any of the dust samples analysed.

Figure 23: Peptides detected from MS data from dust from (A) maternal bed-sheet (P77), (B) infant play-area (Y175) and (C) infant bed-sheet (Y231).

Red spots indicate peptides detected using a decoy database generated by the PEAKS software, blue spots peptides detected using the peanut sequence database. Red boxes indicate the range of likely significant peptides hits. A roasted commercial peanut flour comparator (D) was used as positive control.



The detection of individual peanut allergens and the sequence accessions (from the database UniProt) which comprised them is shown in Table 7. The percentage protein coverage shown was calculated using the complete sequences as given in UniProt based on translational start, and therefore included sequences not expected in the final protein (e.g. signal peptides). Dust from the maternal bed-sheet (P77) with peanut levels < LLQ on ELISA analysis contained poor coverage of the major peanut allergens Ara h 1, 2 and 3. As the percentage coverage of isoforms representing these allergens was consistently low the likelihood of peanut being present in this sample was low. Conversely, dust samples from the infant bed-sheet (Y231) and play-area (Y175) contained significant coverage; Y175 yielding higher protein coverage than Y231 which

was also suggested in Figure 23 and from the ELISA analysis performed previously where Y175 had 870 µg/g and Y231 had 797.5 µg/g peanut protein. Peptides corresponding to the allergens Ara h 1, 2, 3, 6, 7, 8, 9, 10 and 11 could be detected in dust Y175. The detection of Ara h 8 (Bet v 1 homologue) was poor and in no samples could the profilin allergen Ara h 5 be detected.

Table 7. Detection of allergen sequence isoforms by MS and % of total protein sequence detected (%). ND = not detected.

Allergen	**UniProt Accession (peptide sequence)**	**Protein coverage (%)**		
		Dust P77	**Dust Y175**	**Dust Y231**
Cupin barrel family				
Ara h 1	P43238	10.2	60.5	35.9
	P43237	10.3	65.3	36.8
	E5G076	ND	49.6	28.4
	Q6PSU3	10.9	ND	39.0
	Q6PSU4	ND	ND	ND
	Q6PSU5	ND	63.9	ND
	Q6PSU6	ND	ND	ND
Ara h 3	O82580	17.0	55.2	30.0
	Q5I6T2	19.4	66.5	41.2
	B5TYU1	17.7	65.5	36.4
	Q9FZ11	16.3	62.8	46.3
	Q647H4	19.4	72.6	49.8
	Q647H3	20.3	66.1	48.4
	Q8LKN1	ND	ND	ND
	Q6T2T4	19.4	ND	49.8
	Q9SQH7	17.4	46.0	37.4
	Q0GM57	7.8	54.5	ND
	E5G077	7.8	ND	24.8
	Q647H2	ND	21.3	ND
	A1DZF0	17.4	67.3	39.9
	Q6IQG5	ND	ND	ND
Prolamin family				
Ara h 2	Q6PSU2	8.1	57.0	40.1
	Q6PSU2-2	ND	ND	ND
	Q6PSU2-3	ND	ND	ND
	Q6PSU2-4	ND	ND	ND
Ara h 6	Q647G9	ND	64.1	37.9
	A1DZE9	ND	37.9	ND
Ara h 7	Q9SQH1			
	Q647G8	ND	38.6	ND

	B4XID4	ND	37.2	15.2
Ara h 9	B6CEX8	ND	ND	ND
	B6CG41	ND	76.1	ND
Bet v 1 family				
Ara h 8	Q6VT83	ND	ND	ND
	B2ZGS2	ND	ND	ND
	B1PYZ4	ND	ND	ND
	Q0PKR4	ND	ND	ND
	Q2YHR1	ND	ND	ND
	B0YIU5	ND	16.3	ND
Oleosin family				
Arah10,11	Q647G5	5.3	21.9	ND
	Q647G4	ND	ND	ND
	Q9AXI0	ND	14.2	ND
	Q9AXI1	ND	14.2	ND
	Q6J1J8	ND	14.2	ND
Profilin family				
Ara h 5	Q9SQI9	ND	ND	ND
	D3K177	ND	ND	ND
	L7QH52	ND	ND	ND

7.7 Publication 3: FLG loss-of-function mutation increase the impact of EPE on PS and PA

Brough H.A., Simpson A., Makinson K., Hankinson J., Brown S., Douiri A., Belgrave D.C.M., Penagos M., Srephens A.C. Mclean W.H.I., Turcanu V. Nicolaou N., Custovic A., Lack G. Peanut allergy: Impact of EPE in children with a filaggrin loss-of-function mutation. J Allergy Clin Immunol. 2014;134:867-75

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Peanut allergy: Effect of environmental peanut exposure in children with filaggrin loss-of-function mutations

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Background: Filaggrin *(FLG)* loss-of-function mutations lead to an impaired skin barrier associated with peanut allergy. Household peanut consumption is associated with peanut allergy, and peanut allergen in household dust correlates with household peanut consumption.
Objective: We sought to determine whether environmental peanut exposure increases the odds of peanut allergy and whether *FLG* mutations modulate these odds.
Methods: Exposure to peanut antigen in dust within the first year of life was measured in a population-based birth cohort. Peanut sensitization and peanut allergy (defined by using oral food challenges or component-resolved diagnostics [CRD]) were assessed at 8 and 11 years. Genotyping was performed for 6 *FLG* mutations.
Results: After adjustment for infantile atopic dermatitis and preceding egg skin prick test (SPT) sensitization, we found a strong and significant interaction between natural log *(ln [loge])* peanut dust levels and *FLG* mutations on peanut sensitization and peanut allergy. Among children with *FLG* mutations, for each *ln* unit increase in the house dust peanut protein level, there was a more than 6-fold increased odds of peanut SPT sensitization, CRD sensitization, or both in children at ages 8 years, 11 years, or both and a greater than 3-fold increased odds of peanut allergy compared with odds seen in children with wild-type *FLG*. There was no significant effect of exposure in children without *FLG* mutations. In children carrying an *FLG* mutation, the threshold level for peanut SPT sensitization was 0.92 μg of peanut protein per gram (95% CI, 0.70-1.22 μg/g), that for CRD sensitization was 1.03 μg/g (95% CI, 0.90-1.82 μg/g), and that for peanut allergy was 1.17 μg/g (95% CI, 0.01-163.83 μg/g).
Conclusion: Early-life environmental peanut exposure is associated with an increased risk of peanut sensitization and allergy in children who carry an *FLG* mutation. These data support the hypothesis that peanut allergy develops through transcutaneous sensitization in children with an impaired skin barrier. (J Allergy Clin Immunol 2014;134:867-75.)

Key words: FLG *loss-of-function mutations, filaggrin, skin barrier, peanut sensitization, peanut allergy, environmental peanut exposure, dust, threshold*

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Abbreviations used
AD: Atopic dermatitis
CRD: Component-resolved diagnostics
FLG: Filaggrin
GEE: Penalized generalized estimating equations methodology
ISU: ISAC standardized unit
LLQ: Lower limit of quantitation
MAAS: Manchester Asthma and Allergy Study
OFC: Oral food challenge
OR: Odds ratio
sIgE: Allergen-specific IgE
SPT: Skin prick test

There is a clear association between early-onset atopic dermatitis (AD) and food allergy.[1,2] Children with AD have an impaired skin barrier, which might allow antigen to penetrate the skin and sensitize the subject.[3,4] In children with a history of AD, 90% of those who went on to have peanut allergy had been exposed topically to creams containing *Arachis* species (peanut) oil in the first 6 months of life.[2] In mice epicutaneous exposure to food allergens after skin stripping induces a potent allergic T_H2-type response associated with high IL-4, IL-5, and allergen-specific IgE (sIgE) levels and systemic anaphylaxis after oral challenge.[5,6]

Filaggrin is responsible for the strength and integrity of the stratum corneum[7] and regulates the permeability of the skin to water and antigens.[8] Loss-of-function mutations in the gene encoding filaggrin *(FLG)* are present in up to 50% of patients with moderate-to-severe AD[9,10] and have been shown to increase the risk of inhalant allergic sensitization, allergic rhinitis, asthma,[11,12] and peanut allergy.[13] In the flaky tail mouse, which has a 1-bp deletion mutation (5303delA) within the murine *flg* gene (analogous to common human *FLG* loss-of-function mutations), topical allergen application leads to cellular infiltration and allergen-specific antibody response, even without skin stripping.[14] This suggests that filaggrin deficiency, even in the absence of dermatitis, might be sufficient for transcutaneous sensitization.

High consumption of peanut by household members during the child's first year of life is associated with an increased risk of peanut allergy, possibly because of environmental peanut exposure in the child's home[15]; however, in this study questionnaire-based assessment of household peanut consumption was not validated against an objective measure of peanut in the environment and was potentially subject to retrospective bias. We recently showed that peanut protein in household dust is positively correlated with household peanut consumption.[16] In addition, we showed that peanut protein in dust activates basophils from children with peanut allergy in a dose-dependent manner and is thus biologically active.[16]

We hypothesized that peanut sensitization can occur through presentation of environmental peanut antigen through an impaired skin barrier to underlying antigen-presenting cells. To address this hypothesis, we investigated whether early-life environmental peanut exposure measured directly by quantifying peanut antigen in household dust was a risk factor for the development of peanut allergy and whether this relationship was modified by *FLG* genotype. Specifically, we predicted that an increase in the peanut protein concentration in household dust during infancy would be associated with an increase in school-age peanut sensitization and allergy and that this effect would be augmented in children with 1 or more *FLG* loss-of-function mutations.

METHODS

Study population

The Manchester Asthma and Allergy Study (MAAS) is an unselected birth cohort described in detail elsewhere (registration: ICRCTN72673620).[17] In brief, 1184 subjects were recruited prenatally from 1995 to 1997 and followed up at ages 1, 3, 5, 8, and 11 years. The study was approved by the local ethics committee; parents provided written informed consent.

Data sources

Validated questionnaires were interviewer administered to collect information on parentally reported symptoms and physicians' diagnoses. Parental report of a history of AD during infancy was assessed by using a modified International Study of Asthma and Allergies in Childhood questionnaire to apply the UK Working Party's diagnostic criteria for AD.[18] Peanut sensitization was assessed at ages 8 and 11 years by using skin prick tests (SPTs) to whole peanut extract (Hollister-Stier, Spokane, Wash)[19] and by measuring sIgE to whole peanut extract and peanut components Ara h 1, 2, and 3 with ImmunoCAP (age 8 years) or the ISAC Multiplex Immuno Solid-phase Allergen Chip (age 11 years; Thermo Fisher Scientific, Uppsala, Sweden).[20] Maternal peanut consumption during pregnancy and breast-feeding were collected retrospectively (aged 8 years) in a subset of patients assessed for peanut allergy by means of diagnostic oral food challenge (OFC).

Definition of outcomes

Peanut SPT sensitization. Peanut SPT sensitization was defined as a mean wheal diameter of 3 mm or greater than that elicited by the negative control.

Peanut component-resolved diagnostics sensitization. Peanut component-resolved diagnostics (CRD) sensitization was defined as sIgE to the peanut components Ara h 1, 2, or 3 of 0.35 kU_A/L or (8 years) or 0.35 ISAC standardized units (ISU) or greater (11 years).[20] Patients with Ara h 1, 2, or 3 levels of less than 0.35 kU_A/L (8 years) or 0.35 ISU (11 years) were deemed non-CRD sensitized. If no CRD analysis was available, then patients with peanut sIgE levels of less than 0.2 kU_A/L ImmunoCAP were considered not CRD sensitized.

Peanut allergy. All children with evidence of peanut sensitization at age 8 years (peanut SPT response ≥3 mm or sIgE level ≥0.2 kU_A/L) were offered an OFC to peanut to determine allergy versus tolerance.[19] Open OFCs were applied among children who had a history of tolerating peanut on consumption; all other children underwent a double-blind, placebo-controlled OFC.[19] OFC results were considered positive after development of 2 or more objective signs indicating an allergic reaction.[19] Children with a convincing history of an immediate hypersensitivity reaction on exposure to peanut combined with a peanut sIgE level of 15 kU_A/L or greater,[21] an SPT response of 8 mm or greater,[22] or both (age 8 years) were considered to have peanut allergy and did not undergo an OFC. Two children with a convincing history of an immediate hypersensitivity reaction on exposure to peanut and an SPT response of 3 mm or greater who refused consent for OFCs were considered to have peanut allergy based on an Ara h 2 level of 0.35 ISU or greater[19] at subsequent follow-up at age 11 years.

Quantitation of environmental peanut exposure in household dust

Dust samples were collected predominantly at 36 weeks' gestation from the lounge-sofa, as previously described.[23] If no antenatal dust sample was available from the lounge-sofa, then dust samples from 6 or 12 months were analyzed for peanut protein (where available). Dust samples were extracted

TABLE I. Demographics and clinical characteristics of the included group (n = 623) versus the excluded group (n = 561) and whole group (n = 1184)

	Included group* (n = 623)		Excluded group† (n = 561)		Whole group (n = 1184)		*P* value, included (n = 623) vs excluded (n = 561)
	Total no.	No. (%)	Total no.	No. (%)	Total no.	No. (%)	
Peanut SPT sensitization at 8 y	559	30 (5.4)	360	18 (5.0)	920	48 (5.2)	.69
Peanut SPT sensitization at 11 y	450	19 (4.2)	256	13 (5.1)	706	32 (4.5)	.41
Peanut SPT sensitization at age 8 and/or 11 y§	434	35 (8.1)	237	24 (10.1)	710	59 (8.3)	.15
Peanut CRD sensitization at age 8 y	371	13 (3.5)	211	7 (3.3)	584	20 (3.4)	.84
Peanut CRD sensitization at age 11 y	297	12 (4.0)	154	8 (5.2)	451	20 (4.4)	.37
Peanut CRD sensitization at age 8 and/or 11 y§	241	19 (7.9)	116	9 (7.8)	357	28 (7.8)	.94
Peanut allergy at age 8 and/or 11 y	577	20 (3.5)	382	10 (2.6)	959	30 (3.1)	.19
History of AD during infancy	614	207 (33.7)	477	190 (39.8)	1091	397 (36.4)	<.01
No AD on clinical assessment at age 1 y	338	272 (80.5)	173	142 (82.1)	511	414 (81.0)	.46
Mild AD on assessment at age 1 y	338	46 (13.6)	173	25 (14.5)	511	71 (13.9)	.66
Moderate/severe AD at age 1 y	338	20 (5.9)	173	6 (3.5)	511	26 (5.1)	.01
Combined *FLG* loss-of-function mutation	623	57 (9.1)	234	29 (12.4)	857	86 (10.0)	.02
Parental report of "hay fever ever" in the child	569	135 (23.7)	400	105 (26.3)	969	240 (24.8)	.18
Egg SPT sensitization at age 3 y	545	21 (3.9)	398	15 (3.8)	943	36 (3.8)	.92
Male sex	623	311 (49.9)	561	331 (59.0)	1184	642 (54.2)	<.001
Full older siblings (same mother and father)	623	316 (50.7)	532	297 (55.8)	1155	614 (53.2)	.02
Parental atopy (low vs medium/high risk)	621	501 (80.7)	514	443 (86.2)	1135	944 (83.2)	.001
Breast-feeding (yes vs no)	618	443 (71.7)	497	337 (67.8)	1115	780 (70.0)	.03
Peanut consumption during pregnancy (yes vs no)	70	56 (80.0)	41	35 (85.4)	111	91 (82.0)	.28
Peanut consumption during breast-feeding (yes vs no)	59	45 (76.3)	29	24 (82.8)	88	69 (78.4)	.26
House dust mite reduction measures‡	160	88 (55.0)	93	45 (48.4)	253	133 (52.6)	1.00
Maternal age at baseline (y), mean (SD)	615	30.67 (4.74)	499	30.02 (4.81)	1114	30.38 (4.78)	.51
Peanut protein in dust (μg/g) using values below LLQ, median (IQR)	623	0.73 (0.40-1.33)	128	0.78 (0.36-1.40)	751	0.73 (0.38-1.35)	.96
Peanut protein in dust (μg/g) using LLQ/2, median (IQR)	623	0.73 (0.25-1.33)	128	0.78 (0.25-1.40)	751	0.73 (0.25-1.35)	.90

IQR, Interquartile range.

*Included group comprised of white children enrolled in MAAS with available sofa dust within the first year of life and successful *FLG* genotyping.

†Children were excluded for the following reasons: (1) nonwhite ethnicity, (2) lack of available blood sample for *FLG* genotyping or failed genotyping, or (3) no dust extract available for the assessment of environmental peanut allergen exposure.

‡"High-risk" infants (both parents with positive SPT responses) with no pets in the home in MAAS were randomized to house dust mite reduction measures versus control subjects.

§Children who were not peanut sensitized at age 8 or 11 years and missing data at the other time point were classed as having missing sensitization data.

in borate-buffered saline (0.1% Tween 20, pH 8.0) and stored at −20°C until analysis. Peanut protein in dust extracts was determined by using the Veratox polyclonal ELISA against whole peanut protein (Neogen, Lansing, Mich), which has been validated for sensitivity, specificity, and reliability in measuring peanut protein contamination of food,[24,25] dust, and wipe samples.[26] The Veratox ELISA lower limit of quantitation (LLQ) for peanut protein in dust was 100 ng/mL (0.5 μg/g based on a dust sample weighing between 50-100 mg); this variable was analyzed by using a fixed calculation for values of less than this level (LLQ/2; results are shown in Table E1 in this article's Online Repository at www.jacionline.org)[27] and by using all data of less than this value (results in the main body of the article) because the variable with LLQ/2 created 230 (37%) censored data points.[28] Analyses for both forms of the peanut dust variable were compared to determine whether the 2 different ways of dealing with data of less than the LLQ made a material difference to the results obtained. Participant information was blinded from the researcher performing the ELISA-based dust analyses.

Genotyping

FLG genotyping was performed with probes and primers, as previously described.[9] Genotyping for R501X, S3247X, and R2447X loss-of-function mutations was performed with a TaqMan-based allelic discrimination assay (Applied Biosystems, Cheshire, United Kingdom). Mutation 2282del4 was genotyped by sizing of a fluorescently labeled PCR fragment on a 3100 or 3730 DNA sequencer. *FLG* mutations 3673delC and 3702delG were assessed by means of GeneScan analysis of fluorescently labeled PCR products. These 6 *FLG* mutations have been consistently associated with AD in white populations[10]; however, because some of these *FLG* mutations are not found in nonwhite subjects,[29] all nonwhite participants were excluded from analyses that included *FLG* genotype. Data were analyzed as combined carriage of an *FLG* null allele; that is, if a child carried 1 or more of the 6 genetic variations, he or she was considered an *FLG* null allele carrier. Complete *FLG* genotype results (ie, results for all 6 *FLG* loss-of-function mutations screened) were available for 805 (76.0%) of 1059 white participants, 117 samples failed genotype analysis for 1 or more mutations, and no sample was available in 137 participants. In cases with incomplete *FLG* data, the presence of 1 *FLG* mutation defined that case as a carrier; participants with incomplete genotyping data in whom all successfully tested alleles were wild-type alleles were excluded from further analysis because their *FLG* genotype status remained ambiguous.

Statistical analysis

Data were analyzed with STATA 12.1 software (StataCorp, College Station, Tex). Demographics and clinical characteristics were compared between participants and nonparticipants. Count data were compared by using the Pearson χ^2 test. Continuous data were compared with the Student *t* test for normally distributed data and the Mann-Whitney *U* test for nonnormally distributed data. All variables except maternal age and peanut protein in dust were compared by using the Pearson χ^2 test. Maternal age was normally

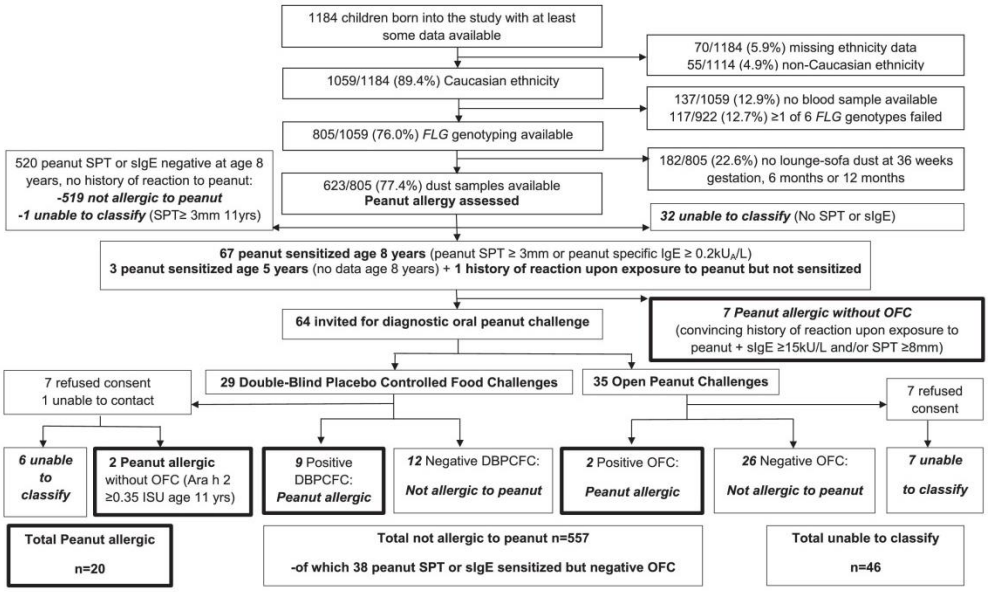

FIG 1. CONSORT diagram outlining participant flow. Peanut allergy outcomes are highlighted in *boxes outlined in boldface*. *DBPCFC,* Double-blind, placebo-controlled food challenge.

distributed and thus was compared with the Student *t* test. Peanut protein in dust (without natural log *[ln]* transformation) was not normally distributed and thus was compared with the Mann-Whitney *U* test. Peanut protein in dust (in micrograms per gram) underwent *ln* transformation for subsequent analyses. Factors associated with peanut allergy at the ages of 8 years, 11 years, or both were assessed by using a penalized logistic regression methodology to account for unbalanced data (20/577 had peanut allergy).[30] Factors associated with peanut sensitization (SPT and CRD results) were assessed by using penalized generalized estimating equations methodology (GEE) through a quasi–least squares approach, with an exchangeable working correlation matrix to account for repeated measures within subjects at 8 and 11 years.[31] Goodness of fit of the GEE statistical model was assessed by using the quasilikelihood under independence model criterion. The goodness of fit of the penalized logistic regression methodology statistical model was assessed by using the Akaike information criterion. We tested whether the effect of environmental peanut exposure on peanut sensitization and allergy was modified by *FLG* genotype by including an interaction term.

The additive effect of *FLG* loss-of-function mutation was calculated by using the exponential of the coefficient (β) of the interaction (*FLG* genotype by peanut dust exposure) minus the baseline coefficient (β) of peanut dust exposure. The predictive probability of peanut sensitization and allergy was calculated from the multivariate regression model. Threshold levels of peanut protein in dust for peanut sensitization and allergy were calculated by using the intersection between wild-type *FLG* versus *FLG* mutation in the multivariate regression model.[30,32] To evaluate the reliability of the thresholds obtained and the uncertainty around them, we conducted bootstrap cross-validation with 1000 replications.

RESULTS

Participants and descriptive data

Details of the participant flow are presented in Fig 1. From 1184 participants, we analyzed data from 623 white children with available *FLG* genotyping and early-life environmental peanut exposure. Of these children, at age 8 years, 32 had no peanut SPT or peanut sIgE data, 70 were peanut sensitized (of these, 3 children were sensitized at age 5 years and had no peanut SPT or sIgE data at age 8 years), 1 was not peanut sensitized but reported a reaction on peanut exposure, and 520 were not peanut sensitized and reported no reactions to peanut (of these, 1 was subsequently peanut sensitized at age 11 years and thus impossible to classify). Seven children with a convincing history of an allergic reaction on peanut exposure and a peanut sIgE level of 15 kU_A/L or greater, an SPT response of 8 mm or greater, or both were classified as having peanut allergy; the remaining 64 sensitized children were invited for an OFC (29 double-blind, placebo-controlled food challenges and 35 open challenges). We were unable to contact 1 subject, and 14 refused consent (of these, 2 were classified as having peanut allergy at age 11 years on the basis of a convincing history of an immediate hypersensitivity reaction on exposure to peanut and an Ara h 2 level ≥0.35 ISU). Thus 20 children were defined as having peanut allergy, 557 were defined as nonallergic, and 46 could not be classified (because of missing SPT and sIgE data or because they declined consent for an OFC).

The demographics of the whole group, both included and excluded children, are shown in Table I. Comparison of the included and excluded groups revealed no differences in peanut sensitization or allergy; we observed small (but statistically significant) differences in parental atopy, *FLG* status, history and severity of AD, sex, breast-feeding, and sibship position. *FLG* loss-of-function mutations were carried by 57 (9.1%) of 623 children (all children; Table I) and 4 (20%) of 20 children with peanut allergy (Table II). A history of infantile AD was present in 207 (33.7%) of 614 (all children) children and 16 (80%) of 20 children with peanut allergy. Of the 16 children with peanut allergy with wild-type *FLG*, 13 (81%) had a history of infantile AD. The median peanut protein concentration in dust was 0.73 μg/g (interquartile range, 0.40-1.33 μg/g); the peanut allergen level was less than the LLQ in 230 (36.9%) of 623 homes.

FLG genotype modifies the effect of early-life environmental peanut on the risk of peanut sensitization and allergy

Factors associated with both peanut sensitization and peanut allergy were history and severity of infantile AD, *FLG*

TABLE II. *FLG* genotype frequencies in 20 children with peanut allergy and 577 children without peanut allergy at ages 8 years, 11 years, or both

	R501X	2282del4	S3247X	R2447X	3673delC	3702delG	Combined *FLG* loss-of-function genotype
No. (%) of peanut allergic children with *FLG* genotype (n = 20)							
Wild-type *FLG*	18 (90.0)	17 (85.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	16 (80.0)
FLG loss-of-function mutation	2 (10.0)	3 (15.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (20.0)*†
Failure of analysis‡	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
No. (%) of children without peanut allergy with *FLG* genotype (n = 557)							
Wild-type *FLG*	533 (95.7)	535 (96.05)	552 (99.1)	551 (98.9)	603 (100.0)	603 (100.0)	507 (91.0)
FLG loss-of-function mutation	23 (4.1)	20 (3.6)	4 (0.7)	5 (0.9)	0 (0.0)	0 (0.0)	50 (9.0)*§
Failure of analysis‡	1 (0.2)	2 (0.35)	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)	

*There were no mutant allele homozygotes for any allele tested.
†This includes 1 compound heterozygote (R501X/2282del4).
‡Although individual *FLG* genotypes failed, if a child had incomplete data but had a mutant *FLG* allele, they were included as a case in the combined loss-of-function genotype. If they had incomplete data but all alleles successfully tested were wild-type alleles, they were excluded because this could indicate a false-negative result.
§This includes 2 compound heterozygotes (R501X/2282del4).

TABLE III. Clinical and demographic factors associated with peanut SPT and CRD sensitization and peanut allergy on univariate GEE and penalized logistic regression methodology analysis

	Peanut SPT sensitization adjusted for age at assessment (8 + 11 y; GEE; n = 584)			Peanut CRD sensitization adjusted for age at assessment (8 + 11 y; GEE; n = 437)			Peanut allergy at age 8 y, 11 y, or both (LR; n = 577)		
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value
History of AD during infancy	10.5	4.2-26.1	**<.001**	11.9	3.3-43.1	**<.001**	8.9	2.9-26.9	**<.001**
AD severity, no AD at 1 y	Reference category			Reference category			Reference category		
Mild AD on assessment at 1 y	2.2	0.6-8.4	.25	3.4	0.7-16.5	.13	5.0	1.1-23.2	**.04**
Moderate-to-severe AD at 1 y	20.8	4.1-62.4	**<.001**	16.6	3.2-86.6	**.001**	28.0	6.6-118.8	**<.001**
Combined *FLG* loss-of-function mutations	3.5	1.5-8.3	**<.01**	4.0	1.4-11.4	**<.01**	2.5	0.8-7.9	.11
Parental report of "hay fever ever" in the child	3.4	1.6-7.3	**.001**	3.4	1.3-9.2	**.02**	4.2	1.6-11.1	**<.01**
Egg SPT sensitization at age 3 y	12.3	4.5-33.6	**<.001**	16.4	4.8-56.0	**<.001**	25.5	8.4-77.0	**<.001**
Male sex	2.2	1.0-4.6	**.04**	1.8	0.7-4.8	.22	1.6	0.6-3.9	.33
Full older siblings (same mother and father)	0.9	0.4-1.8	.72	0.5	0.2-1.4	.19	0.7	0.3-1.8	.46
Parental atopy, low vs medium/high risk	6.9	0.9-51.4	.06	1.9	0.4-8.3	.42	4.7	0.6-35.5	.13
Breast-feeding (yes vs no)	1.0	0.5-2.2	.99	2.7	0.6-11.9	.19	1.6	0.5-4.8	.43
Peanut consumption during pregnancy (yes vs no)	1.0	0.3-2.8	.93	0.8	0.2-2.8	.72	0.5	0.2-1.9	.32
Peanut consumption during breast-feeding (yes vs no)	0.8	0.3-2.3	.65	0.8	0.2-2.7	.70	0.6	0.2-2.0	.38
House dust mite reduction measures	1.0	0.3-3.2	.95	0.8	0.2-4.4	.81	0.7	0.1-2.6	.57
Maternal age at baseline (y)	1.0	1.0-1.1	.31	1.1	1.0-1.1	.06	1.0	0.9-1.1	.79
Peanut protein in dust (*ln* transformed μg/g)*	1.3	0.9-1.7	.16	1.2	0.8-1.8	.33	1.2	0.8-1.8	.47
Age at assessment (8 or 11 y)	0.8	0.5-1.1	.10	1.0	0.7-1.5	1.00	NA	NA	NA

Values in boldface are significant.
LR, Penalized logistic regression methodology; *NA*, not applicable.
*Peanut protein in dust: values less than the LLQ were used in this analysis.

loss-of-function mutation (trend for allergy), egg SPT sensitization at age 3 years, and parental report of "hay fever ever" in the child on univariate analysis (Table III). Peanut protein levels in dust were not associated with peanut sensitization or allergy overall; however, there was a strong and significant interaction on univariate analysis between *FLG* genotype and early-life environmental peanut exposure on peanut SPT sensitization (odds ratio [OR], 5.3; 95% CI, 1.8-15.3; $P < .01$) and peanut CRD sensitization (OR, 4.5; 95% CI, 1.5-13.5; $P < .01$) and a trend toward peanut allergy (OR, 2.7; 95% CI, 0.9-8.0; $P = .07$) (Table IV). Given the low number of children with peanut allergy outcomes, we were conservative in the selection of covariates in the multivariate model and used 2 covariates (egg SPT sensitization at age 3 years and a history of infantile AD) that were both highly associated with peanut SPT/CRD sensitization and allergy. In the multivariate analysis, with the inclusion of an interaction variable for *FLG* genotype*(*ln* peanut exposure), we found a strong and significant change in *FLG* genotype divergence with early-life environmental peanut exposure on both peanut sensitization and allergy (Table IV). These interactions were consistent for peanut SPT sensitization (OR, 5.2; 95% CI, 2.1-13.1; $P < .001$; Fig 2, *A*), peanut CRD sensitization (OR, 5.3; 95% CI, 1.9-14.8; $P = .001$; Fig 2, *B*), and clinically confirmed peanut allergy (OR, 3.2; 95% CI, 1.1-9.8; $P = .04$; Fig 3). Analysis of the peanut dust variable with LLQ/2 did not show a material difference in results (see Table E1). The additive effect of each *ln* unit increase in house dust peanut in children with 1 or more *FLG* loss-of-function mutations was 6.1-fold for peanut SPT sensitization, 6.5-fold for peanut CRD sensitization, and 3.3-fold for peanut allergy in the multivariate model. In children with a wild-type *FLG*

TABLE IV. GEE for peanut sensitization using quasilikelihood under independent model criterion goodness-of-fit analyses

	GEE peanut SPT sensitization adjusted for clustering at age 8 + 11 y (n = 584)					GEE peanut CRD sensitization adjusted for clustering at age 8 + 11 y (n = 437)					LR for peanut allergy at age 8 y, 11 y, or both (n = 577)				
	No.*	OR	95% CI	*P* value	QIC§	No.†	OR	95% CI	*P* value	QIC§	No.‡	OR	95% CI	*P* value	AIC§
Combined *FLG* loss-of-function mutation	584	3.5	1.5-8.3	**<.01**	386.6	437	4.0	1.4-11.4	**<.01**	215.3	577	2.54	0.82-7.88	.11	175.6
Age at assessment (8 or 11 y)		0.8	0.5-1.1	.10			0.9	0.6-1.4	.69			NA			
Combined *FLG* loss-of-function mutation	584	3.6	1.5-8.2	**<.01**	386.7	437	4.0	1.4-11.0	**<.01**	216.5	577	2.5	0.8-7.9	.11	177.1
Peanut protein in dust (*ln* transformed μg/g)‖		1.3	0.9-1.7	.15			1.2	0.8-1.8	.27			1.2	0.8-1.8	.46	
Age at assessment (8 or 11 y)		0.7	0.5-1.1	.10			0.9	0.6-1.4	.66			NA			
Combined *FLG* loss-of-function mutation	584	2.4	0.7-8.6	.17	370.7	437	2.6	0.6-11.0	.20	207.9	577	2.2	0.6-8.2	.23	175.6
Peanut protein in dust (*ln* transformed μg/g)‖		0.9	0.6-1.3	.52			0.8	0.5-1.4	.38			0.9	0.6-1.6	.82	
Interaction *FLG**peanut in dust		5.3	1.8-15.3	**<.01**			4.5	1.5-13.5	**<.01**			2.70	0.9-8.0	.07	
Age at assessment (8 or 11 y)		0.7	0.5-1.1	.10			0.9	0.6-1.4	.66			NA			
Combined *FLG* loss-of-function mutation	516	1.8	0.4-7.5	.41	303.9	396	1.3	0.2-7.6	.78	176.7	511	1.1	0.3-5.2	.87	132.5
Peanut protein in dust (*ln* transformed μg/g)‖		0.9	0.6-1.3	.50			0.8	0.5-1.5	.53			0.98	0.5-1.9	.98	
Interaction *FLG**peanut in dust		6.8	2.6-17.5	**<.001**			6.6	2.3-18.9	**<.001**			3.9	1.3-11.8	**.02**	
Egg SPT sensitization at age 3 y		16.2	4.5-59.0	**<.001**			25.1	5.2-122.1	**<.001**			34.84	9.9-122.4	**<.001**	
Age at assessment (8 or 11 y)		0.7	0.5-1.1	.14			0.9	0.6-1.6	.82			NA			
Combined *FLG* loss-of-function mutation	516	1.1	0.3-5.2	.87	279.4	396	1.0	0.2-5.5	.95	167.6	511	0.8	0.2-3.9	.83	129.3
Peanut protein in dust (*ln* transformed μg/g)‖		0.9	0.6-1.3	.45			0.8	0.5-1.4	.46			1.0	0.5-1.8	.95	
Interaction *FLG**peanut in dust		5.2	2.1-13.1	**<.001**			5.3	1.9-14.8	**.001**			3.2	1.1-9.8	**.04**	
Egg SPT sensitization at age 3 y		8.8	2.2-34.5	**<.01**			13.0	2.3-75.3	**<.01**			19.95	5.4-74.0	**<.001**	
History of AD during infancy		7.5	2.4-23.2	**<.001**			5.4	1.2-24.2	**.03**			4.04	1.2-14.1	**.03**	
Age at assessment (8 or 11 y)		0.7	0.4-1.1	.12			1.0	0.6-1.7	.90			NA			

Values in boldface are significant.

AIC, Akaike information criterion; *LR*, penalized logistic regression methodology; *NA*, not applicable; *QIC*, quasilikelihood under independent model criterion.

*†‡White children enrolled in MAAS with available sofa dust within the first year of life, successful *FLG* genotyping, and peanut SPT* or CRD† sensitization or peanut allergy‡ assessment.

§Reductions in quasilikelihood under independent model criterion (GEE) and Akaike information criterion (LR) values denote improved goodness of fit of the statistical model.

‖Peanut protein in dust: values less than the LLQ were used in this analysis.

genotype, there was no association between early-life environmental peanut exposure and subsequent peanut sensitization or allergy.

Threshold environmental peanut levels in dust for peanut sensitization and allergy

In children carrying 1 or more *FLG* loss-of-function mutations, the threshold environmental peanut allergen level for peanut SPT sensitization was −0.079 *ln* transformed units (0.92 μg of peanut protein/gram of dust; 95% CI, 0.70-1.22 μg/g), that for CRD sensitization was 0.032 *ln* transformed units (1.03 μg/g; 95% CI, 0.90-1.82 μg/g), and that for peanut allergy was 0.156 *ln* transformed units (1.17 μg/g; 95% CI, 0.01-163.83 μg/g).

DISCUSSION

This study demonstrates a gene-environment interaction on the development of peanut sensitization and clinically proven peanut allergy. In children carrying 1 or more *FLG* loss-of-function mutations, there was a dose-response relationship between early-life environmental exposure to peanut protein in household dust and subsequent peanut sensitization and allergy; each *ln* unit (2.7-fold) increase in house dust peanut exposure during infancy was associated with a more than 6-fold increase in the odds of school-age peanut sensitization and a 3.3-fold increase in the odds of school-age peanut allergy. Therefore we demonstrated a consistent interaction between *FLG* genotype and peanut dust exposure for peanut SPT sensitization, major allergen sensitization, and clinically proven peanut allergy. Previous studies have also shown a stronger effect of *FLG* loss-of-function mutations on peanut sensitization than peanut allergy.[33] The interaction between *FLG* genotype and environmental peanut exposure was significant after adjusting for infantile AD and preceding egg sensitization; thus the modifying effect of *FLG* genotype was independent of AD or other atopy markers.

Among *FLG* mutation carriers, peanut protein levels in dust reached a maximum of 14.78 μg/g; thus an increase in peanut dust exposure from the LLQ (0.5 μg/g) to 14.78 μg/g equated

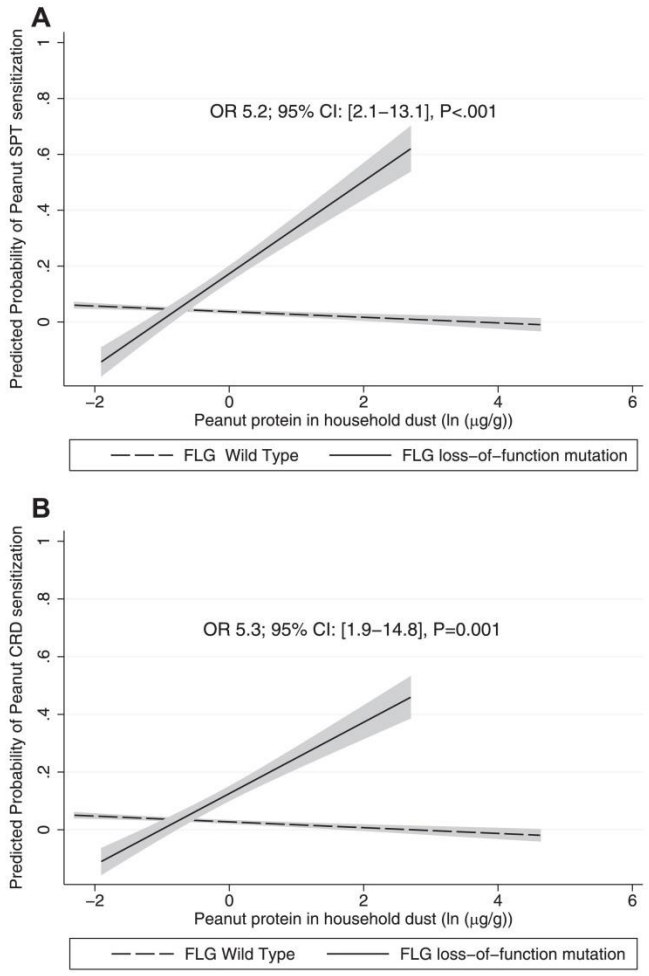

FIG 2. Mean predictive probability of peanut sensitization over 8 and 11 years on GEE analysis with increasing environmental peanut exposure (defined by *ln* transformed peanut protein in micrograms per gram of dust) for children with 1 or more *FLG* loss-of-function mutations versus those with wild-type *FLG*. The model was adjusted for a history of infantile AD and egg SPT sensitization at age 3 years. Interaction ORs and 95% CIs displayed between peanut protein in dust and *FLG* loss-of-function mutations on peanut sensitization are shown. Predictive probability is only shown within the observable environmental peanut exposure data obtained. **A,** Peanut SPT sensitization. **B,** Peanut CRD sensitization.

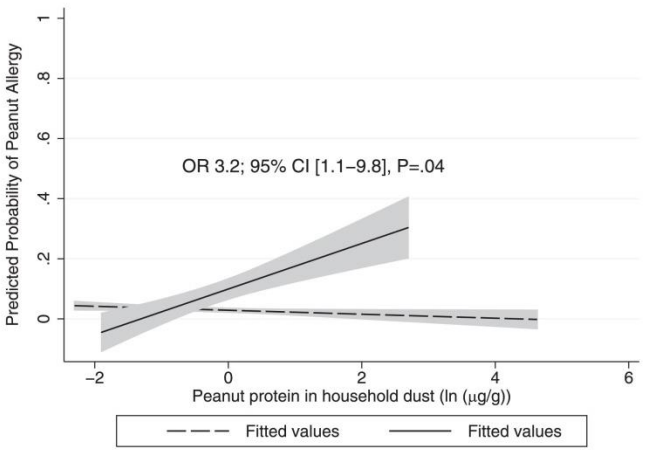

FIG 3. Mean adjusted predictive probability of peanut allergy at 8 years, 11 years, or both on multivariate penalized logistic regression analysis with increasing environmental peanut exposure (defined by *ln* transformed peanut protein in micrograms per gram of dust) in children with 1 or more *FLG* loss-of-function mutations versus those with wild-type *FLG*. Interaction ORs and 95% CIs are displayed between peanut protein in dust and *FLG* loss-of-function mutations on peanut allergy. Predictive probability is only shown within the observable environmental peanut exposure data obtained.

to an almost 30-fold increase (3.4 *ln* scales), which is equivalent to a 58-fold ($3.3^{3.4}$) increase in the odds of peanut allergy. These results suggest that the level of early-life environmental peanut exposure in children who carry *FLG* loss-of-function variants might critically influence the development of peanut sensitization and, importantly, clinical peanut allergy; however, future work is required to ensure the linearity of peanut protein data over the entire range of peanut protein in dust. In contrast, no association was seen between environmental peanut exposure and peanut sensitization or allergy in children without *FLG* mutations. In children carrying an *FLG* mutant allele, the mean threshold peanut protein level in dust for peanut sensitization and allergy was around twice the LLQ of the ELISA (0.50 μg/g). Thus on the basis of our findings in this white United Kingdom population, minimal quantities of peanut protein in the environment could lead to peanut sensitization and allergy in children who carry *FLG* loss-of-function mutations, but the risk markedly increases with increasing exposure.

Previous studies have shown gene-environment interactions between *FLG* loss-of-function mutations and other atopic diseases.[34] Among children carrying an *FLG* mutation, those whose families owned a cat had an approximately 4-fold odds of having AD compared with those whose families did not own a cat; there was no effect of cat ownership among children without *FLG* mutations.[34] Contact allergy to nickel is twice as common in adults with the *FLG* frameshift mutation 2282del4,[35] and in murine models *flg* loss-of-function mutations lead to increased bidirectional paracellular penetration of water-soluble tracers and reduced inflammatory threshold to allergens.[36] There is a significant association between *FLG* mutations and development of asthma and allergic sensitization but only in children with preceding AD.[37] This has been used as an argument for the role of *FLG* loss-of-function mutations as a predisposing factor for allergic sensitization after epicutaneous exposure to allergens. Peanut protein in environmental dust and surfaces could penetrate disrupted skin because of impaired filaggrin production and could be taken up by Langerhans cells, leading to a T_H2 response and IgE production by B cells.[38,39] Studies are investigating the role of thymic stromal lymphopoietin produced by keratinocytes in response to environmental antigens in patients with AD.[40] Thymic stromal lymphopoietin in combination with enhanced allergen penetration through a damaged epidermis could lead to a T_H2-type milieu; it would be interesting to review this in the context of filaggrin-deficient children with high levels of environmental peanut exposure.

There are certain limitations to this study. We were unable to include all MAAS participants because of the availability of early-life dust samples and *FLG* genotyping. Because the 6 *FLG* loss-of-function mutations assessed have been associated with AD in white populations,[12] we excluded all nonwhite participants. Given that 95% of MAAS participants were white, this is unlikely to lead to bias. On comparing the groups of included versus excluded children, there were some small differences in their demographic characteristics, but importantly, there were

no significant differences in peanut sensitization or allergy rates; therefore these are unlikely to have influenced the results. Peanut allergen levels in lounge-sofa dust might not be the best index of infant exposure; however, we have shown previously that there is high within-home correlation of peanut protein levels in dust, particularly between an infant's bed and play area.[26] In our previously published work the infant play area was usually in the lounge, which was also the location of the sofa in the MAAS study. There were no available data on the amount of peanut the infant was consuming; however, given that the majority of dust collected was antenatal, these peanut dust levels would not have been due to the infant consuming peanut.

We acknowledge that there are small numbers of subjects with confirmed peanut allergy in whom *FLG* genotype and early-life peanut exposure data are available. This reflects the complexities of measuring all necessary predictors over the life course in children with robustly ascertained clinical outcomes that are themselves relatively uncommon (*FLG* loss-of-function mutations and clinical peanut allergy). We emphasize that the findings of an interaction between *FLG* loss-of-function genotype and environmental peanut exposure for sensitization (however measured) and peanut allergy are consistent, in keeping with previous gene-environment interactions for *FLG*, and biologically plausible.

It is important to consider how peanut allergen in dust might lead to sensitization to assess the clinical applicability of our findings; although this might lead to epicutaneous sensitization through direct skin contact, we cannot exclude the possibility of inhalation of dust particles containing peanut allergen. Although filaggrin is not expressed in the lung[41] or inferior nasal turbinates,[42] it is expressed in the cornified epithelium in the vestibular nasal lining.[11] However, several studies suggest that peanut is poorly aerosolizeable[26,43] and report that allergic symptoms after inhalation of peanut have not been replicated on blinded challenges.[44] It is also important to determine how peanut protein gets into household dust. Peanut protein is present on hand wipes and in saliva up to 3 hours after peanut consumption and thus might be amenable to transfer through this route.[26] Fox et al[15] found that household consumption of peanut butter was more highly associated with peanut allergy in infants than household consumption of covered forms of peanut-containing foods. They hypothesized that peanut butter was more likely to lead to sensitization through hand-to-hand contact because it is sticky and thus more likely to be transferred onto surfaces (and dust) or people. Peanut protein persists on table surfaces and sofa-pillow dust, despite usual cleaning measures,[26] and thus might be an important source of exposure.

Although our study focused on peanut sensitization and allergy, *FLG* loss-of-function mutations might confer susceptibility to environmental exposure to other food allergens in dust, such as fish, egg, and cow's milk.[45] The dual-allergen-exposure hypothesis postulates that food allergy develops through transcutaneous exposure to allergen through a disrupted skin barrier, whereas oral exposure leads to tolerance induction.[38] Our findings of a dose-response effect for peanut allergen in dust on the development of peanut allergy in children genetically predisposed to a skin barrier defect support this hypothesis. Furthermore, our study raises the intriguing possibility of identifying a group of children with *FLG* loss-of-function mutations and targeting them in interventional studies through early environmental modification.

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Clinical implications: Children with *FLG* loss-of-function mutations are at an increased risk of peanut sensitization and allergy if they are exposed to peanut antigen in household dust in early life. Interventional studies to assess a causal relationship are required.

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TABLE E1. GEE for peanut sensitization using the quasilikelihood under independent model criterion goodness-of-fit analyses

	GEE for peanut SPT sensitization adjusted for clustering at age 8 and 11 y (n = 584)					GEE for peanut CRD sensitization adjusted for clustering at age 8 and 11 y (n = 437)					LR for peanut allergy at age 8 y, 11 y, or both (n = 577)				
	No.*	OR	95% CI	*P* value	QIC§	No.†	OR	95% CI	*P* value	QIC§	No.‡	OR	95% CI	*P* value	AIC§
Combined *FLG* loss-of-function mutation	584	3.5	1.5-8.3	**<.01**	386.6	437	4.0	1.4-11.4	**<.01**	215.3	577	2.5	0.8-7.9	.11	175.6
Age at assessment (8 or 11 y)		0.8	0.5-1.1	.10			0.9	0.6-1.4	.69			NA			
Combined *FLG* loss-of-function mutation	584	3.6	1.5-8.2	**<.01**	386.5	437	3.9	1.4-10.9	**<.01**	215.9	577	2.6	0.8-7.9	.11	176.8
Peanut protein in dust (*ln* transformed μg/g)‖		1.2	0.9-1.7	.15			1.3	0.90-1.8	.19			1.2	0.8-1.8	.35	
Age at assessment (8 or 11 y)		0.7	0.5-1.1	.10			0.9	0.6-1.4	.65			NA			
Combined *FLG* loss-of-function mutation	584	2.8	0.8-9.8	.17	373.2	437	2.6	0.6-11.1	.20	208.2	577	2.3	0.6-8.4	.21	175.5
Peanut protein in dust (*ln* transformed μg/g)‖		0.9	0.6-1.3	.58			0.9	0.5-1.4	.51			1.0	0.6-1.6	.97	
Interaction *FLG**peanut in dust		4.3	1.4-12.8	**.01**			4.0	1.4-11.4	**.01**			2.5	0.9-7.1	.08	
Age at assessment (8 or 11 y)		0.7	0.5-1.1	.10			0.9	0.6-1.4	.66			NA			
Combined *FLG* loss-of-function mutation	516	2.1	0.5-8.4	.30	307.1	396	1.2	0.2-7.2	.78	177.0	511	1.1	0.2-5.1	.91	132.4
Peanut protein in dust (*ln* transformed μg/g)‖		0.9	0.6-1.3	.59			0.9	0.6-1.5	.75			1.1	0.6-1.9	.83	
Interaction *FLG**peanut in dust		5.5	2.0-14.7	**.001**			6.0	2.2-16.2	**<.001**			3.7	1.3-10.7	**.02**	
Egg SPT sensitization at age 3 y		15.9	4.4-57.8	**<.001**			26.3	5.3-130.2	**<.001**			35.9	10.1-127.7	**<.001**	
Age at assessment (8 or 11 y)		0.7	0.5-1.1	.13			0.9	0.6-1.6	.81			NA			
Combined *FLG* loss-of-function mutation	516	1.3	0.3-5.8	.71	279.4	396	0.9	0.2-5.3	.93	167.9	511	0.8	0.2-3.9	.81	129.3
Peanut protein in dust (*ln* transformed μg/g)‖		0.9	0.6-1.3	.52			0.9	0.6-1.4	.67			1.0	0.6-1.8	.89	
Interaction *FLG**peanut in dust		4.3	1.7-11.0	**<.01**			4.8	1.8-12.6	**<.01**			3.1	1.1-8.9	**.04**	
Egg SPT sensitization at age 3 y		8.6	2.2-33.6	**<.01**			13.5	2.3-79.2	**<.01**			20.4	5.5-76.3	**<.001**	
Atopic eczema during infancy		7.6	2.5-23.3	**<.001**			5.4	1.2-24.2	**<.01**			4.1	1.2-14.1	**.03**	
Age at assessment (8 or 11 y)		0.7	0.4-1.1	.12			1.0	0.6-1.7	.91			NA			

Values in boldface are significant.

AIC, Akaike information criterion; *LR*, penalized logistic regression methodology; *NA*, not applicable; *QIC*, quasilikelihood under independent model criterion.

*†‡White children enrolled in the MAAS with available sofa dust within the first year of life, successful *FLG* genotyping, and peanut SPT* or CRD† sensitization or peanut allergy‡ assessment.

‖Peanut protein levels in dust less than the LLQ were assigned an LLQ/2 calculation.

§Reductions in QIC (GEE) and AIC (LR) values denote improved goodness-of-fit of statistical model.

7.8 Publication 4: Eczema increases the impact of EPE on PS and likely PA

Brough H.A., Liu A., Sicherer S., Makinson K., Douiri A., Brown S.J., Stephens A.C., McLean W.H.I. Turcanu V., Wood R., Jones S.M., Burks W., Dawson P., Stablein D., Sampson H., Lack G. Atopic dermatitis increases the impact of exposure to peanut antigen in dust on peanut sensitization and likely peanut allergy. J Allergy Clin Immunol. 2015; 135(1): 164-70.[396]

Atopic dermatitis increases the effect of exposure to peanut antigen in dust on peanut sensitization and likely peanut allergy

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Background: History and severity of atopic dermatitis (AD) are risk factors for peanut allergy. Recent evidence suggests that children can become sensitized to food allergens through an impaired skin barrier. Household peanut consumption, which correlates strongly with peanut protein levels in household dust, is a risk factor for peanut allergy.

Objective: We sought to assess whether environmental peanut exposure (EPE) is a risk for peanut sensitization and allergy and whether markers of an impaired skin barrier modify this risk.

Methods: Peanut protein in household dust (in micrograms per gram) was assessed in highly atopic children (age, 3-15 months) recruited to the Consortium of Food Allergy Research Observational Study. History and severity of AD, peanut sensitization, and likely allergy (peanut-specific IgE, ≥5 kU_A/mL) were assessed at recruitment into the Consortium of Food Allergy Research study.

Results: There was an exposure-response relationship between peanut protein levels in household dust and peanut skin prick

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test (SPT) sensitization and likely allergy. In the final multivariate model an increase in 4 log_2 EPE units increased the odds of peanut SPT sensitization (1.71-fold; 95% CI, 1.13- to 2.59-fold; $P = .01$) and likely peanut allergy (PA; 2.10-fold; 95% CI, 1.20- to 3.67-fold; $P < .01$). The effect of EPE on peanut SPT sensitization was augmented in children with a history of AD (OR, 1.97; 95% CI, 1.26-3.09; $P < .01$) and augmented even further in children with a history of severe AD (OR, 2.41; 95% CI, 1.30-4.47; $P < .01$); the effect of EPE on PA was also augmented in children with a history of AD (OR, 2.34; 95% CI, 1.31-4.18; $P < .01$).

Conclusion: Exposure to peanut antigen in dust through an impaired skin barrier in atopically inflamed skin is a plausible route for peanut SPT sensitization and PA. (J Allergy Clin Immunol 2015;135:164-70.)

Key words: *Atopic dermatitis, peanut sensitization, peanut allergy, environmental peanut exposure, dust*

Abbreviations used
AD: Atopic dermatitis
CoFAR: Consortium of Food Allergy Research
EPE: Environmental peanut exposure
FLG: Filaggrin
IQR: Interquartile range
LLQ: Lower limit of quantitation
LR: Logistic regression
OR: Odds ratio
PA: Peanut allergy
sIgE: Specific IgE
SPT: Skin prick test

Skin barrier dysfunction plays an important role in the development of atopic dermatitis (AD),[1,2] and AD is often cited as the first step in the allergic march.[3,4] There is a clear association between early-onset AD and food allergy[5,6] and a growing body of evidence that epicutaneous exposure to peanut through an impaired skin barrier increases the risk of peanut sensitization and clinically confirmed peanut allergy.[7-9] Among children with peanut allergy with AD in the Avon Longitudinal Study of Parents and Children, 90% had been exposed to creams containing *Arachis* (peanut) oil in the first 6 months of life.[6] In BALB/c mice epicutaneous peanut exposure has been shown to induce a potent allergic T_H2-type response and anaphylaxis after a single oral antigen challenge[7-9]; however, in these studies this was only achieved if skin stripping, leading to skin barrier impairment and inflammation, was performed before antigen application. In flaky tail mice that carry a mutation within the murine *flg* gene, topical application of ovalbumin leads to a cellular infiltrate and antigen-specific antibody response, even without skin stripping.[10]

We have shown that early exposure to peanut antigen in household dust is a risk factor for the development of peanut sensitization and clinically confirmed peanut allergy in children who carry a filaggrin *(FLG)* null mutation in the Manchester Asthma and Allergy Study cohort.[11] In another study environmental exposure to peanut measured indirectly based on household peanut consumption was associated with peanut allergy, particularly when compared with atopic children.[12] Peanut protein in household dust was not objectively quantified in this study; however, other studies have measured peanut allergens in dust,[13,14] and we have shown that peanut allergen levels in dust from the infant's bed and play area correlate with household peanut consumption and stimulate an allergic response in effector cells of patients with peanut allergy.[15]

We hypothesized that an impaired skin barrier in children with AD or *FLG* null mutations would modify the effect of environmental peanut exposure (EPE), as defined by peanut protein in household dust (in micrograms per gram), on peanut sensitization and allergy. If proved, this hypothesis would support the notion that a primary mode leading to the development of peanut sensitization and allergy occurs through presentation of environmental peanut antigen through an impaired skin barrier to underlying antigen-presenting cells. The purpose of this study was to assess whether early EPE increases the risk of peanut sensitization and allergy in young atopic children.

METHODS

Participants were from the National Institutes of Health–sponsored Consortium of Food Allergy Research (CoFAR). The design and methodology are described elsewhere.[16] In brief, 512 children less than 15 months of age were recruited with a convincing clinical history of cow's milk allergy, egg allergy, or both and a positive skin prick test (SPT) response to cow's milk, egg, or both, respectively, or with moderate-to-severe AD with a positive SPT response to cow's milk, egg, or both but without known peanut allergy. Study procedures were reviewed and approved by a National Institute of Allergy and Infectious Diseases Data Safety Monitoring Board and by local institutional review boards, and written signed informed consent was obtained. The analyses included 359 (70.1%) of 512 participants who provided enough dust to analyze approximately 10 mg for peanut protein.

SPTs were performed with the GreerPick (Greer Laboratories, Lenoir, NC) on the infant's back. Results were obtained after 15 minutes, and the average mean wheal diameter (after subtraction of the saline negative control) was recorded. Children with peanut SPT responses of 3 mm or greater were described as peanut SPT sensitized, and children with peanut SPT responses of less than 3 mm were described as not sensitized. Children with serum specific IgE (sIgE) to peanut (ImmunoCAP system; Thermo Fisher Scientific, Uppsala, Sweden) of 0.35 kU_A/mL or greater were described as peanut sIgE sensitized. Children with serum sIgE levels to peanut of 5 kU_A/mL or greater were described as having a serologic diagnosis of likely peanut allergy (PA); this was postulated as in previous studies, 70% to 90% of 5- to 7-year-old children had positive diagnostic peanut challenge results with this level of peanut sIgE.[17-19] Children were defined as not peanut allergic if they had a history of tolerating eating peanut (regardless of sensitization status) or if they were not sensitized to peanut, even if there was no history of peanut ingestion. Peanut-sensitized children (peanut SPT response ≥3 mm or peanut sIgE level of between 0.35 and 5 kU_A/mL) without a history of peanut ingestion were excluded from the PA analysis because they did not undergo a peanut challenge at baseline and thus could not be defined as having peanut allergy or peanut tolerance. Of 359 subjects with available living room dust, 150 (41.8%) children had no history of ingestion of peanut and peanut SPT responses of 3 mm or greater or sIgE levels of 0.35 kU_A/mL or greater and thus were excluded from the PA analysis. Of the remaining children, 89 (42.6%) of 209 were considered to have a serologic diagnosis of PA because of a peanut sIgE level of 5 kU_A/mL or greater. There were 120 children considered not to have peanut allergy who either reported peanut consumption without a reaction (n = 20/209 [9.6%]) or who were not sensitized to peanut (n = 100/209 [47.8%]).

FLG genotyping was performed with genomic DNA extracted from blood. The *FLG* null mutations R501X, 2282del4, S3247X, and R2447X were assessed with a TaqMan-based allelic discrimination assay (Applied

of disease (by history), and (3) intensity of disease (disturbance of night's sleep by itching), each on a 3-point scale, as previously described.[22] The rule of 9 is used to calculate the area of the body's skin affected for SCORAD score assessment, where the head and neck amount to 9%, the upper limbs amount to 9% each, the lower limbs amount to 18% each, the anterior trunk amounts to 18%, the back amounts to 18%, and the genitals amount to 1%.[23]

EPE was quantified from dust collected at baseline from the family's living room floor. Families were asked to avoid vacuuming their living room floors for 3 days before obtaining dust. Participants were provided with a DUSTREAM adaptor and collector (Indoor Biotechnologies, Warminster, United Kingdom), a nylon collection filter, a disposable template, and instructions for vacuuming. The living room floor was vacuumed for 2 minutes within a 1-m^2 surface area. Dust samples were sieved, and fine dust was extracted in a proportional volume of extraction solution.[24] Peanut protein in dust was determined by using the Veratox polyclonal ELISA against whole peanut protein (Neogen, Lansing, Mich), which has been validated for sensitivity, specificity, and reliability in measuring peanut protein in food[25,26] and dust.[24] The lower limit of quantification (LLQ) of the assay was defined as 100 ng/mL whole peanut (25 ng/mL peanut protein), and samples of less than this value were defined as LLQ/2 (12.5 ng/mL peanut protein, which equated to between 1.05 and 1.23 μg/g depending on the weight of dust obtained).[27] There were 16 (4.5%) of 359 living room dust samples with peanut protein levels of less than the LLQ. Results were converted from nanograms per milliliter into micrograms of peanut protein per gram of dust. Participant information was kept blind from the researcher performing the ELISA dust analyses. Dust samples were also obtained from the infant's bed dust; details are described in the Methods section in this article's Online Repository at www.jacionline.org.

Statistical analysis

Data were entered into SPSS (SPSS 19.0; SPSS, Chicago, Ill) and STATA (STATA/IC 12.1; StataCorp, College Station, Tex) spreadsheets for analysis. Associations between demographic, clinical, and household factors and peanut SPT sensitization and PA were assessed by using a logistic regression (LR) model for children with available dust for analysis. Peanut protein levels in dust (micrograms per gram) underwent $\log_2$ transformation to normalize data. EPE spanned approximately 12 $\log_2$ scales (1.05-3761.68 μg/g), and therefore we showed the effect of 4 $\log_2$ unit increases in EPE on peanut SPT sensitization and PA. In a stepwise process all factors with a trend toward an association with peanut SPT sensitization or PA on univariate analysis ($P < .15$) were included in the multivariate model, and then only those covariates with a P value of less than .05 were included in the final multivariate model. The same covariates were included in the multivariate analysis for all children, children with a history of AD, and children with a history of severe AD. We assessed EPE as a continuous variable and as quartiles by dividing the span of continuous EPE into 4 equal groups. Visual graphs were inspected, and the linearity of the logit ($p/[1-p]$) and $\log_2$ continuous peanut protein level was reasonable for both peanut SPT sensitization and PA on univariate analysis. Overlapping 95% CIs of odds ratios (ORs) among EPE quartiles supported the linearity of the exposure-response relationship between $\log_2$ EPE and the logit of Prob (peanut SPT sensitization = positive) and Prob (PA = positive). Therefore we used continuous EPE as the optimum representation of the primary exposure variable throughout the article.

The effect of EPE on peanut sensitization or PA was assessed in a univariate and multivariate LR model in all children and subgroups of children without a history of AD, with a history of AD, or with a history of severe AD. We subsequently included an interaction term with EPE and a history of AD (vs no AD) or history of severe AD (vs no AD). To establish the relationship between EPE during the child's early life and maternal peanut consumption in pregnancy, peanut protein levels in living room dust (in micrograms per gram) were compared in homes in which mothers either avoided or consumed

RESULTS

EPE is associated with peanut SPT sensitization and PA

ORs (95% CIs) of factors possibly associated with peanut SPT sensitization or PA (peanut sIgE, ≥5 kU_A/mL) are displayed in Tables I (univariate LR analysis), II, and III (multivariate LR analyses). There was a significant association between a 4-unit $\log_2$ increase in EPE and peanut SPT sensitization both on univariate analysis (n = 359; OR, 1.52; 95% CI, 1.08-2.14; $P = .01$) and multivariate LR analysis (n = 292; OR, 1.71; 95% CI, 1.13-2.59; $P = .01$), adjusting for parental report of hay fever ever in the child, egg SPT wheal diameter (in millimeters), and maternal peanut consumption during pregnancy and breast-feeding (which were also associated with peanut SPT sensitization at $P < .05$). There was a trend toward an association between EPE and PA on univariate analysis (n = 209; OR, 1.46; 95% CI, 0.92-2.29; $P = .11$) and a significant association on multivariate LR analysis (n = 209; OR, 2.10; 95% CI, 1.20-3.67; $P < .01$), adjusting for ethnicity, egg SPT wheal diameter, and cow's milk SPT wheal diameter (which were also associated with PA at $P < .05$). The relationship between peanut protein in the infants' bed and peanut SPT sensitization and PA is described in this article's Online Repository at www.jacionline.org.

History of AD modifies the effect of EPE on peanut SPT sensitization and PA

On stratified univariate analysis, the effect of increasing EPE on peanut SPT sensitization and PA was augmented in children with a history of AD and severe AD (Fig 1, *A*: peanut SPT sensitization; Fig 1, *B*: PA). On univariate analysis, there was a significant interaction between EPE and AD on the risk of peanut SPT sensitization (OR, 1.41; 95% CI, 1.01-1.97; $P < .05$) per $\log_2$ unit EPE increase; this further increased when comparing the interaction between EPE and a history of severe AD (OR, 1.46; 95% CI, 1.04-2.07; $P < .05$). The interaction between EPE and a history of AD did not reach statistical significance for PA. There was no association between EPE and peanut SPT sensitization (OR, 0.81; 95% CI, 0.59-1.12) or PA (OR, 0.95; 95% CI, 0.60-1.49) in children without a history of AD.

On multivariate LR analysis, the exposure-response relationship of EPE was augmented in children with a history of AD for peanut SPT sensitization (OR, 1.97; 95% CI, 1.26-3.09; $P < .01$) and PA (OR, 2.34; 95% CI, 1.31-4.18; $P < .01$; Table IV). For peanut SPT sensitization, the effect of EPE was further augmented in children with a history of severe AD (OR, 2.41; 95% CI, 1.30-4.47; $P < .01$); however, a similar increase was not observed for PA. In the multivariate predictive probability figures, the association between EPE and peanut SPT sensitization and PA remained; however, there was no longer a clear differentiation of the effect of EPE among all children, children with a history of AD, and children with a history of severe AD (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org: peanut SPT sensitization; see Fig E1, *B*: PA).

The interaction between EPE and a history of AD for the risk of peanut SPT sensitization remained significant in the multivariate

TABLE I. Unadjusted ORs and 95% CIs measuring associations between peanut SPT sensitization and likely PA and log_2 EPE units and subject demographic, clinical, and household factors*

	Peanut SPT sensitization (n = 359 [54.6% positive])			Likely PA (n = 209 [42.6% positive])		
	OR	95% CI	*P* value	OR	95% CI	*P* value
4 log_2 EPE (μg/g)†	1.52	1.08-2.14	**.01**	1.46	0.92-2.29	.11
History of infantile AD	1.83	0.82-4.06	.14	1.87	0.63-5.51	.26
Maximum AD severity before entry						
No AD (0)		Reference category			Reference category	
Mild (3-4)	2.46	0.88-6.89	.09	1.31	0.31-5.53	.71
Moderate (5-6)	1.77	0.75-4.19	.19	1.91	0.60-6.08	.28
Severe (7-9)	1.77	0.78-4.01	.17	1.94	0.64-5.88	.24
Nonwhite ethnicity	1.73	0.44-2.21	.23	1.93	1.04-3.60	**.04**
FLG null mutation (excluding nonwhite subjects)	0.72	0.37-1.39	.32	1.29	0.54-3.08	.56
Parental report of hay fever ever in the child	3.07	1.28-7.32	**.01**	1.52	0.57-4.20	.39
Male sex	0.82	0.53-1.28	.38	1.49	0.81-2.73	.20
Maternal history of atopy or asthma	1.29	0.83-2.02	.26	1.26	0.69-2.28	.45
Paternal history of atopy or asthma	1.00	0.65-1.54	1.0	1.00	0.56-1.78	1.0
Maternal history of AD	0.94	0.56-1.59	.82	1.13	0.56-2.28	.73
Paternal history of AD	0.74	0.42-1.30	.29	0.57	0.25-1.28	.17
Peanut consumption in pregnancy	1.67	0.94-2.26	.08	1.49	0.65-3.40	.34
Peanut consumption while breast-feeding	0.69	0.43-1.10	.12	0.51	0.27-0.94	.03
Peanut butter in house while breast-feeding	1.04	0.64-1.69	.88	0.94	0.49-1.78	.84
Older siblings	1.34	0.87-2.04	.18	1.11	0.63-1.95	.73
Egg SPT wheal diameter (mm)	1.15	1.10-1.21	**<.01**	1.26	1.17-1.35	**<.01**
Cow's milk SPT wheal diameter (mm)	1.07	1.03-1.11	**<.01**	1.21	1.09-1.35	**<.01**
Duration of breast-feeding (mo)	1.05	1.00-1.10	.08	1.12	1.04-1.20	**<.01**
Maternal age at baseline (y)	1.02	0.99-1.07	.12	1.02	0.97-1.08	.40
Child's age at baseline assessment (mo)	1.11	1.04-1.19	**<.01**	1.04	0.96-1.14	.34

Statistically significant values ($P < .05$) are shown in boldface.

*Descriptive statistics of subject factors and EPE are shown in Table E1.

†ORs for EPE reflect a 4-unit increase in log_2 EPE. ORs for other continuous factors reflect a 1-unit increase in the factor unit. These include egg SPT wheal diameter, cow's milk, duration of breast-feeding, maternal age at baseline, and child's age at baseline. ORs for AD severity compare each severity level with the level "no AD." All other factors are dichotomous. ORs compare yes with no, ever with never, or male with female.

TABLE II. Adjusted peanut sensitization (OR [95% CI]) measuring associations between EPE and subject factors (n = 292)*

	OR	95% CI	*P* value
4 log_2 EPE (μg/g)	1.71	1.13-2.59	**.01**
Egg SPT wheal diameter (mm)	1.17	1.11-1.24	**<.001**
Maternal peanut consumption in pregnancy	2.77	1.24-6.20	**.01**
Maternal peanut consumption while breast-feeding	0.46	0.25-0.85	**.01**
Parental report of hay fever ever in the child	3.88	1.35-11.15	**.01**

Subject factors and EPE values are significant at the 5% level (in boldface). The OR of EPE represents an increase of 4 log_2 EPE units (in micrograms per gram).

*Sample size was reduced from 359 to 292 because of missing data for some factors in the multivariate analysis.

model; the OR was 1.48 (95% CI, 1.01-2.17; $P < .05$) per log_2 unit EPE increase in children with a history of AD versus those with no AD, and the OR was 1.56 (95% CI, 1.04-2.34, $P = .03$) in children with a history of severe AD versus those with no AD. In the final multivariate model there was a trend toward an interaction between EPE and a history of AD for PA with an OR of 1.68 (95% CI, 0.91-3.12; $P = 0.10$) and an OR of 1.68 (95% CI, 0.85-3.31; $P = .14$) in children with a history of severe AD versus those with no AD.

FLG genotype on peanut sensitization and PA

The prevalence of *FLG* null mutations in white children with AD (with dust available) was 14.9% (41/275); of these children, 37 had *FLG* heterozygote mutations, 3 had a combined heterozygote mutations, and 1 had a 2282del4 homozygous mutation. There was no significant association between *FLG* heterozygous or compound heterozygous/homozygous mutations and peanut SPT sensitization/PA; there was also no interaction between *FLG* genotype and EPE.

Comparisons of the included group (n = 359) with available living room dust and the excluded group (n = 153)

There was no difference in the rate of peanut sensitization or PA between subjects with (n = 359) versus those without (n = 153) available dust; however, there were small but significant differences in the rate of severe AD, ethnicity, number of older siblings, maternal history of AD, maternal peanut consumption during breast-feeding, and peanut present in the home while breast-feeding (see Table E1 in this article's Online Repository at www.jacionline.org).

DISCUSSION

In this high-risk atopic cohort we found that EPE, as assessed by log_2 transformed peanut protein (in micrograms) per gram of living room dust was a risk factor for peanut SPT sensitization and PA (peanut sIgE, ≥5 kU_A/mL). After adjustment, an increase in 4 log_2 EPE units increased the odds of peanut SPT sensitization 1.71-fold (95% CI, 1.13- to 2.59-fold) and the odds of PA 2.10-fold (95% CI, 1.20- to 3.67-fold). The effect of EPE on

TABLE III. Adjusted likely PA (OR [95% CI]) measuring associations between EPE and subject factors (n = 209)*

	OR	95% CI	*P* value
4 $\log_2$ EPE (μg/g)	2.10	1.20-3.67	**<.01**
Egg SPT wheal diameter (mm)	1.25	1.15-1.36	**<.001**
Nonwhite ethnicity	2.59	1.21-5.58	**.02**
Cow's milk SPT wheal diameter (mm)	1.14	1.06-1.22	**<.001**

Subject factors and EPE values are significant at the 5% level (in boldface). The OR of EPE represents an increase of 4 $\log_2$ EPE units (in micrograms per gram).
*The sample size was reduced from 359 to 209 because of missing data for some factors in the multivariate analysis.

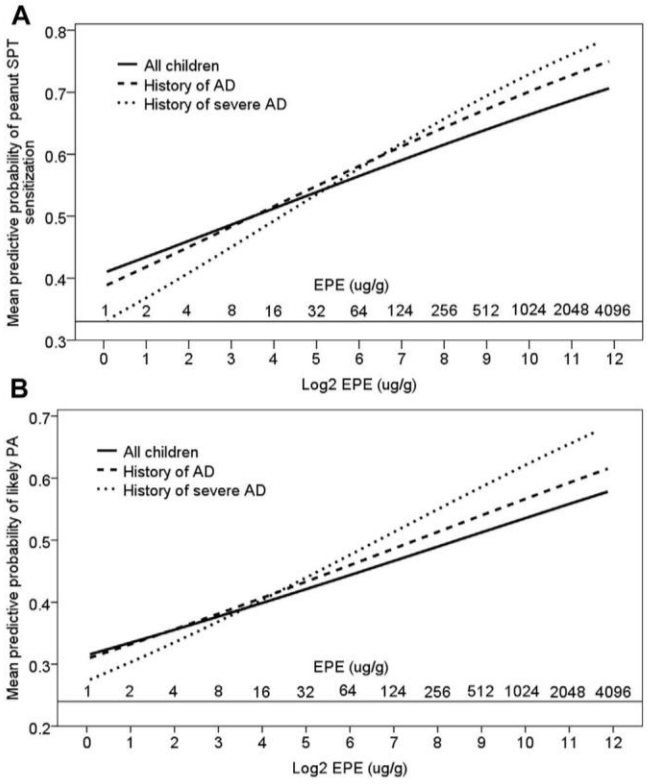

FIG 1. A, Univariate stratified predictive probability for the effect of EPE (displayed in $\log_2$ [microgram per gram] units and untransformed [microgram per gram] units) for peanut SPT sensitization in all children, children with a history of AD, and children with a history of severe AD. **B,** Univariate stratified predictive probability for the effect of EPE (displayed in $\log_2$ [microgram per gram] units and untransformed [microgram per gram] units) for likely PA in all children, children with a history of AD, and children with a history of severe AD.

peanut SPT sensitization and PA increased in an exposure-dependent manner in children with a history of AD, with an increase in odds of 1.97 and 2.34, respectively. The effect of EPE on peanut SPT sensitization was further augmented in children with a history of severe AD; however, this was not the case for PA, which might be due to the smaller sample size of this group. There was a significant interaction between EPE and the history and severity of AD for peanut SPT sensitization, with a trend toward an AD-EPE interaction for PA. Given that peanut sensitization and allergy are more common in children with a history of AD,[5,6] these data suggest that environmental exposure to peanut through an impaired skin barrier is a plausible route for peanut sensitization and allergy. The relationship between peanut protein in the infants' bed and peanut SPT sensitization and PA is discussed in this article's Online Repository at www.jacionline.org.

The egg-induced SPT wheal diameter was also associated with peanut SPT sensitization and PA. Egg allergy is known to be a strong predictor of peanut sensitization and allergy.[28] The cow's milk–induced SPT wheal diameter was also associated with peanut SPT sensitization and PA; however, it lost significance on multivariate analysis for peanut SPT sensitization, and this might just be another marker of atopy. Environmental exposure to peanut was not a risk factor for egg SPT sensitization or milk SPT sensitization, confirming the specificity of environmental peanut levels on peanut sensitization rather than food sensitization in general. Nonwhite ethnicity (black, Asian, and other nonwhite races combined) was associated with a peanut sIgE level of 5 kU_A/L or greater but not peanut SPT sensitization. This supports the findings of the Learning Early About Peanut (LEAP) study, in which black race was associated with a higher peanut sIgE level but a lower peanut SPT response in the baseline screening data from the LEAP study.[28]

FLG null mutations were not associated with peanut sensitization or PA. This differs from previous published findings; children with 1 of more *FLG* null mutations were found to have an increased risk of challenge-proven peanut allergy in white individuals from 4 different populations (United Kingdom, Irish, Dutch, and Canadian).[29] The lack of association with *FLG* genotype might be because in CoFAR children already had a 92.5% history of AD and a 54.3% history of severe AD; thus the skin barrier was already impaired, irrespective of whether children had *FLG* null mutations. In addition, the rate of *FLG* null mutations was surprisingly low in this cohort (14.9%) given the high rate and severity of AD; previous studies have shown that *FLG* null mutations are present in up to 56% of children with moderate-to-severe AD.[21,30] This might reflect a more varied genetic background in the white American population.[31] Another potential explanation for the low *FLG* mutation rate in this cohort is that 104 children with known PA or peanut sIgE >5 kU_A/L were excluded from the CoFAR study before enrollment. If these children had been included, we would have expected a higher rate of *FLG* null mutations, given the known association between peanut allergy and *FLG* null mutations.[29] A further explanation could be that in children with cow's milk and egg allergy (one of the inclusion criteria for the CoFAR observational cohort), exposure to cow's milk or egg allergens through breast milk or small quantities in food might have led to more severe AD.

Previously, the CoFAR study showed that frequent (≥2 times weekly) maternal peanut consumption during the last trimester of pregnancy was a risk factor for a peanut sIgE level of 5 kU_A/L or greater (OR, 2.9; 95% CI, 1.7-4.9).[32] In the subgroup of children with available dust samples (n = 359), maternal peanut consumption during pregnancy (any trimester) was associated with peanut SPT sensitization (adjusted OR, 2.66; 95% CI, 1.18-5.99) but not with a peanut sIgE level of 5 kU_A/L or greater ($P > .3$); frequent maternal peanut consumption (≥2 times weekly) in the last trimester showed only a trend toward an association with a peanut sIgE level of 5 kU_A/L or greater ($P = .15$). The lack of significance for this might be due to the smaller sample size of children with available dust; however, maternal peanut consumption during pregnancy might simply be an indirect marker of EPE. Levels of peanut protein in living room dust were significantly greater in households in which mothers

TABLE IV. Stratified LR analysis of the effect of 4 $\log_2$ EPE units on peanut SPT sensitization and PA in all children, children with a history of AD, and children with a history of severe AD

	All participants			Participants with history of AD			Participants with history of severe AD		
	No‡	OR (95% CI)	*P* value	No.	OR (95% CI)	*P* value	No.	OR (95% CI)	*P* value
Peanut SPT sensitization*	292	1.71 (1.13-2.59)	**.01**	269	1.97 (1.26-3.09)	**<.01**	158	2.41 (1.30-4.47)	**<.01**
Likely PA†	209	2.10 (1.20-3.67)	**<.01**	192	2.34 (1.31-4.18)	**<.01**	114	2.05 (0.98-4.29)	.06

Subject factors and EPE values are significant at the 5% level (in boldface).
*Adjusted for parental report of hay ever in the child, egg SPT wheal diameter, maternal peanut consumption during pregnancy, and breast-feeding.
†Adjusted for ethnicity, egg, and milk SPT wheal diameter.
‡The sample size was reduced from 359 to 292 (peanut SPT sensitization) and 209 (likely PA) because of missing data for some factors in the multivariate analysis.

consumed peanuts during pregnancy (median, 45.2 μg/g; interquartile range [IQR], 17.5-161.8 μg/g) versus households in which mothers avoided peanuts during pregnancy (median, 16.6 μg/g; IQR, 4.3-72.2 μg/g; $P = .001$). A prospective study would be required in which maternal peanut consumption during pregnancy was controlled and household peanut consumption was subsequently compared with peanut protein in household dust throughout early childhood to tease out the effect of maternal peanut consumption during pregnancy and EPE during infancy.

The limitations of this study included missing living room dust samples in 153 (30%) of 512 participants, which might have introduced an element of bias. A serologic diagnosis of PA (sIgE, ≥5 kU_A/L) rather than one based on oral food challenges was used, which meant that 150 children were excluded because of uncertainty about their peanut allergy outcome; this could also have introduced bias. Children with known peanut allergy were excluded at baseline; these children might have had even higher peanut protein levels in living room dust and thus even steeper predictive probability curves for peanut sensitization and PA. Subjects recruited who did not have moderate-to-severe AD had either cow's milk or egg allergy; this might have led to an unusual association between EPE and peanut SPT sensitization or PA in children with no history of AD. The dust sample obtained was a single baseline collection from one area of the home and thus might be prone to variation; however, previous studies have shown high within-home correlation of peanut protein levels in dust, and peanut protein levels from a single dust collection have been shown to correlate strongly with household peanut consumption over the previous 6-month period.[15] Peanut protein levels in dust from the living-room floor were positively correlated with those found in the infants' bed (see Fig E2 in this article's Online Repository at www.jacionline.org). There was no detailed assessment of infant peanut consumption, which could potentially protect a child from high EPE, as per the findings of Fox et al,[12] who showed that children who consumed peanut in the first year of life were not affected by high household peanut consumption. Animal data suggest that oral allergen exposure prevents induction of allergy,[33] whereas epicutaneous exposure prevents induction of oral tolerance.[8] The role of early high-dose peanut consumption on the prevention of peanut allergy is currently being investigated[28] but has already been suggested in cross-sectional observational studies.[34]

In summary, these findings demonstrate a positive association between exposure to peanut protein in dust and peanut SPT sensitization and PA in atopic children. The effect of EPE on peanut sensitization and PA was augmented in children with a history of AD and severe AD for peanut sensitization after adjusting for other covariates. This provides biological plausibility that EPE might be sensitizing children through an impaired skin barrier, thus supporting the hypothesis of epicutaneous sensitization. We demonstrated the specificity of EPE on peanut SPT sensitization and PA by showing that EPE does not increase the risk of egg or cow's milk SPT sensitization; however, it would be interesting to assess the effect of other food allergens in dust and respective sensitization and allergy to these foods. Routes of exposure to food antigens appear to be crucial in determining whether food allergy or tolerance develops as per the dual-allergen exposure hypothesis.[35,36] Although early consumption of food will inevitably lead to higher environmental exposure to foods, there are currently studies in place assessing the role of oral tolerance induction in young children (www.leapstudy.co.uk and www.eatstudy.co.uk); should these strategies fail to prevent the development of food sensitization and allergy, the alternative strategy of reducing environmental exposure to food allergens could be considered.

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Key messages

- **Increased environmental exposure to peanut protein is associated with an increased risk of sensitization and likely allergy to peanut in atopic children.**
- **The effect of peanut dust exposure on peanut sensitization is augmented in children with a history of and increasing severity of AD.**
- **The data are consistent with the hypothesis that allergic sensitization to peanut occurs through an impaired and inflamed skin barrier.**

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METHODS

EPE was also quantified from dust collected at baseline from the infant's bed. Families were asked to avoid changing their infant's bed sheet for 3 days before obtaining dust. Peanut protein levels in bed dust versus living room dust (in micrograms per gram) were compared by using the Mann-Whitney *U* test and the Spearman rank correlation coefficient *(r_s)*; additionally, Pearson correlation was used to compare $\log_2$ transformed peanut protein levels (in micrograms per gram) in dust from the infant's bed versus living room dust.

RESULTS

There were only 173 (33.8%) of 512 infant bed dust samples available for peanut protein analysis in the CoFAR observational study. This was because of a large proportion of homes in which no or less than 5 mg of dust was obtained from the infant's bed. Median peanut protein concentrations in the infant's bed (10.7 μg/g; IQR, 2.5-42.38 μg/g) were also significantly lower than peanut protein levels obtained from living room dust, where the concentration was approximately 4 times higher (39.1 μg/g; IQR, 13.4-150.60 μg/g; $P < .001$). However, peanut protein levels in the infant's bed and living room floor were positively correlated (n = 138; $r_s = 0.52$; 95% CI, 0.39-0.63; $P < .001$; see Fig E2).

There was a trend toward a significant association between bed dust EPE values and peanut SPT sensitization on univariate analysis (n = 172; OR, 1.08; 95% CI, 0.97-1.21; $P = .16$); however, this was lost on multivariate analysis (n = 132; OR, 1.05; 95% CI, 0.92-1.20; $P = .49$) adjusting for ethnicity, egg SPT wheal diameter, and cow's milk SPT wheal diameter. There was no significant association between infant bed dust EPE and PA on univariate LR analysis for EPE (n = 109; OR, 0.93; 95% CI %, 0.80-1.06; $P = .27$). There was no interaction between AD or AD severity and bed dust EPE on peanut SPT sensitization or likely allergy.

DISCUSSION

The lack of association between peanut protein levels in infant bed dust and peanut sensitization/PA is not surprising because peanut protein levels in bed dust correlate best with individual peanut consumption[E1] and most infants recruited to CoFAR would not have been eating peanut at this stage. This is reflected in the lower median peanut protein concentration found in the infant's bed compared with that in living room dust. The living room is the area that reflects the passage of most members of the family and thus the contribution of household peanut consumption to EPE. Furthermore, with the low number of dust samples and complete data sets we had using infant bed dust samples, we were underpowered to show an effect.

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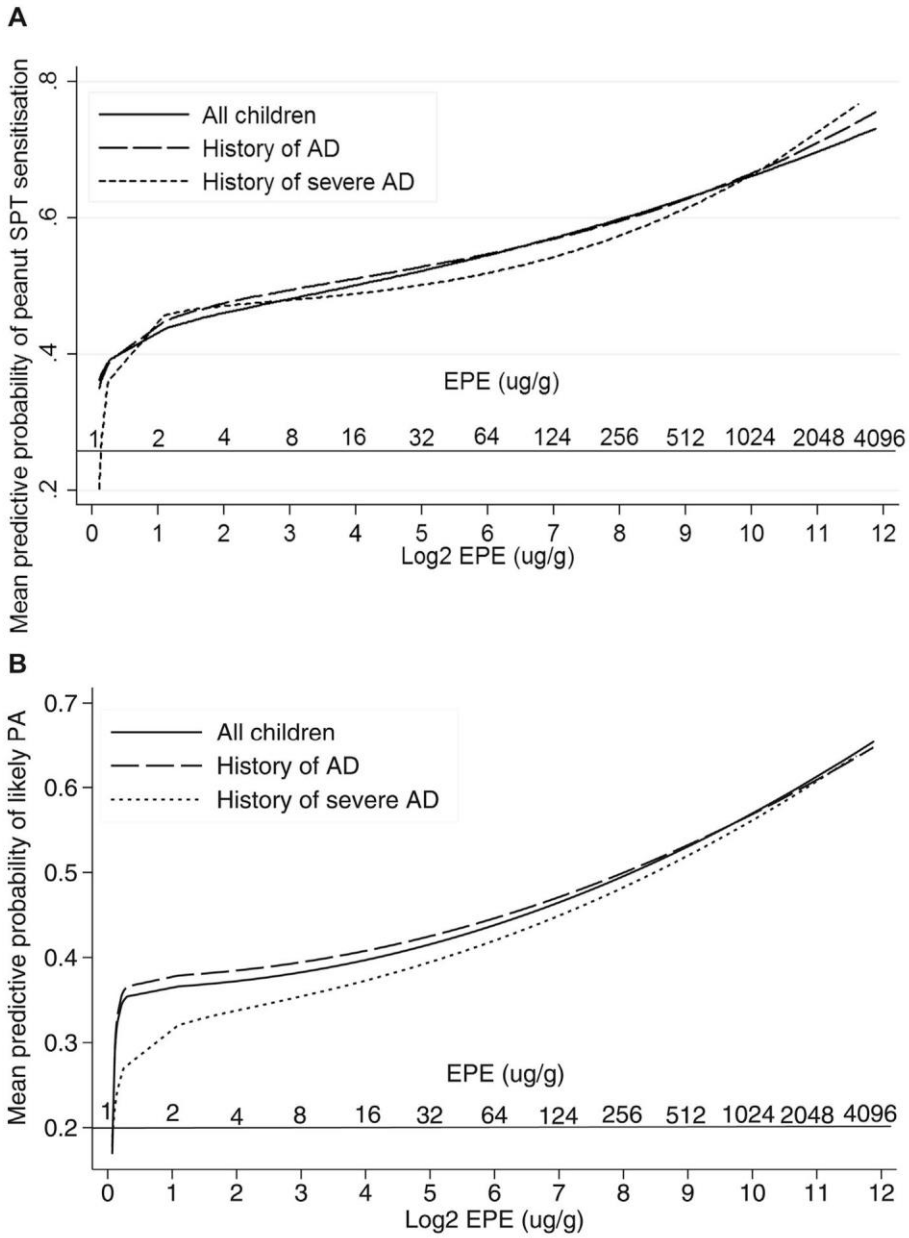

FIG E1. A, Multivariate stratified predictive probability for the effect of EPE (displayed in $\log_2$ [microgram per gram] units and untransformed [microgram per gram] units) for peanut SPT sensitization in all children, children with a history of AD, and children with a history of severe AD. Results are adjusted for egg SPT wheal diameter, hay fever, maternal peanut consumption during pregnancy, and breast-feeding. **B,** Multivariate stratified predictive probability for the effect of EPE (displayed in $\log_2$ [microgram per gram] units and untransformed [microgram per gram] units) for peanut SPT sensitization in all children, children with a history of AD, and children with a history of severe AD. Results are adjusted for egg and milk wheal diameter and ethnicity.

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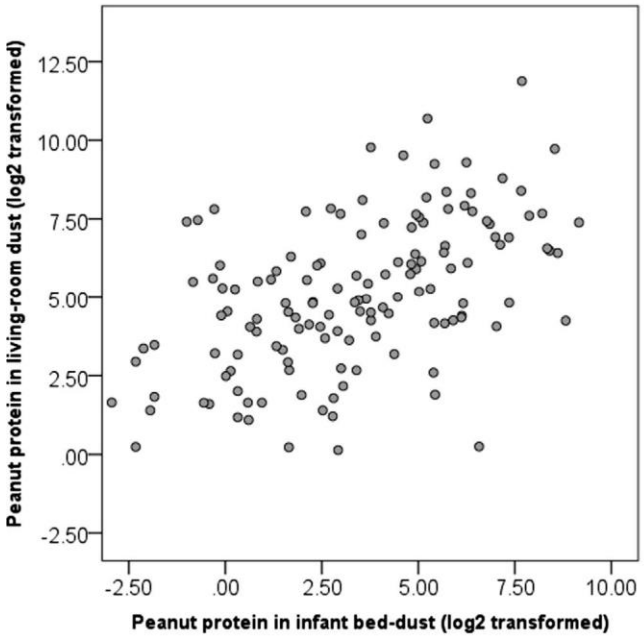

FIG E2. Scatter plot of peanut protein concentration in bed versus living room dust. The Spearman tank correlation coefficient *(r_s)* was 0.521 ($P < .001$).

TABLE E1. CoFAR demographics from the included group (n = 359) with available living room dust versus the excluded group (n = 153) and whole cohort (n = 512)*

	Included group	Variable included group	Percentage of variable included group	Excluded group	Variable excluded group	Percentage of variable excluded group	*P* value	Whole CoFAR cohort	Variable whole cohort	Percentage of variable in cohort
Peanut SPT sensitization ≥3 mm	359	196	54.6%	153	80	52.3%	.38	512	276	53.9%
Peanut sIgE ≥0.35 kU/mL	359	214	59.6%	153	91	59.5%	.96	512	305	59.6%
Likely PA (sIgE ≥5 kU/ml)	209	89	42.6%	99	45	45.5%	.40	308	134	43.5%
History of infantile AD	359	332	92.5%	153	139	90.8%	.28	512	471	92.0%
Maximum AD severity before study entry										
No AD (0)	359	27	7.5%	153	14	9.2%	.28	512	41	8.0%
Mild (3-4)	359	35	9.7%	153	17	11.1%	.41	512	52	10.2%
Moderate (5-6)	359	102	28.4%	153	47	30.7%	.34	512	149	29.1%
Severe (7-9)	359	195	54.3%	153	75	49.0%	**.04**	512	270	52.7%
Nonwhite ethnicity	359	81	22.6%	153	51	33.3%	**<.01**	512	380	74.2%
FLG null mutation (excluding nonwhite subjects)	275	41	14.9%	101	15	14.9%	.98	376	56	14.9%
Parental report of hay fever ever in the child	353	31	8.8%	148	11	7.4%	.33	501	42	8.4%
Male sex	357	240	67.2%	153	103	67.3%	.97	512	345	67.4%
Maternal history of atopy or asthma	356	243	68.3%	153	102	66.7%	.52	509	345	67.8%
Paternal history of atopy or asthma	350	217	62.0%	150	89	59.3%	.31	500	306	61.2%
Maternal history of AD	245	87	35.5%	97	28	28.9%	**.02**	342	115	33.6%
Paternal history of AD	215	71	33.0%	85	27	31.8%	.69	300	98	32.7%
Peanut consumption during pregnancy	352	295	83.8%	152	124	81.6%	.28	504	419	83.1%
Peanut consumption during breast-feeding	299	179	59.9%	133	64	48.1%	**<.01**	432	243	56.3%
Peanut present in house while breast-feeding	358	271	75.7%	153	107	69.9%	**.02**	511	378	74.0%
Older siblings	359	148	41.2%	153	54	35.3%	**.02**	512	202	39.5%
Egg SPT wheal diameter, median (IQR)	358	7.0 (4.0-10.5)		153	7.5 (4.3-11.0)		.32	511	7.5 (4.0-10.5)	
Cow's milk SPT wheal diameter, median (IQR)	356	5.0 (0.0-9.0)		155	4.0 (0.0-9.0)		.69	511	5.0 (0.0-9.0)	
Breast-feeding duration (mo), median (IQR)	359	5.0 (1.0-9.0)		153	5.0 (2.0-9.0)		.15	512	5.0 (2.0-9.0)	
Maternal age at baseline (y), median (IQR)	357	32.0 (28.0-35.0)		153	32.0 (28.5-35.0)		.78	510	32.0 (28.0-35.0)	
Child's age at baseline assessment (mo), median (IQR)	359	9.0 (7.0-12.0)		153	9.0 (7.0-12.0)		.64	512	9 (7.0-12.0)	
EPE (μg/g) in living room dust	359	39.1 (13.4-150.6)		153	Not available			359	39.1 (13.4-150.6)	
EPE (μg/g) in infant bed dust	172	10.7 (2.5-42.8)		340	Not available			172	10.7 (2.5-42.8)	

Statistically significant values ($P < .05$) are shown in boldface.

*Numbers and percentages of count data or medians (IQRs) of continuous factors and EPE values are shown.

7.9 BAMSE study results

7.9.1 Demographics and clinical characteristics

Demographic and clinical characteristics included are described in Table 8. Median peanut protein [IQR] in dust was 4.07µg/gram [1.58, 11.86]. Peanut sIgE sensitization was 6.6% at 4 years (n=103/1572) and 8.6% at 8 years (n=161/1876). Peanut CRD sensitization (Ara h 1,2 or 3≥0.35kU/L) at 8 years was 4.1% (n=75/1854), of which 74/75 (99%) had Ara h 2 ≥0.35kU/L.

Table 8: Demographics of BAMSE cohort with dust and *FLG* genotyping

	Number of cases	No. data points	Percentage
Peanut sIgE ≥ 0.35kU/L at 4 years	103	1572	6.55%
Peanut sIgE ≥ 0.35kU/L at 8 years	161	1876	8.58%
Ara h 1, 2 or 3 ≥0.35kU/L at 8 years vs. Ara h 1, 2 or 3 <0.35kU/L or peanut sIgE <0.35kU/L (excluding peanut sIgE ≥0.35kU/L with no components)	75	1854	4.05%
Avoiding peanut because of previous adverse reaction	73	1878	3.89%
History of infantile atopic eczema	313	1840	17.01%
At least one *FLG* mutation (R501X, 2282del or R2447x)	134	1878	7.14%
Allergic rhinitis (ISAAC definition) (age 4 years)	215	1828	11.76%
Allergic rhinitis (ISAAC definition) (age 8 years)	288	1865	15.44%
Egg sIgE≥ 0.35IU/ml at 4 years of age	82	1575	5.21%
Male sex	973	1871	52.00%
Caucasian ethnicity	1677	1842	91.04%
Biological siblings	1695	1870	90.64%
Parental atopy – (asthma/AR/AD)	838	1858	45.10%
Maternal age (mean, SD)	30.9 (SD4.49)	1871	N/A
Peanut protein in maternal bed-dust (µg/g): median [IQR]	4.07 [1.58-11.86]	1878	N/A
Exclusive breastfeeding duration (months): mean (SD)	5.19 (2.43)	1838	N/A
Total breastfeeding duration (months): mean (SD)	8.73 (3.31)	1825	N/A

7.9.2 Nested case control analysis for peanut sIgE and CRD sensitization

Peanut sensitized children were compared against children without PS matched for sex and parental atopy as described in the statistical methods; EPE was a risk factor for both peanut sIgE sensitization at 4 years and peanut CRD sensitization at 8 years with a 23% and 29% increased risk of PS per natural log (*ln*) unit increase in EPE respectively (Table 9). In children who were peanut sIgE sensitized but not peanut CRD sensitized, there was only a trend towards an association between EPE and PS (OR=1.20, 95% CI: 0.97-1.48, *P*=0.09). Differential relationship between EPE and primary peanut CRD sensitization (Ara h 1,2 or 3 ≥0.35kU/L) and non-clinically significant PS (peanut sIgE ≥0.35kU/L but negative results for Ara h 1,2 or 3<0.35kU/L) is displayed in Figure 24.

Figure 24: Mean predictive probability of peanut CRD sensitization versus non-clinically significant PS as a function of EPE

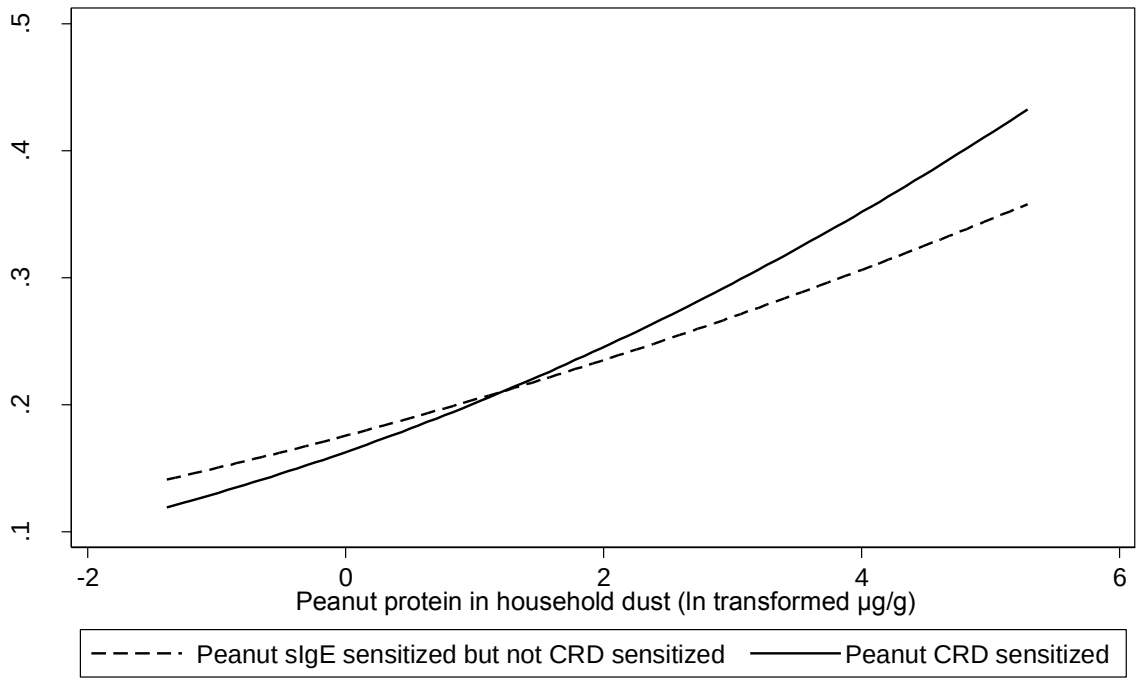

Table 9: Univariate conditional LR incorporating matching for peanut sensitized cases versus non-sensitized controls.

Peanut sIgE sensitization (≥0.35kU/L) at age 4 years and peanut CRD sensitization (Ara h 1, 2 or 3 ≥0.35kU/L) at age 8 years.

	Peanut sIgE sensitized at age 4 years (n=411)			Peanut CRD sensitized at age 8 years (n=195)		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
EPE (µg/gram ln transformed)	1.23	1.06-1.43	**<.01**	1.29	1.04-1.61	**.02**
No. of biological siblings	1.38	0.63-3.03	.42	2.50	0.77-8.16	.13
≥1 *FLG* loss-of-function mutation (excluding non-Caucasians)	2.67	1.18-6.04	**.02**	3.00	0.84-10.75	.09
History of infantile eczema	7.35	4.20-12.83	**<.001**	6.39	2.95-13.83	**<.001**
Egg sIgE sensitization: 4 years	18.91	7.50-47.66	**<.001**	12.85	3.78-43.67	**<.001**
Allergic rhinitis: 4 years (ISAAC)	3.44	1.90-6.23	**<.001**	2.53	1.04-6.18	**.04**
Allergic rhinitis: 8 years (ISAAC)	11.44	5.68-23.07	**<.001**	12.97	4.54-37.05	**<.001**
Exclusively breastfed (months)	0.95	0.88-1.03	.22	0.98	0.87-1.11	.77
Breastfed (excl/partial) (months)	0.99	0.93-1.05	.74	1.01	0.93-1.11	.76
Maternal age at baseline (in years)	0.99	0.94-1.04	.65	1.05	0.99-1.12	.12

Median peanut protein in dust was higher in peanut sIgE sensitized children (3.39 µg/g, IQR 1.41-11.01, n=137) than non-peanut IgE sensitized controls (2.28µg/g, IQR 0.88-5.14, n=274) ($P<.01$); and higher in peanut CRD sensitized children (4.64µg/g, IQR 1.58-12.77, n=65) than non-peanut CRD sensitized controls (2.24µg/g, IQR 0.88-4.74, n=130) ($P<.01$).

On multivariate conditional LR analysis, EPE was significantly associated with peanut sIgE and peanut CRD sensitization with a 1.41- and 2.1-fold increased risk per unit increase EPE respectively (Table 10). Infantile eczema and egg sensitization were both significantly associated with peanut sIgE and CRD sensitization. *FLG* loss-of-function mutations increased the risk of peanut sIgE sensitization 3.78-fold and peanut CRD sensitization 7.33-fold. Allergic rhinitis at age 4 and 8 years was associated with both peanut sIgE at age 4 years and peanut CRD sensitization at age 8 years in the multivariate analysis. The number of biological siblings, duration of exclusive and total breastfeeding and maternal age at baseline were not risk factors for PS. There was no significant interaction between EPE and infantile eczema or egg sensitization on peanut CRD sensitization, and only a slight trend for an interaction with *FLG* loss-of-function mutations (OR=3.28, 95% CI: 0.51-21.29, $P=0.21$).

Table 10: Multivariate conditional LR incorporating matching for peanut sensitized cases versus non-sensitized controls.

Peanut sIgE sensitization (≥0.35kU/L) at age 4 years and peanut CRD sensitization (Ara h 1, 2 or 3 ≥0.35kU/L) at age 8 years. Non-Caucasians were excluded due to inclusion of *FLG* mutations as a covariate.

	Peanut sIgE sensitized at age 4 years (excluding non-Caucasians) (n=237) *AIC: 90.78			Peanut CRD sensitized at age 8 years (excluding non-Caucasians) (n=143) *AIC: 49.19		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
EPE (µg/gram ln transformed)	1.41	1.05-1.90	**.02**	2.11	1.38-3.22	**.001**
≥1 *FLG* loss-of-function mutation	3.78	0.93-15.36	.06	7.33	1.21-44.21	**.03**
History of infantile eczema	2.53	0.98-6.51	**.05**	3.93	1.03-15.00	**.05**
Egg sIgE sensitization: 4 years	28.00	5.24-149.39	**<.001**	39.9	1.47-1081.68	**.03**
Allergic rhinitis: 8 years (ISAAC)	12.57	4.164-37.98	**<.001**	28.93	3.77-221.61	**<.001**

*Smaller AIC is better

7.9.3 Whole cohort analysis for peanut sIgE and CRD sensitization

On univariate (Table 11) and multivariate analysis (Table 12, p235) infantile eczema, egg sensitization, *FLG* loss-of-function mutation (trend for CRD) and allergic rhinitis at age 8 years were associated with peanut sIgE and CRD sensitization; however, EPE was no longer associated with PS. There was no interaction between EPE and *FLG* mutations, infantile eczema, egg sensitization or parental atopy on PS. On subgroup analysis peanut CRD sensitized children were compared against children who were sensitized to egg (sIgE≥0.35kU/L at age 4 years) but did not go on to develop peanut CRD sensitization.

Table 11: Factors associated with peanut sIgE sensitization at age 4 and 8 years and peanut CRD sensitization at age 8 years.

	Univariate GEE for peanut sIgE sensitization at 4 and 8 years			Univariate peanut CRD sensitization at age 8 years		
	OR	95% CI	P-value	OR	95% CI	*P*-value
EPE (µg/gram ln transformed)	0.95	0.86-1.06	.39	1.01	0.86-1.19	.89
Sex	1.14	0.83-1.56	.43	1.41	0.88-2.26	.15
Full older siblings	1.08	0.63-1.85	.77	0.86	0.40-1.81	.68
≥1 *FLG* loss-of-function mutations (excluding non-Caucasians)	1.85	1.08-3.16	**.03**	1.76	0.82-3.77	.15
History of infantile eczema	6.49	4.66-9.02	**.001**	7.94	4.91-12.83	**<.001**
Egg sIgE sensitization at 4 years	13.58	8.55-21.58	**<.001**	11.04	6.13-19.90	**<.001**
Allergic rhinitis aged 4 years (ISAAC)	2.82	1.92-4.13	**<.001**	2.16	1.22-3.83	**<.01**
Allergic rhinitis aged 8 years (ISAAC)	8.05	5.78-11.22	**<.001**	5.87	3.66-9.42	**<.001**
Parental atopy (asthma and/or hay-fever)	1.77	1.28-2.44	**<.001**	1.77	1.10-2.83	**.02**
Non-Caucasian ethnicity	1.58	0.97-2.57	.07	1.09	0.49-2.43	.79
Exclusively breastfed in months	0.98	0.94-1.06	.92	0.97	0.88-1.06	.49
Breastfed (exclusive/partial) in months	0.99	0.94-1.03	.57	0.97	0.91-1.04	.43
Maternal age at baseline (in years)	1.02	0.98-1.05	.44	1.02	0.97-1.07	.53

Table 12: Multivariate analysis of peanut sIgE sensitization aged 4 and 8 years.

Generalised estimating equations (GEE) used to account for repeated measures within individuals at 4 and 8 years and LR analysis for peanut CRD sensitization at age 8 years in the BAMSE cohort excluding non-Caucasians. Goodness of fit was assessed by QIC and AIC.

	Multivariate peanut sIgE sensitization (excluding non-Caucasians) (n=1388) QIC:1094			Multivariate peanut CRD sensitization (excluding non-Caucasians) (n=1375) AIC:767		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
EPE (µg/gram ln transformed)	0.98	0.84-1.13	.75	1.08	0.88-1.32	.48
≥1 *FLG* loss-of-function mutations	2.37	1.22-4.60	**.01**	1.82	0.72-4.57	.20
History of infantile eczema	3.81	2.44-5.94	**<.001**	5.51	3.03-10.03	**<.001**
Egg sIgE sensitization at 4 years	8.12	4.35-15.15	**<.001**	6.61	3.16-13.80	**<.001**
Allergic rhinitis aged 8 years (ISAAC)	5.57	3.57-8.71	**<.001**	3.05	1.65-5.63	**<.001**

EPE was a risk factor for peanut CRD sensitization on both univariate (OR=1.33 95% CI: 1.01-1.74, *P*<.05, n=134) (Table 13) and multivariate analysis (OR=1.56, 95% CI: 1.14-2.12, *P*<.01, n=132, AIC: 331) adjusting for allergic rhinitis at age 8 years and infantile eczema. Peanut levels in household dust were also significantly higher in peanut CRD sensitized children (n=75, median 4.79µg/g, IQR 1.63-12.00) versus egg sensitized, non-peanut CRD sensitized children (n=59, median 2.35µg/g, IQR 1.23-6.15) (*P*=.03). The impact of EPE on PS was compared against other high-risk groups of children who did not become peanut sensitized (*FLG* mutations, infantile eczema, parental atopy) and there was no significant association.

Table 13: Univariate LR analysis of factors associated with peanut CRD sensitization versus non peanut CRD sensitization in children with preceding egg sensitization.

	Peanut CRD sensitization at aged 8 years compared against children who are not peanut CRD sensitized patient at aged 8 years but who were sensitized to egg at 4 years (n=134)		
	OR	95% CI	*P*-value
EPE (µg/gram ln transformed)	1.33	1.01-1.74	**<0.05**
Sex	1.90	0.95-3.80	0.07
Full older siblings	0.61	0.17-2.13	0.44
≥1 *FLG* loss-of-function mutations (excluding non-Caucasians)	1.09	0.33-3.56	0.89
History of infantile eczema	2.51	1.24-5.08	**0.01**
Allergic rhinitis aged 4 years (ISAAC)	0.87	0.38-1.96	0.73
Allergic rhinitis aged 8 years (ISAAC)	2.65	1.26-5.56	**0.01**
Parental atopy (asthma and /or hay-fever)	1.34	0.67-2.65	0.41
Non-Caucasian ethnicity	2.55	0.93-6.98	0.07
Exclusively breastfed in months	0.89	0.77-1.04	0.14
Breastfed (exclusive/partial) in months	0.99	0.89-1.09	0.76
Maternal age at baseline (in years)	1.06	0.99-1.14	0.11

7.10 Publication 5: IL9 is a key component of memory Th cell peanut-specific responses from peanut allergic children

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IL-9 is a key component of memory T_H cell peanut-specific responses from children with peanut allergy

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Background: Differentiation between patients with peanut allergy (PA) and those with peanut sensitization (PS) who tolerate peanut but have peanut-specific IgE, positive skin prick test responses, or both represents a significant diagnostic difficulty. Previously, gene expression microarrays were successfully used to identify biomarkers and explore immune responses during PA immunotherapy.

Objective: We aimed to characterize peanut-specific responses from patients with PA, subjects with PS, and atopic children without peanut allergy (NA children).

Methods: A preliminary exploratory microarray investigation of gene expression in peanut-activated memory T_H subsets from 3 children with PA and 3 NA children identified potential PA diagnostic biomarkers. Microarray findings were confirmed by using real-time quantitative PCR in 30 subjects (12 children with PA, 12 children with PS, and 6 NA children). Flow cytometry was used to identify the T_H subsets involved.

Results: Among 12,257 differentially expressed genes, *IL9* showed the greatest difference between children with PA and NA children (45.59-fold change, $P < .001$), followed by *IL5* and then *IL13*. Notably, *IL9* allowed the most accurate classification of children with PA and NA children by using a machine-learning approach with recursive feature elimination and the random forest algorithm. Skin- and gut-homing T_H cells from donors with PA expressed similar T_H2- and T_H9-associated genes. Real-time quantitative PCR confirmed that *IL9* was the highest differentially expressed gene between children with PA and NA children (23.3-fold change, $P < .01$) and children with PS (18.5-fold change, $P < .05$). Intracellular cytokine staining showed that IL-9 and the T_H2-specific cytokine IL-5 are produced by distinct T_H populations.

Conclusion: In this study *IL9* best differentiated between children with PA and children with PS (and atopic NA children). Mutually exclusive production of IL-9 and the T_H2-specific cytokine IL-5 suggests that the IL-9–producing cells belong to the recently described T_H9 subset. (J Allergy Clin Immunol 2014;134:1329-38.)

Key words: *Peanut allergy, peanut sensitization, gene expression, real-time quantitative PCR, microarray, IL-9, T_H9, IL-5, IL-13, T_H2*

The incidence of peanut allergy (PA) has increased over the last decades so that its prevalence in childhood is currently estimated at 1.4%[1] in the United States and reaches 1.8% in the United Kingdom.[2,3] Because PA is rarely outgrown and is responsible for a significant proportion of severe anaphylactic reactions to foods, it represents a major population health concern.[4]

Mechanistically, PA is driven by T_H cells, the determinant role of which was confirmed by the observation that allergy can be transferred from donors with PA to children without peanut allergy (NA children) through solid organ transplantation, which entails T_H cell transfer.[5] Conversely, PA resolution has been observed after bone marrow transplantation.[6] We[7] and others[8,9] showed that the *in vitro* peanut-specific T_H cell response in patients with PA is dominated by a T_H2-polarized population characterized by the production of T_H2 cytokines, such as IL-4, IL-5, and IL-13. However, we also observed that a significant number of *in vitro* peanut-responding T_H cells did not produce T_H2 (nor T_H1-specific) cytokines, even when restimulated with phorbol ester and calcium ionophore, suggesting that they might belong to distinct T_H cell subsets.[10]

Indeed, the discovery of regulatory T (Treg), T_H17, T_H22, and, more recently, T_H9 cells shows that T_H cell responses go beyond the binary T_H1/T_H2 paradigm.[11] Treg cells suppress IgE production[12] and block mast cell, basophil, and eosinophil activation,[13] whereas in mouse models of asthma, T_H17 cells increase eosinophilia and IgE production.[14] T_H9 was recently identified as a distinct T_H cell subset induced by the combination of IL-4 and TGF-β.[15] IL-9, which is the T_H9 subset–defining cytokine, is a mast cell growth factor that increases cytokine production in activated mast cells and enhances IL-4–driven IgE production by B cells. IL-9 is highly expressed in the lungs of asthmatic human subjects,[16] and in an experimental mouse model neutralizing anti–IL-9 antibodies were shown to ameliorate T_H9-mediated asthma.[17,18]

In the present study we aimed to further dissect the peanut-responding T_H cell subsets by separating the skin-homing T_H

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Abbreviations used
B7: $\alpha_4\beta_7$ Integrin
CCL1: Chemokine (C-C motif) ligand 1
CISH: Cytokine-inducible SH2-containing protein
CLA: Cutaneous lymphocyte antigen
FACS: Fluorescence-activated cell sorting
ICCS: Intracellular cytokine staining
IL17RB: IL-17 receptor B
IQR: Interquartile range
IRF4: Interferon regulatory factor 4
NA: Without peanut allergy
OVA: Ovalbumin
PA: Peanut allergy
PCA: Primary component analysis
PS: peanut sensitization
RF: Random forest
RQ: Relative quantitation
RT-qPCR: Real-time quantitative PCR
Treg: Regulatory T

cells, which express the cutaneous lymphocyte antigen (CLA),[19] and the gut-homing memory T_H cells, which express the $\alpha_4\beta_7$ integrin (B7) gut-homing marker, respectively.[20] Skin exposure to peanut antigens after skin stripping to mimic an eczema phenotype leads to strong T_H2-skewed responses in mice, supporting the concept of epicutaneous sensitization.[21]

Furthermore, we recently showed that peanut-specific proliferation is quantitatively higher in the skin-homing than gut-homing T_H cells in patients with PA.[22] Conversely, oral exposure to antigens underlies oral tolerance and is associated with higher levels of antigen-specific proliferation of gut-homing T cells.[20] This has led to the dual-allergen exposure hypothesis being proposed in that the timing and balance of cutaneous versus oral exposure to an allergen might determine whether the child has allergy or tolerance.[23]

In this study we aimed to assess differential gene expression in PBMCs cultured with peanut from children with PA, children with PS, and NA children in both skin- and gut-homing T_H cells and fluorescence-activated cell sorting (FACS) analysis of intracellular cytokine production.

METHODS

Study participants

Donors with PA, donors with PS, and atopic NA donors were recruited from a tertiary referral allergy center at St Thomas Hospital Children's Allergy Unit, London, United Kingdom. Informed consent was obtained before participation. Ethical approval for this study was obtained from the St Thomas Research Ethics Committee (reference 10/H0802/45). Three children with PA and 3 atopic NA children were recruited for gene expression analysis, and 36 microarrays were carried out on various T-cell populations derived from their peanut-stimulated and unstimulated PBMCs (as negative controls). Participants recruited for the gene microarray work were all male to avoid differential gene expression linked to sex. Forty patients recruited for subsequent real-time quantitative PCR (RT-qPCR) and FACS analysis did not have sex as a selection criterion. PA was defined as having a convincing history of an immediate hypersensitivity reaction after exposure to peanut and a skin prick test response to peanut extract of 8 mm or greater or a peanut-specific IgE level of 15 kU/L or greater.[24] Peanut tolerance was defined as regular peanut consumption (ie, eating an age-appropriate amount of peanut in 1 meal without any allergic symptoms within the last month); some of these subjects had PS, as defined by an SPT response of 3 mm or greater or a peanut-specific IgE level of 0.35 kU/L or greater. If there was no history of peanut consumption, children underwent a double-blind, placebo-controlled food challenge. The demographic and clinical characteristics of all study participants are described in Table E1 in this article's Online Repository at www.jacionline.org.

Details of the methodology are described in the Methods section in this article's Online Repository at www.jacionline.org.

Overview of methods

PBMCs from 3 children with PA and 3 NA children were cultured with peanut antigens for 18 hours. Subsequently activated ($CD69^+$) skin-homing (CLA^+) memory T_H cells ($CD4^+CD45RO$), and respectively activated ($CD69^+$) gut-homing ($B7^+$) memory T_H cells were sorted by means of FACS into Trizol LS (Life Technologies, Paisley, United Kingdom) (see Fig E1 in this article's Online Repository at www.jacionline.org). mRNA was extracted with the miRNeasy Mini kit (Qiagen, Manchester, United Kingdom), and cDNA was synthesized with the NuGEN Ovation Pico WTA system v2 kit (NuGEN, San Carolos, Calif). After data normalization and quality checks (see Figs E2 and E3 in this article's Online Repository at www.jacionline.org), differential gene expression between donors with PA and NA donors was determined by using the Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, Calif). Microarray results were analyzed with the Partek Genomics Suite (Partek, St Louis, Mo) and with an automatic classification approach, as currently used in the data-mining/machine-learning fields. Microarray findings were subsequently confirmed at a gene level by using RT-qPCR on whole PBMCs from 30 children (12 children with PA, 12 children with PS, and 6 NA children) and at a protein level by using FACS intracellular cytokine staining (ICCS) in 5 children with PA and 5 children with PS.

RESULTS

Microarray analysis of gene expression in peanut-activated memory T_H cells from donors with PA versus NA donors

The level of gene expression in peanut-activated memory T_H cells from donors with PA and atopic NA donors is displayed as a volcano plot by using the Partek Genomics Suite (Fig 1); the horizontal axis shows the fold-change difference of gene expression between children with PA and NA children, whereas the vertical axis displays the statistical significance of the difference of gene expression between children with PA and atopic NA children, taking into account gene expression variability within the children with PA and the NA children groups, respectively. The genes appearing in the top right area were upregulated in children with PA versus NA children; the most statistically significant differently expressed gene was *IL9* (45.59-fold change, $P < .001$), followed by the T_H2 gene cluster containing *IL5* and *IL13*. Conversely, the genes appearing in the top left area (eg, *SULT1B1* and *RGS18*) were upregulated in atopic NA donors.

Peanut-specific memory T_H cells from donors with PA express typical T_H2-specific genes, as well as T_H9-associated genes

A hierarchical clustering heat map of the data set (Fig 2) consisting of 48 differentially expressed genes selected through a filter criteria of at least 2-fold changes of expression with a P value of less than .05 was produced with the Partek Genomics Suite. There was notable differential expression between

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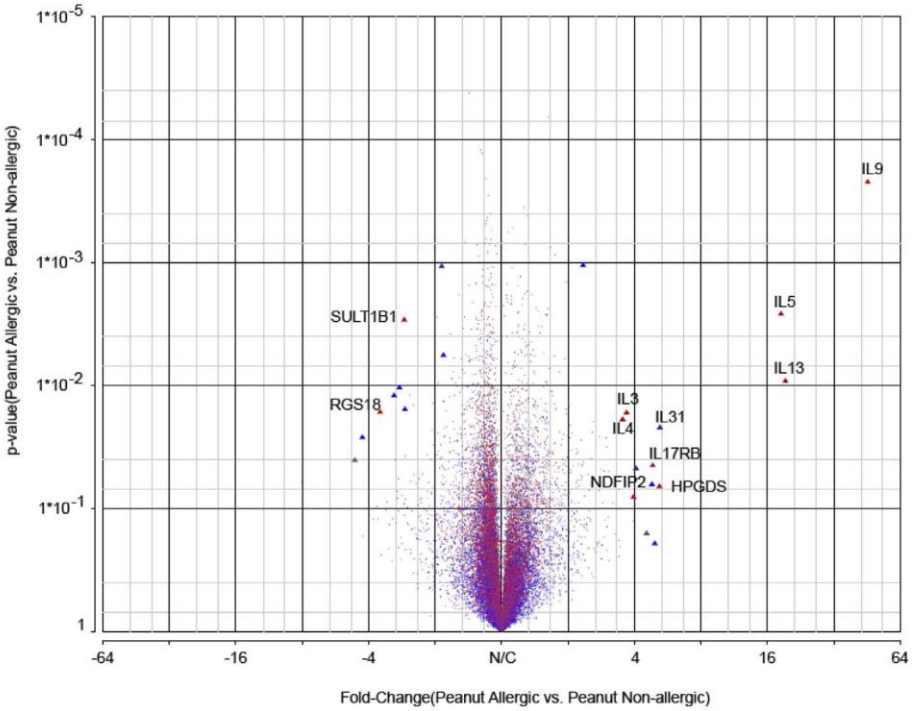

FIG 1. Volcano plot of gene expression fold change of significantly differentially expressed genes in peanut-activated memory T_H cells from 3 donors with PA and 3 NA donors. *Horizontal axis*, Fold-change differential gene expression between children with PA and NA children: positive values indicate higher expression in children with PA, and negative values reflect higher expression in NA children. *Vertical axis*, Statistical significance of differential gene expression between children with PA and NA children, adjusting for gene expression variability within the PA and NA groups, respectively. *Points* represent individual genes.

peanut-stimulated activated ($CD69^+$) T_H cells from donors with PA and NA donors for T_H2 signature genes (*IL4*, *IL5*, and *IL13*) and T_H9-associated genes (*IL9*, interferon regulatory factor 4 *[IRF4]*, and IL-17 receptor B *[IL17RB]*).[25] Chemokine (C-C motif) ligand 1 *(CCL1)*, *IL31*, and *IL3* were preferentially expressed in skin-homing T_H cells from donors with PA. Other genes differentially expressed in children with PA versus NA children and their functions and involvement in allergy are described in Table I.[25-40] Most genes preferentially expressed in NA children versus children with PA were as yet not functionally understood, and only *SULT1B1*, *GCNT4*, and *RGS18* could be identified (Table I).

ANOVA of genes expressed in T_H subsets from PA and NA children

To compare the basal nonstimulated level of gene expression in skin- and gut-homing peanut-activated memory T_H cells, we performed 36 microarrays (displayed in Table E2 in this article's Online Repository at www.jacionline.org) in 3 types of T_H cell subsets isolated from donors with PA and NA donors, respectively:

I. *Peanut activated*: $CD69^+$ T_H cells from PBMCs cultured in peanut protein;
II. *Peanut nonactivated*: $CD69^-$ T_H cells that were not activated but were isolated from the same PBMCs cultured with peanut protein (internal negative controls); and
III. *No peanut (unstimulated)*: $CD69^-$ T_H cells isolated from PBMCs to which no peanut antigen was added (external negative controls).

Differential gene expression of peanut-stimulated (P+) activated ($CD69^+$) T_H cells between children with PA and atopic NA children is displayed in Fig 3; we also include results for internal negative controls (peanut-stimulated and nonactivated [$CD69^-$] cells) and external negative controls (unstimulated and nonactivated [$CD69^-$] cells) in Fig E4 in this article's Online Repository at www.jacionline.org. There was differential gene expression of *IL9* and the associated genes *IL17RB* and *IRF4*, as well as classical T_H2 cytokines (*IL5*, *IL13*, and *IL4*) and cytokine-inducible SH2-containing protein *(CISH)* in donors with PA versus NA donors. *CCL1* and *IL31* levels were preferentially increased in skin-homing $CD69^+$ memory T_H2 cells from children with PA; however, T_H2/T_H9 cytokines were not expressed preferentially in skin- or gut-homing T_H cells. All external negative controls (unstimulated $CD69^-$ T_H cells) in donors with PA and NA donors had low expression of all genes assessed. There was very little background expression of *IL9*, *IL4*, *IL5*, *IL13*, *IL31*, and *CCL1* in internal negative controls (peanut-stimulated $CD69^-$ cells) in PA and NA donors; however, there was some background expression of *IL17RB*, *IRF4*, and *CISH* in peanut-stimulated $CD69^-$ T_H cells (see Fig E4).

IL9 allows differentiation between children with PA and NA children when used in the frame of a random forest classifier

We used a random forest (RF) algorithm to classify the peanut-stimulated activated ($CD69^+$) T_H cell responses in children with PA versus NA children. We built 250, 500, and 750 trees (analysis pathways); the number of analysis pathways is dictated by a

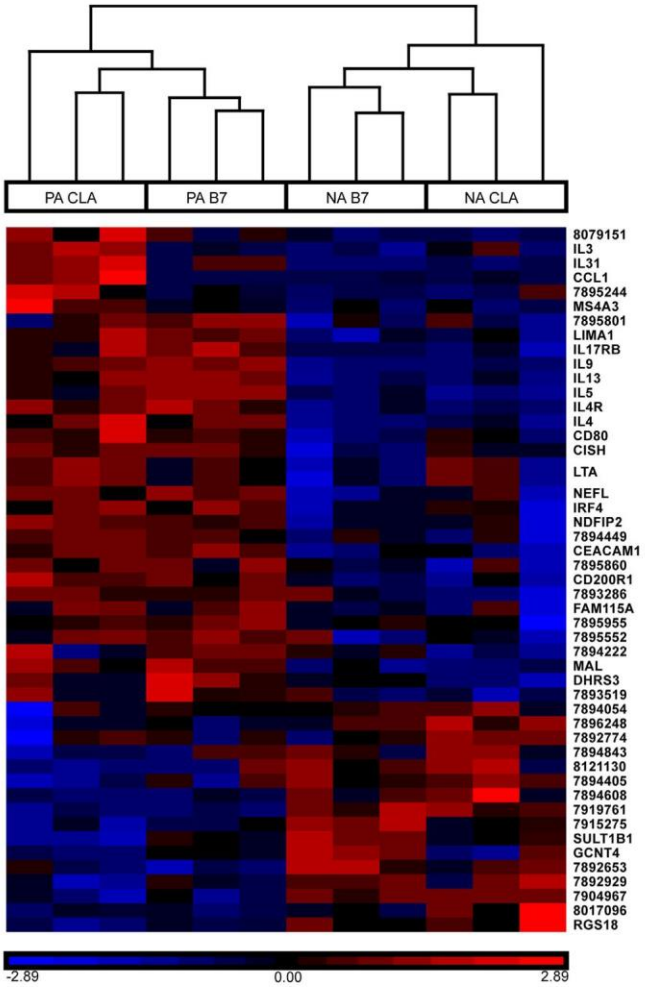

FIG 2. Heat map of 48 differentially expressed genes in skin- and gut-homing peanut-activated memory T_H cells from donors with PA (n = 3) and NA donors (n = 3; ≥2 fold changes of expression, $P < .05$). Each *column* represents T_H cell gene expression from an individual donor, and each *row* shows a single gene. Upregulated genes are red ("hot"), and downregulated genes are blue ("cold").

tradeoff between the efficiency (computing speed and memory consumption) of the classifier and the quality of its predictions. By using this approach, RF was able to differentiate between children with PA versus NA children with 100% accuracy, even when the gene selection was limited to sets of 10, then 5, and then only 2 genes. In this final analysis the 2 genes that are able to accurately distinguish between PA and NA samples were *IL9* and *HSPA5* for 250 trees, *IL9* and *8069610* for 500 trees, and *IL9* and *HUWE1* for 750 trees (Fig 4). From these sets of genes, *IL9* expression was the best discriminating gene between memory T_H cells from children with PA and NA children.

Differential gene expression of T_H2 and T_H9 cytokines between children with PA and NA children is confirmed by using RT-qPCR

Expression of *IL5* and *IL13* (signature genes for T_H2 responses) and *IL9* and *IRF4* (reflecting T_H9 responses), respectively, was higher in PBMCs isolated from donors with PA than in those from NA donors (Fig 5). Relative quantitation (RQ) for *IL9* was 25.40 (interquartile range [IQR], 4.09-65.33) in children with PA versus 1.09 (IQR, 0.96-2.10) in NA children, which equated to a 23.30-fold change in *IL9* expression. RQ for *IL5* was 61.73 (IQR, 4.29-167.87) in children with PA versus 2.86 (IQR, 1.80-4.97) in NA children (21.6-fold change), and RQ for *IL13* was 53.96 (IQR, 31.68-121.14) in children with PA versus 4.54 (IQR, 1.80-7.15) in NA children (11.89-fold difference). Thus *IL9* had the highest fold differential gene expression between donors with PA and NA donors. Significant differential gene expression (RQ) was also observed for other genes known to be involved in allergic responses, such as *IL31*, *CISH*, and *CCL1* ($P < .01$) and the T_H2/T_H9-specific transcription factor *IRF4* ($P < .05$). On RT-qPCR analysis, there was no significant differential *IL4* and *IL17RB* expression between donors with PA and NA donors.

Differential gene expression of T_H2 and T_H9 cytokines between children with PA and children with PS by using RT-qPCR

After the finding that *IL9* had the highest differential expression in children with PA versus NA children, we sought to determine whether this would also be a useful biomarker to differentiate between children with PA and children with PS (Fig 5). RQ for *IL9* was 25.40 (IQR, 4.09-65.33) in children with PA versus 1.37 (IQR, 1.21-3.88) in children with PS (18.54-fold change). RQ for *IL5* was 61.73 (IQR, 4.29-167.87) in children with PA versus 3.40 (IQR, 1.14-14.29) in children with PS (18.16-fold change), and RQ for *IL13* was 53.96 (IQR, 31.68-121.14) in children with PA versus 7.56 (IQR, 4.08-22.86) in children with PS (7.14-fold difference). Thus *IL9* also had the highest fold differential gene expression between donors with PA and donors with PS. *CCL1*, *CISH*, and *IL31* were also differentially expressed between donors with PA and donors with PS. One child with PS who was eating whole peanuts 1 to 2 times per month had higher *IL9*, *IL5*, and *IL31* gene expression levels, which is represented by the green points for these 3 RQ box plots (Fig 5).

Different T_H populations secrete IL-9 and IL-5 cytokines

ICCS of peanut-stimulated memory T_H cells demonstrated that IL-5 and IL-9 are produced by distinct T_H cell subsets (Fig 6 and see Table E3 in this article's Online Repository at www.jacionline.org); memory T_H cells from all donors with PA produced more IL-9 and IL-5 than those from children with PS, and the pattern of cytokine expression was mutually exclusive.

DISCUSSION

We used the well-established Affymetrix microarray method to determine gene expression profiles of memory T cells responding to peanut antigens *in vitro* to characterize differential gene expression and the different T_H cell subsets involved in PA. Microarray gene expression data found evidence of high *IL9* expression in activated memory T_H cells from donors with PA in addition to the expected T_H2 gene signature. Applying an RF algorithm, we found that *IL9* was the best gene classifier that allows differentiation between children with PA and NA children with 100% accuracy. Using RT-qPCR and FACS, we confirmed the presence of *IL9* at a gene and protein level and showed that *IL9* was the best gene to distinguish between children with PA and NA children

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TABLE I. List of genes that were significantly different ($P < .05$) between donors with PA and NA and outline of their known roles in allergic responses

Gene	*P* value	Biological functions of genes and involvement in allergy
IL9	.0002	*IL9* is produced by T_H9 cells, mast cells, and eosinophils; its main targets are T cells, B cells, and mast cells. TGF-β in the presence of *IL4* reprograms T_H2 cell differentiation and leads to the development of T_H9 cells, which produce IL-9. Thus IL-4 blocks the generation of TGF-β–induced Foxp3$^+$ Treg cells and induces T_H9 cells. *IL9* is involved in T_H2 inflammatory reactions, promotes the production of IL-4–induced IgE, induces chemokine and mucus secretion by bronchial epithelial cells, and leads to mast cell proliferation.[25]
IL5	.0024	*IL5* is an eosinophil and B-cell growth factor mainly produced by T_H2 cells, activated eosinophils, mast cells, CD8$^+$ Tc2 cells, NK, cells and NKT cells. *IL5* promotes proliferation, activation, differentiation, survival, and adhesion of eosinophils.[25]
SULT1B1	.0029	Sulfotransferase family cytosolic 1B, member 1, enzyme catalyzes the sulfate conjugation of hormones, neurotransmitters, and drugs.[26]
IL13	.0086	*IL13* is secreted by activated T_H2 cells, mast cells, basophils, eosinophils, and NKT cells. *IL13* targets are B cells, mast cells, epithelial cells, eosinophils, smooth muscle cells, and macrophages. IL-13 contributes to asthma and allergic rhinitis late-phase responses.[25]
IL4	.0134	*IL4* is produced mainly by T_H2 cells, basophils, eosinophils, mast cells, and a subset of NKT cells. The main *IL4* targets are T and B cells to induce the differentiation of antigen-stimulated naive T cells into T_H2 cells, increase the expression of class II MHC molecules in B cells, enhance expression of CD23, induce IgE class-switching, and upregulate expression of IL-4 receptor.[25]
LIMA1	.0135	LIM domain and actin binding 1 is a cytoskeleton-associated protein that inhibits actin filament depolymerization.[27]
IL31	.0137	*IL31* is a cytokine related to the IL-6–type cytokines. *IL31* is produced mainly by activated T_H2 cells and CD8$^+$ T cells. *IL31* expression is increased in skin biopsy specimens of patients with eczema and allergic contact dermatitis. Serum *IL31* levels are correlated with eczema severity.[25]
IL3	.0149	*IL3* is expressed by T cells, macrophages, stromal cells, NK cells, mast cells, and eosinophils. Among other functions, *IL3* also increases the activation and release of mediators from eosinophils and basophils in response to IgE FcεR cross-linking.[25]
CD200R1	.0153	Cell-surface glycoprotein CD200 receptor 1 is a receptor for the OX-2 membrane glycoprotein. Chronic infection drives expression of the inhibitory receptor CD200R and its ligand CD200 by mouse and human CD4 T cells.[36]
RGS18	.0176	Regulator of G-protein signaling 18 is a protein implicated in lymphocyte function by modulating signaling through 7-transmembrane receptors, in chemokine signaling and homing, and in differentiation and transformation.[28]
FAM115A	.0237	Family with sequence similarity 115, member A, is a protein with unknown function.[33]
IRF4	.0243	Interferon regulatory factor 4 regulates a large number of genes relevant to T_H cell differentiation.[29] IRF4 is also essential for the developmental program of T_H9 cells.[30]
CEACAM1	.0277	Carcinoembryonic antigen-related cell adhesion molecule 1 is a cell-cell adhesion molecule detected on leukocytes, epithelia, and endothelia. Its multiple cellular activities include apoptosis, tumor suppression, and modulation of innate and adaptive immune responses.[31]
IL4R	.0281	There are 2 types of IL-4–binding receptors: type I IL-4R, which predominates in hematopoietic cells and is responsible for IL-4 signaling in T cells, and type II IL-4R, which is expressed on hematopoietic and nonhematopoietic cells but not T cells.[25]
GCNT4	.0289	Glucosaminyl (N-acetyl) transferase 4 is an enzyme that mediates core 2 O-glycan branching, which is important in mucin-type biosynthesis.[32]
MAL	.0300	Myelin and lymphocyte protein has been localized to the endoplasmic reticulum of T cells and is a candidate linker protein in T-cell signal transduction. It is increased in house dust mite–stimulated T_H cells from atopic subjects.[37]
CCL1	.0337	Chemokine (C-C motif) ligand 1 is secreted by activated T cells and IgE-activated human and mast cells and binds to the CCR8 receptor. CCR8 is expressed by dendritic cells, monocytes, NK cells, and immature B cells and is associated with T_H2 cells.[38]
CISH	.0385	*CISH* controls IL-2 signaling and can be induced by IL-2, IL-3, GM-CSF, and eosinophil peroxidase in hematopoietic cells. *CISH* expression is increased in house dust mite–stimulated T cells from atopic subjects[37] and OVA-stimulated PBMCs from patients with egg allergy.[40]
IL17RB	.0410	*IL17RB* binds IL-17B and IL-17E (IL-25). IL-17 amplifies delayed-type reactions by increasing chemokine production to recruit monocytes and neutrophils to the site of inflammation.[34]
MS4A3	.0486	Membrane-spanning 4-domains subfamily A, member 3, likely plays a role in signal transduction in lymphoid cells. *MS4A3* levels are increased in patients with latex allergy, vegetable food allergy, or both.[35]

Genes are listed in order of statistical significance. All genes were upregulated in children with PA versus NA children, except *SULT1B1*, *GCNT4*, and *RGS18*, which were upregulated in NA children versus children with PA.
Foxp3, Forkhead box protein 3; *NK*, natural killer.

(23.3-fold difference), as well as between children with PA and children with PS (18.5-fold difference), thus making *IL9* a good biomarker for clinical PA in this study. The differential expression of T_H2 signature genes (*IL5* and *IL13*) and T_H9-associated genes (*IL9* and the transcription factor *IRF4*) in children with PA versus NA children, as well as the dichotomous pattern of *IL5* and *IL9* production in peanut-stimulated activated memory T cells suggests the involvement of both T_H2 and a distinct T_H9 subset in children with PA.

Jabeen and Kaplan[41] reviewed the recently discovered T_H cell subset T_H9, which produces IL-9 but not the other T_H2-defining cytokines, such as IL-5 and IL-13. T_H9 cells have been described in the inflamed skin of patients with atopic eczema[42] and in the bronchial mucosa in mouse models of allergic asthma,[16] and T_H9 is emerging as an important T-cell subset in human respiratory allergic disease.[15,41] *IL9* has previously been described as the top-ranking gene (from 1482 differentially expressed genes) for discriminating between atopic and nonatopic responses to house dust mite.[37] IL-9 production is also important for *in vivo* allergic responses in patients with seasonal allergic rhinitis because successful specific immunotherapy with grass pollen led to the decrease in *IL9* mRNA and IL-9 protein levels in the

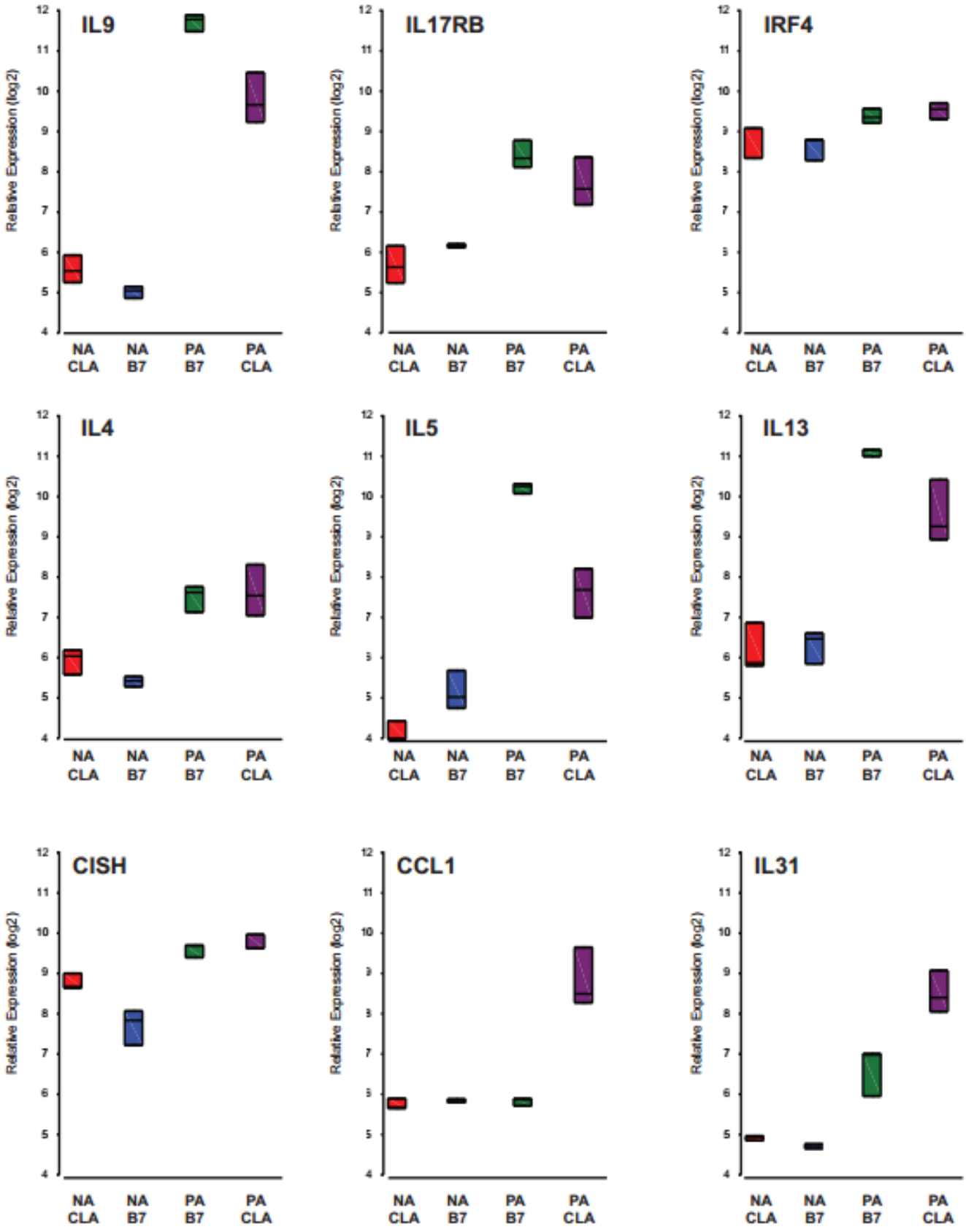

FIG 3. Expression of T_H2/T_H9 subset–specific genes and other genes known to be involved in allergic responses in skin-homing (CLA^+) and gut-homing ($B7^+$) peanut-activated memory T_H cells from donors with PA (n = 3) and NA donors (n = 3).

ALL genes (32321)
↓
Genes after distribution filter (12257)
↓
Random Forrest selection of best classifier genes

Genes after distribution filter: there are 4 possible 2-gene combinations which all classify with 100% accuracy:

1) 750 trees
8114256 IL9
8172914 HUWE1 (HECT, UBA and WWE domain containing 1)

2) 500 trees
8114256 IL9
8069610 hypothetical LOC100271832 (RNA, Ro-associated Y5 pseudogene 10; RNA, Ro-associated Y1; RNA, Ro-associated Y4 pseudogene 7; RNA, Ro-associated Y4 pseudogene 19; RNA, Ro-associated Y3; hypothetical LOC100132111; RNA, Ro-associated Y4)

3) 250 trees
8114256 IL9
8164165 HSPA5 (LOC400750 hypothetical gene supported by AF216292; NM_005347; heat shock 70kDa protein 5, glucose-regulated protein, 78kDa)

FIG 4. RF algorithm for identifying classifier genes that differentiate between peanut-activated memory T_H cells from 3 donors with PA and 3 NA donors.

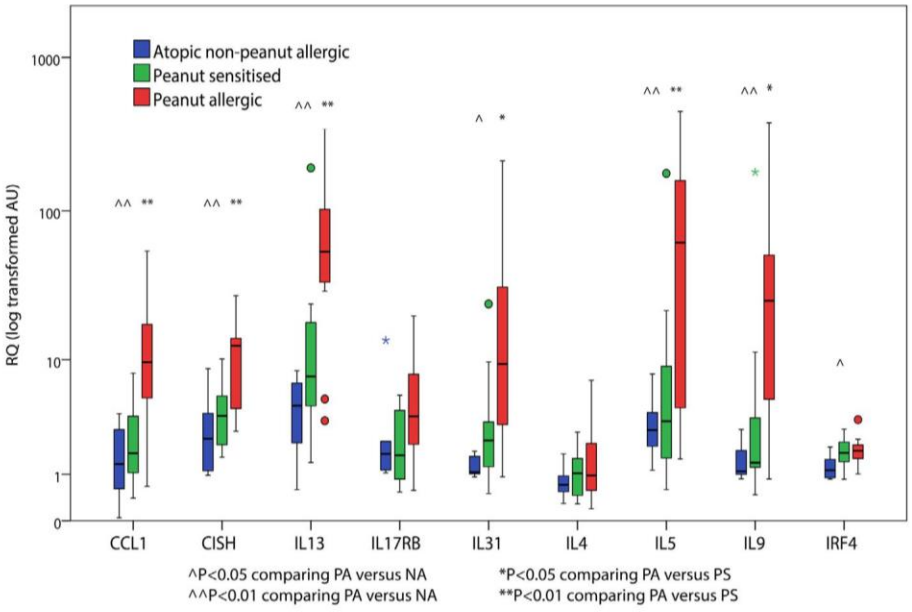

FIG 5. Differential RT-qPCR gene expression in PBMCs from children with PA (n = 12), children with PS (n = 12), and atopic NA children (n = 6) comparing target gene relative to the endogenous gene *18s* (ΔCT) in peanut-stimulated versus unstimulated cultures (ΔΔCT) converted into RQ in log-transformed arbitrary units *(AU)*.

patients' nasal mucosa.[43] IRF4 is necessary for the differentiation of T_H9 cells[29,30] and has previously been described by using gene expression microarrays in allergen-stimulated PBMCs from patients with allergic rhinitis.[44]

In mice, oral antigen-induced anaphylaxis to ovalbumin (OVA) is IgE mediated and predominantly IL-9 and IL-9 receptor pathway dependent.[45] *Il9*$^{-/-}$ and *Il9r*$^{-/-}$ mice had OVA-specific IgE levels after intraperitoneal OVA/alum immunization but did not have anaphylaxis after OVA oral gavage and had reduced intestinal mast cell proliferation and degranulation. This might explain the role of IL-9 in IgE-mediated food allergy versus only IgE-specific sensitization.

In a recent study in human subjects, *IL9* was the best gene to discriminate between peanut-stimulated PBMCs from adults with PA versus NA children (28-fold difference on RT-qPCR); however, *IL9* expression was not assessed in adults with PS.[46] Other differences between that article and ours were that (1) they assessed adults rather than children; (2) their peanut-tolerant group were generally nonatopic, whereas the majority of our peanut-tolerant group was atopic, thus accounting for genes upregulated because of atopy rather than PA *per se*; (3) exploratory microarray analysis was not used to determine candidate genes, and thus other important differentiation genes might have been excluded; and (4) *IL9* gene expression was initially

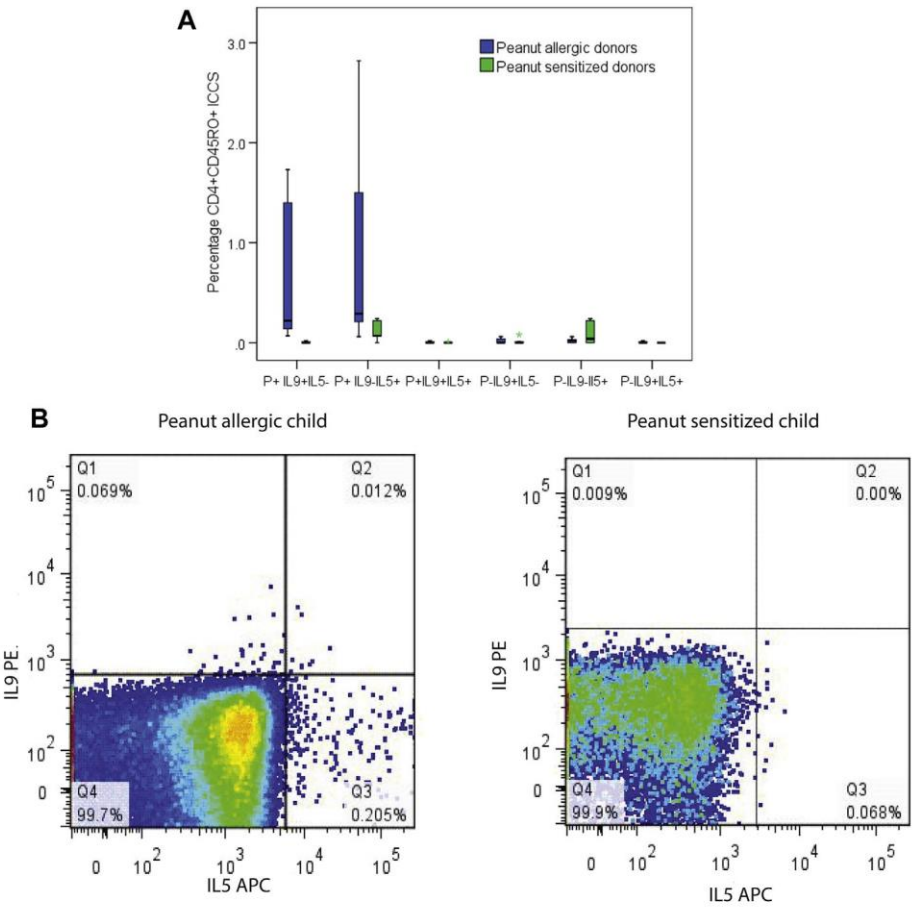

FIG 6. A, Box plot of FACS-sorted memory T_H cells and ICCS of IL-5 and IL-9 expressed in peanut-stimulated *(P+)* versus unstimulated *(P−)* memory T_H cells ($CD4^+CD45RO^+$) from 5 donors with PA and 5 donors with PS. **B,** Example of FACS analysis of peanut-stimulated memory T_H cells from the first donor with PA and PS.

investigated in the entire PBMC population rather than in different T-cell subsets to determine which cells were producing IL-9. However, importantly, they showed significantly increased IL-5 and IL-9 levels in the 5-day peanut culture supernatants of children with PA versus NA children (by using ELISA).

We confirmed differential gene expression between children with PA and NA children for *IL9* and its transcription factor *IRF4*, signature T_H2 genes (*IL5* and *IL13*), and other genes important for allergen-specific responses (*CISH*, *IL31*, and *CCL1*) using RT-qPCR. Confirmation of microarray findings was performed with RT-qPCR of whole PBMCs rather than FACS-sorted cells because if IL-9 is to be useful as a diagnostic biomarker, then it should be able to distinguish between patients with PA and NA subjects in unseparated cells rather than in very small T_H cell subsets that involve cumbersome experimental procedures requiring highly skilled personnel. The IL-25 receptor *(IL17RB)* and *IL4* were no longer significantly differentially expressed between children with PA and NA children on RT-qPCR. The IL-25 receptor *(IL17RB)* is expressed in T_H9 cells generated *in vitro* in the presence of TGF-β and IL-4.[47] The difference between findings in the microarray and RT-qPCR might be because the microarray was performed on peanut-cultured activated memory T_H cells, whereas the RT-qPCR was performed on whole PBMCs, and thus the signal might not be as strong in a mixed cell culture. Furthermore, on microarray analysis, *IL17RB* and *IL4* were not as highly differentially expressed as *IL9*, *IL5*, and *IL13* in children with PA versus NA children (Fig 1). *CISH* was upregulated in children with PA versus NA children and children with PS and has been shown to be differentially expressed in house dust mite–stimulated T cells from atopic subjects versus nonatopic subjects[37,48] and in OVA-stimulated PBMCs from patients with egg allergy versus those without egg allergy.[40]

A notable finding in this study was the lack of differential expression of T_H2- and T_H9-related genes between skin- and gut-homing memory T_H cell subsets in children with PA, given our previous findings.[22] Additionally, there was no significant difference in the number of CLA^+ versus $B7^+$ activated memory T_H cells sorted from peanut-stimulated culture in children with PA versus NA children, although there was a trend toward higher responses to peanut from the gut-homing versus skin-homing memory T_H subset in NA donors ($P = .05$). One potential explanation for this is that the children with PA who we investigated had long-term, well-established PA; therefore it is plausible that although the original T_H2 and T_H9 gene expression profile might have been limited only to the skin-homing T_H cell subset, in the long term the T_H2 and T_H9 responses became dominant in all lymphocyte-homing compartments as a consequence of allergic subjects' repeated exposure to peanut, either through eczematous skin or accidental oral exposure. Future experiments could further identify the differences between peanut-specific immune

responses of skin- and gut-homing subsets in children with PS if this were carried out longitudinally in a prospective approach as the children progress from PS to PA.

Nonetheless, other genes were differentially expressed in skin- versus gut-homing T_H cells; *CCL1*, *IL31*, and *IL3* were upregulated in skin-homing T_H cells from donors with PA compared with gut-homing T_H cells from donors with PA and gut- and skin-homing T_H cells from NA donors. *CCL1* is secreted by activated T cells and IgE-activated human mast cells and binds to the CCR8 receptor expressed by T_H2 cells, dendritic cells, monocytes, natural killer cells, and immature B cells.[41] Notably, CCR8 is expressed by approximately 70% of T_H cells recruited in the airways of asthmatic subjects, which is why oral forms of the small-molecule CCR8 antagonists are currently being developed for therapeutic purposes.[39] *IL31* is produced mainly by activated T_H2 cells, and its levels are increased in skin biopsy specimens of patients with eczema and contact dermatitis.[25] *IL3* increases the activation and release of mediators from eosinophils and basophils in response to IgE FCεRI cross-linking. The differential expression of these genes reflects the contrast between the skin- and gut-homing T_H cell subsets observed in the primary component analysis (PCA; see Fig E3 in this article's Online Repository at www.jacionline.org); however, the differential expression of homing-related genes was not mirrored by a differential expression of T_H2 and T_H9 subset–defining cytokine genes. The lack of such a difference suggests that in patients with well-established PA, allergen-specific T-cell populations comprise T_H2 and T_H9 cells, regardless of their homing phenotype.

Another unexpected finding was the absence of an identifiable Treg gene signature in the peanut-responding memory T_H cells from NA donors; however, the microarray did identify 3 genes, *SULT1B1*, *GCNT4*, and *RGS18*, and other as-yet-unidentified genes that were differentially expressed in NA children versus children with PA. The absence of classical Treg cell biomarkers suggests that a suppressor cytokine environment is not actively induced after only short-term (18-hour) *in vitro* stimulation with antigens from tolerated foods. Another potential explanation for this finding is that peanut-specific ($CD69^+$) Treg cells in NA children do not express CLA or B7 homing markers and were thus not gated and isolated on FACS analysis.

In this study *IL9* was the best discriminator for children with PA versus NA children and also, importantly, children with PS. Mutually exclusive production of T_H9-specific (IL-9) and T_H2-specific (IL-5) cytokines suggest that IL-9–producing cells belong to the distinct T_H9 subset population. The use of gene expression microarrays to generate hypotheses by evaluating the overall immune response to an allergen in a small number of patients, followed by further in-depth investigations, has previously been successfully applied to elucidate the mechanisms underlying peanut oral immunotherapy[49] and for predicting the efficacy of venom immunotherapy.[50] Future research into the interplay between the T_H9 and T_H2 subsets might clarify whether the success of preventative therapeutic approaches aimed at PA resolution could be evaluated on the basis of IL-9 secretion and/or T_H9 suppression in peanut-specific *in vitro* responses. Prospective studies should further evaluate *IL9* as a biomarker for PA and as a potential target for the prevention and treatment of PA.

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Clinical implications: The *IL9* gene might be a useful biomarker to distinguish between children with PA and children with PS. Further prospective studies are required to validate these findings and determine whether *IL9* responses could be targeted in treating and preventing PA.

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7.10.1 Online repository for Publication 5

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METHODS

PBMC isolation and peanut stimulation *in vitro*

Anticoagulated blood was centrifuged for 5 minutes at 620*g* at room temperature to collect autologous plasma. After the plasma layer was collected, cells were diluted with a volume of PBS equal to the volume of plasma removed and then decanted into cell-separation tubes over a Histopaque-1077 layer. These were centrifuged at 1200*g* for 15 minutes at room temperature, and then the PBMCs at the interface were collected and washed 3 times with PBS. PBMCs were counted with a Nucleocounter and then resuspended in serum free AIM V medium (containing 10 μg/mL gentamicin sulfate and 50 μg/mL streptomycin sulfate) plus 2- mercaptoethanol (both from Invitrogen–Life Technologies). PBMCs were cultured for 18 hours to detect the early allergen-specific response, as previously described.[E1] Cells were distributed in 24-well plates at a concentration of 3 million cells in 1 mL per well to ensure optimal cell-to-cell contact[E2] and were either unstimulated or stimulated with 200 μg/mL whole defatted peanut protein extract (ALK-Abelló, Hørsholm, Denmark).

Cell labeling and flow cytometric sorting

Cells were aspirated from the peanut-stimulated and unstimulated cultures, respectively, and washed and stained on ice for 20 minutes with antibodies specific for CD4 as a T_H cell marker (CD4-allophycocyanin; BD Biosciences, Oxford, United Kingdom) and CD45RO as a memory cell marker (CD45RO–Pacific Blue; BioLegend, London, United Kingdom). CLA, which is an adhesion molecule expressed by skin-homing cells, was used as a skin-homing marker (CLA–fluorescein isothiocyanate, BioLegend). β7 integrin, which is expressed by gut-homing cells, was used as a gut-homing marker (B7-PerCP/Cy5.5, BioLegend). CD69 labeling (CD69-PECy7, BioLegend) was used as a marker of cell activation to identify peanut-responding cells because its expression is rapidly upregulated on T cells after activation and remains stable for up to 72 hours.[E3] Thus skin- and gut-homing, activated and nonactivated memory T_H cells (ie, $CD4^+CD45RO^+CLA^+B7^-CD69^+$, $CD4^+CD45RO^+CLA^+B7^-CD69^-$, $CD4^+CD45RO^+CLA^-B7^+CD69^+$, and $CD4^+CD45RO^+CLA^-B7^+CD69^-$, respectively) were 4-way sorted from both peanut-stimulated and unstimulated PBMCs cultures with a FACSAria II Cell Sorter (BD Biosciences, San Jose, Calif). We display the FACS experiments for all 3 donors with PA and 3 NA donors in Fig E1. Selected cells were sorted directly into TRIzol LS Reagent (Life Technologies) with a 3:1 ratio of TRIzol LS Reagent to volume of sample. Samples were vortexed and stored at −80°C until RNA extraction was performed. The number of cells undergoing FACS for each condition is displayed in Table E2.

RNA extraction

RNA was extracted with the miRNeasy Mini kit (Qiagen), according to the manufacturer's protocol, by using TRIzol LS instead of QIAzol. We performed a DNase digestion step to remove any traces of contaminating DNA and carried out water elution of the RNA from the column twice to enhance the RNA yield. The amount and quality of RNA and cDNA obtained from the FACS-sorted cells from donors with PA and NA donors are displayed in Table E2.

cDNA synthesis

The quality and quantity of total RNA were first examined on an Agilent 2100 Bioanalyser with the Agilent RNA 6000 Pico Kit (Agilent Technologies, Wokingham, United Kingdom). On confirming that the RNA integrity was of good quality, cDNA was synthesized with the NuGEN Ovation Pico WTA system v2 kit, according to the manufacturer's protocol. Briefly, 500 pg to 50 ng of total RNA in 5 μL of water was reverse transcribed into first-strand cDNA. Second-strand cDNA was then synthesized, which served as a template for amplification of transcripts through a proprietary Ribo-SPIA amplification step.

cDNA fragmentation, biotin labeling, and hybridization

Three micrograms of amplified cDNA was fragmented and biotin labeled with the NuGEN Encore Biotin Module, according to the manufacturer's protocol. The hybridization cocktail was then prepared from the fragmented biotin-labeled cDNA by using the Affymetrix Hybridization, Wash and Stain kit and hybridized to the Affymetrix GeneChip Human Gene 1.0 ST Array for 18 hours at 45°C and 60 rpm rotation. On completion of hybridization, washing and staining steps were performed on an Affymetrix GeneChip Fluidics Station 450, and arrays were scanned with an Affymetrix GeneChip 3000 7G scanner.

Microarray data normalization and quality checks

A robust multichip average method was used for data normalization. The array signal before and after normalization is shown in Fig E2, *Ai* and *Aii*. All arrays passed the basic quality checks, including a uniform hybridizing signal of the spike-in hybridization control at the staggered concentration of CreX > BioD > BioC > BioB (Fig E2, *B*) and a high positive versus negative area under the curve ("pos_vs_neg_auc") that ranged between 0.82 and 0.90 (data not shown). PCA of peanut-induced gene expression was carried out with the Partek Genomics Suite software on normalized data from the peanut-activated memory T_H cells (n = 6 [3 CLA^+ and 3 $B7^+$ samples] microarrays in children with PA and n = 6 [3 CLA^+ and 3 $B7^+$ samples] microarrays in NA children). PCA shows the variation in all the genes expressed in one 3-dimensional image. The Partek software took all genes (variables) and plotted them against each other, then ranked genes into orders of variability (ie, genes that do not change very much are ranked low and genes that do change are ranked high). Genes were then assembled into groups of genes with similar variability of variation. Groups of genes with the most variability were grouped together (PC1), followed by PC2 then PC3. Thus the PCA resulted in 3 arms of groups of genes based on the variability of gene expression and the similarity of patterns of gene expression. CLA^+ and $B7^+$ samples from donors with PA (red) and NA donors (blue) are shown in Fig E3. The first 3 components (PC1 = 20.7%, PC2 = 14.3%, and PC3 = 10.7%) account for 45.7% of global differential gene expression. Applying PCA on these samples leads to a clear-cut clustering of CLA^+ skin-homing T_H cells (green centroid), distinguishing them from the B7 gut-homing T_H cells (yellow centroid) on the basis of their gene expression.

Partek Suite analysis of microarray

To create a heat map of differentially expressed genes, we used a 4-way ANOVA with peanut allergic status, T_H homing (skin vs gut), donor (to adjust for donor effect), and date of microarray (to adjust for batch effect). The heat map (Fig 1) was based on all genes with 2-fold differential expression and a *P* value of less than .05. The actual *P* values obtained by using this 4-way ANOVA are described in Table I, and box plots for individual differential gene expression are shown in Fig 3. To perform a detailed analysis of differential gene expression in the 36 PA and 36 NA samples (including internal and external negative controls), we subsequently used a 6-way ANOVA using stimulation (peanut stimulated versus unstimulated), both $CD69^+$ activation status and PA status, T_H homing (skin vs gut), donor, and date of microarray. The box plots for individual gene expression using the 6-way ANOVA analysis are displayed in Fig E4.

Microarray gene expression classification approach

Microarray data were analyzed by using an automatic classification approach, as currently used in data-mining/machine-learning fields; in these types of analyses the number of samples to be worked on is usually much larger than the number of data points ("features") used to describe those samples. Because in our study the number of genes (features) in each microarray (n = 32,020) was much larger than the number of microarrays (n = 36 [samples]), a basic filter was first applied based on the fact that most genes show very small variability in their expression levels because they are involved in the basal cellular metabolism. The IQR of individual gene expression in all 6 patients (3 children with PA and 3 NA children) was used to identify those genes with expression levels that vary most between the PA and NA phenotypes. The global IQR of gene expression in each microarray was also calculated, and any genes with a variability of less than 20% of that value were removed. This filter was applied by using the open-source statistical software

R (www.r-project.org/), and it narrowed down the analysis from 32,020 to 12,257 genes, representing about one third of all genes. (Fig 4).

Using this set of 12,257 differentially expressed genes, RF,[E4] which is a state-of-the-art supervised classification algorithm, together with recursive feature elimination, was used to classify all the peanut-activated samples into PA versus NA groups. The RF classifier is an ensemble of multiple classification trees (analysis pathways) and is suitable for our analysis with only 6 samples because its performance is estimated directly, without the need for cross-validation, by the out-of-bag error fraction. The accuracy of each set of genes compared between samples was computed by subtracting this error from 1, its maximum value. Recursive feature elimination is an algorithm used to select a subset of relevant data points or "features" (eg, gene expression levels) for use in model construction; it is usually used with support vector machines to repeatedly remove features with low weights when constructing a classification model.[E5]

An advantage of RF is the fact that it can measure each gene's ("feature's") importance relative to the classification task at hand. Although there are several kinds of such importance measures, the unscaled permutation importance was used in this case because it is recommended for feature selection.[E6] For each gene, it measures the decrease of classification accuracy in the case of random permutation of its expression levels averaged over all trees. The feature selection process that we used in combination with the algorithm is itself a recursive meta-algorithm in which, at each step, a random forest of decision trees was grown and then 50% of the genes (ie, those with the smallest differential gene expression level) were discarded. For a given sample, at each step of the classification process, a feature (ie, gene) was analyzed, and based on its value, a decision was reached to assign that sample to a certain class. The gene selection stopped when the number of remaining genes in the set used for classification decreased to less than a certain initially fixed threshold. All classification tasks were performed with the free software package Random Jungle.[E7]

RT-qPCR confirmation of gene expression

RNA was extracted from peanut-stimulated PBMCs obtained from an independent group of 12 donors with PA and 18 peanut-tolerant donors (of whom 12 had PS) to confirm the microarray findings. Briefly, PBMCs were cultured for 18 hours in serum free AIM V medium (as described above) in the presence of 200 μg/mL whole defatted peanut protein extract (ALK-Abelló) or without any peanut antigen added (as an unstimulated negative control). RT-qPCRs were performed with the following TaqMan MGB probes (Applied Biosystems, Invitrogen, Paisley, United Kingdom), as previously described:[E8] *IL3*, Hs00174117_m1; *IL4*, Hs00929862_m1; *IL5*, Hs00174200_m1; *IL9*, Hs00174125_m1; *IL13*, Hs00174379_m1; *IL31*, Hs01098710_m1; *CCL1*, Hs00171072_m1; *CISH*, Hs00367082_g1; *IRF4*, Hs01056533_m1; *IL17RB*, Hs00218889_m1; and *18s* rRNA, 4319413E. Target gene expression levels were compared against endogenous control 18s ribosomal RNA (ΔCT) and then against the peanut-unstimulated control (ΔΔCT) and converted to RQ ($2^{-[\Delta\Delta CT]}$). Genes with no amplification were assigned a cycle threshold value of 40. The Mann-Whitney test was performed to compare RQ values between NA children and children with PA and between children with PS and children with PA by using SPSS (SPSS 19.0; SPSS, Chicago, Ill).

FACS analysis of intracellular cytokine production

After results obtained from the gene microarray and RT-qPCR, we sought to confirm our gene expression findings on a protein expression level. PBMCs from 5 donors with PA and 5 donors with PS were cultured overnight in RPMI 1640 supplemented with 5% autologous plasma in the presence of 200 μg/mL whole defatted peanut protein extract (ALK-Abelló) or without any antigen added (as an unstimulated negative control) in the presence of monensin (2 μg/mL) and brefeldin A (1 μg/mL) as inhibitors of cytokine secretion. After incubation, PBMCs were fixed, permeabilized, and stained with antibodies specific for CD4, CD45RO, CLA, B7, IL-5, and IL-9, and cytokine production (IL-5 and IL-9) was analyzed by using FACS. IL-5 was used to identify T_H2 cells, whereas IL-9 was used as the hallmark cytokine to identify the T_H9 subset.

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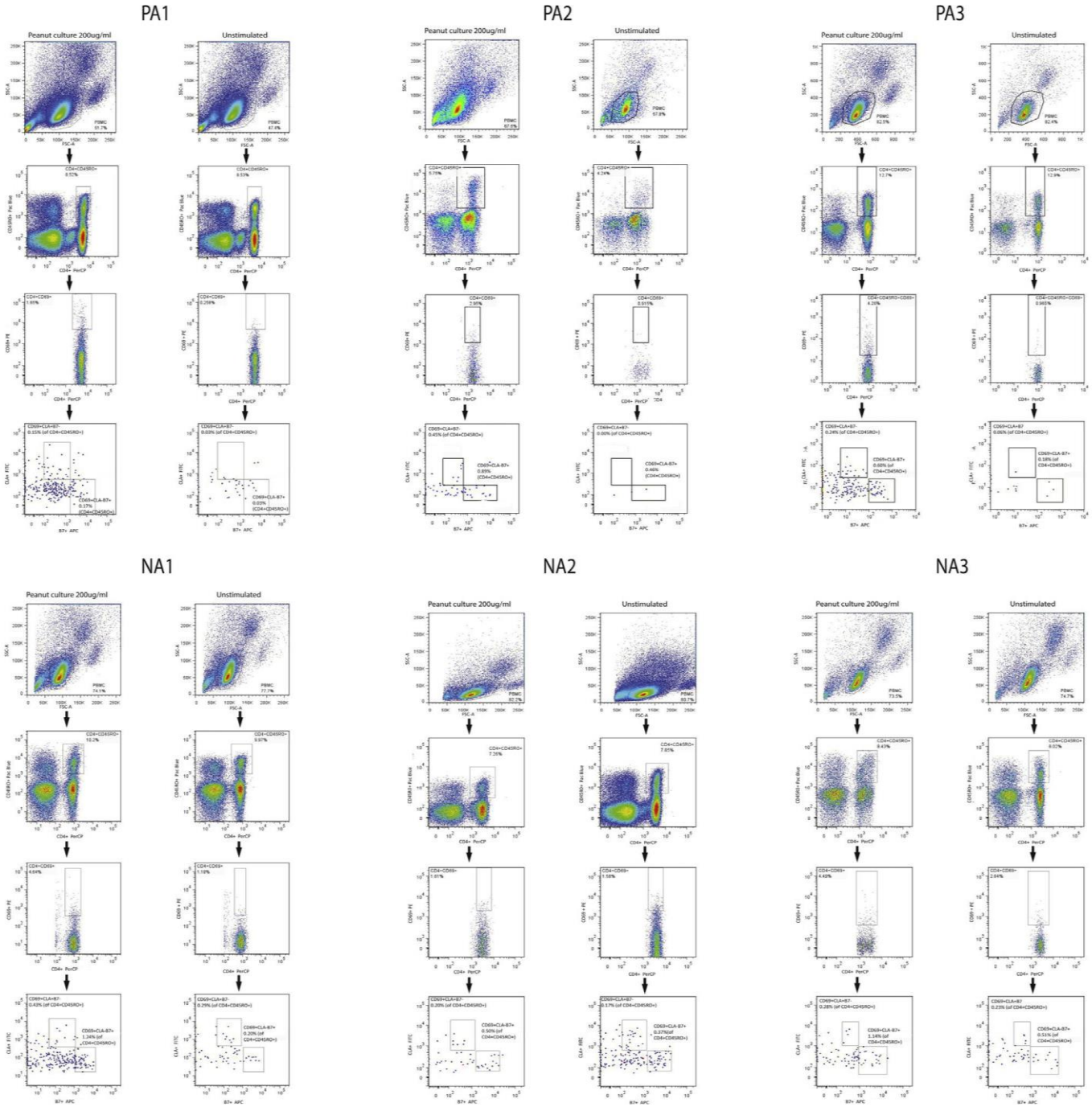

FIG E1. FACS experiments for 3 donors with PA and 3 NA donors, illustrating the isolation of memory T_H cells (expressing CD4 and CD45RO) homing to the skin (CLA^+) or the gut ($B7^+$) and either peanut-activated ($CD69^+$) or nonactivated controls ($CD69^-$).

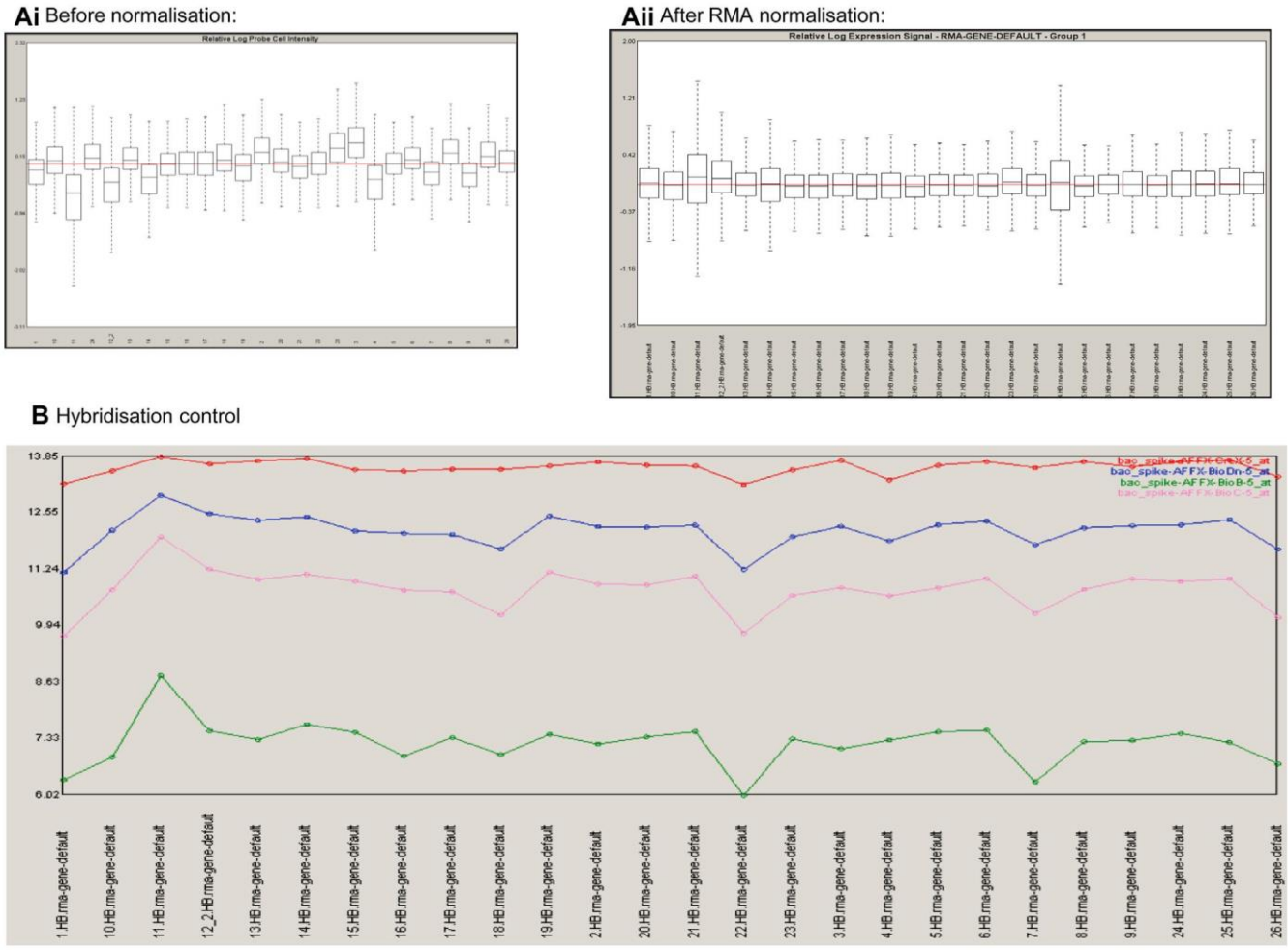

FIG E2. Data normalization and quality checks for microarray hybridization. Box plots of array hybridization signal before and after robust multichip average *(RMA)* normalization are shown in Fig E2, *Ai* and *Aii*, respectively. All arrays had a uniform pattern of hybridization control signal (Fig E2, *B*), which was at the expected intensity in congruence with their staggered concentration of CreX > BioD > BioC > BioB. Red, CreX; blue, BioD; pink, BioC; and green, BioB.

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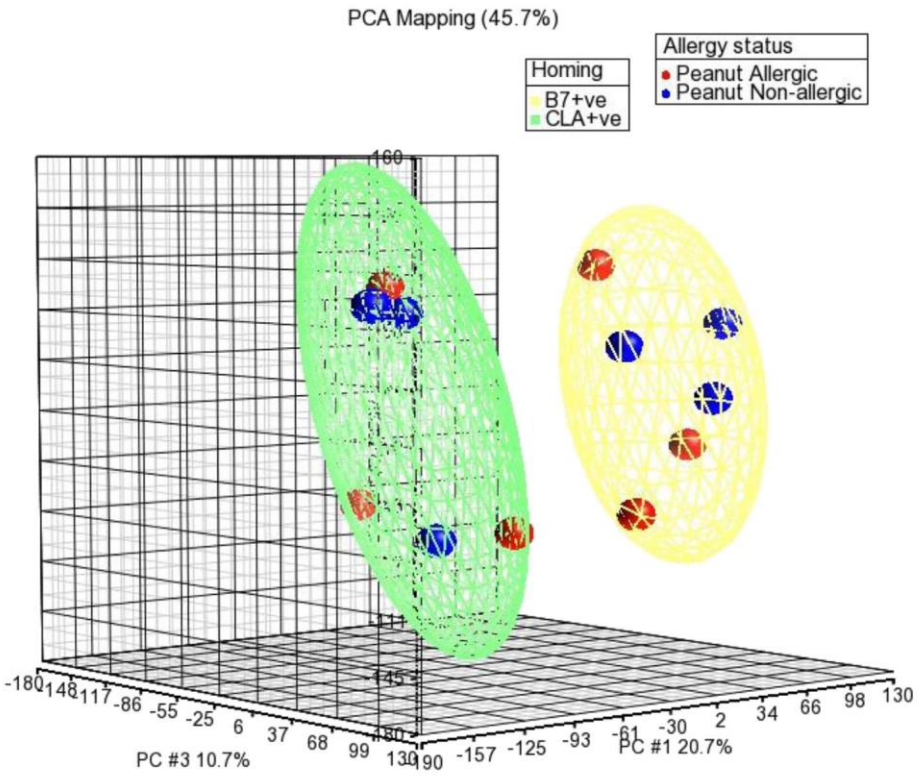

FIG E3. Principal component analysis *(PCA)* of gene expression in the Affymetrix GeneChip Human Gene1.0 ST microarray led to clustering of the CLA^+ skin-homing T_H cells *(green centroid)*, distinguishing them from B7 gut-homing T_H cells *(yellow centroid)*.

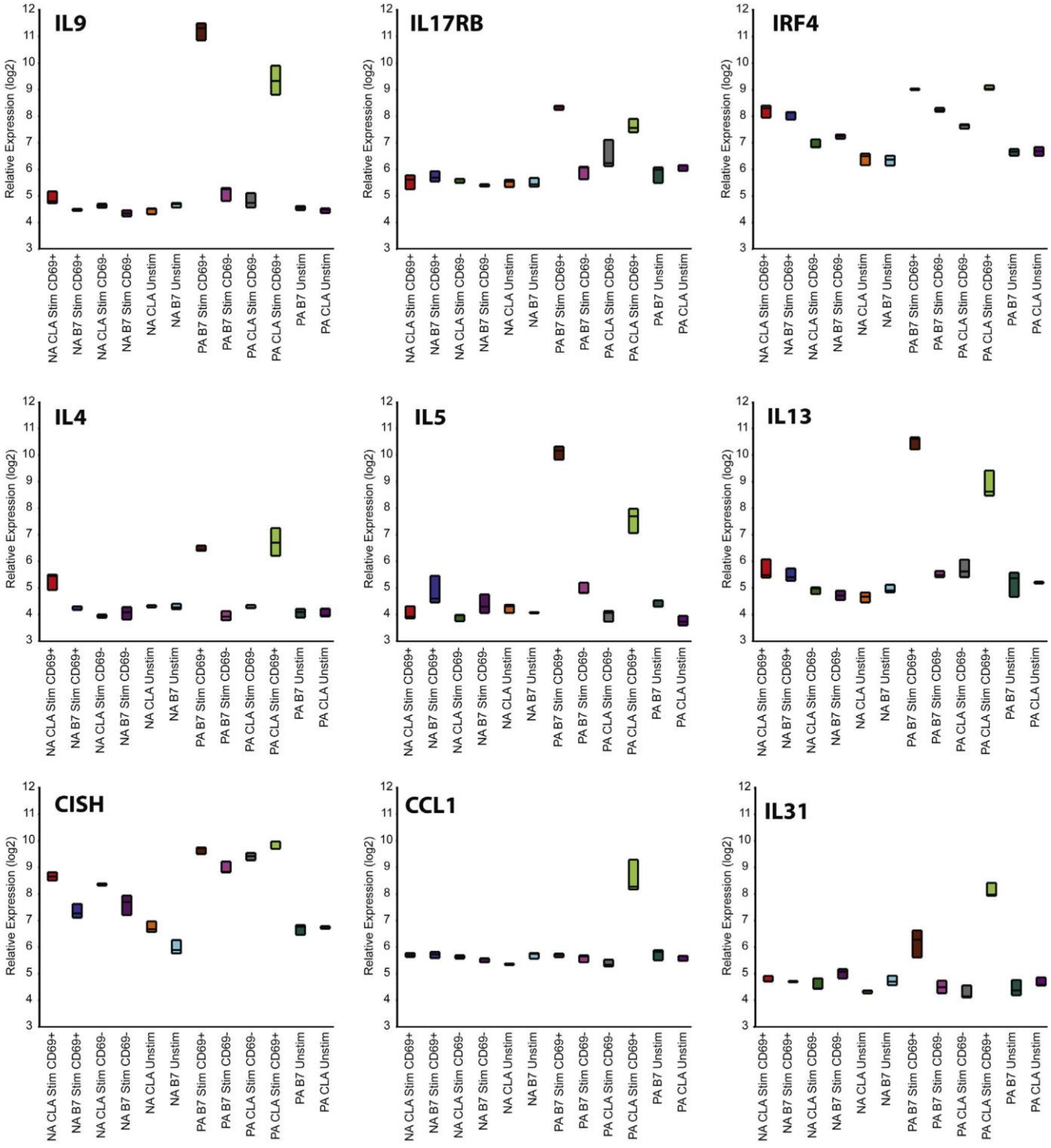

FIG E4. Gene expression in peanut-stimulated *(Stim)* versus unstimulated *(Unstim)* culture conditions in activated ($CD69^+$) and nonactivated ($CD69^-$) skin-homing (CLA^+) and gut-homing ($B7^+$) memory T_H cells in 3 children with PA versus 3 NA children.

TABLE E1. Clinical characteristics of donors with PA and NA donors recruited for gene microarray analysis (n = 6), FACS ICCS (n = 10), and RT-qPCR (n = 30)

Peanut allergic status	Sex	Age (y)	Ethnicity	Last known peanut exposure	Allergic reaction	Skin prick test response (mm)	Peanut-specific IgE (kU/L)	Ara h 2	Ara h 1	Ara h 8	Current eczema	Eczema severity	Asthma	Atopic disease
Gene expression analysis with Affymetrix microarray														
PA	M	9	White	7 y	Hives, facial angioedema, cough, difficulty breathing	10	>100	>100	>100	0.10	Yes	Mild	Yes	AR, tree nut allergy
PA	M	7	Black, British	4 y	Hives, angioedema	12	31.8	213.3	38	27	Yes	Moderate	Yes	Fish allergy, AR, asthma
PA	M	15	White	14 y	Generalized hives, cough and vomiting	22	>100	68	40	N/A	No	Severe	Yes	AR, tree nut sensitized
NA	M	8	Philippines	Eats peanut weekly	No allergic reaction	0	0.09	0.00	0.05	0	Yes	Moderate	Yes	Grass pollen allergy
NA	M	8	White	Eats peanut weekly	No allergic reaction	0	0.01	0.04	0.01	N/A	No	N/A	No	Cow's milk allergy
NA	M	6	White	Eats peanut weekly	No allergic reaction	0	0.01	0.04	0.00	0.00	No	N/A	No	Nonallergic rhinitis
Flow cytometric analysis for intracellular cytokine staining														
PA	F	5	White	4 wk	Angioedema and urticaria	9	1.2	0.00	1.25	0.00	Yes	Moderate	Yes	OAS, kiwi allergy
PA	F	15	White	10 y	Angioedema and hives	8	11.1	N/A	N/A	N/A	Yes	Mild	Yes	OAS, latex allergy, egg, wheat, sesame, mustard, fish allergy
PA	M	12	White	9 y	Contact angioedema	15	38.7	11.8	22.3	0.01	No	N/A	Yes	Tree nut and chickpea allergy
PA	M	14	White	9 y	Positive oral food challenge	10	2.03	2.14	<0.35	1.1	No	N/A	Yes	Nil else
PA	F	11	Mixed black-white	4 y	Angioedema, hives, difficulty breathing	9	81.2	32.1	42.3	22.8	Yes	Mild	Yes	Tree nut allergy and AR
PS	M	8	Mixed black-white	1-2 times/mo	Nil	N/A	7.81	0.18	0.19	1.30	Yes	Moderate	No	Cashew, pistachio, and egg allergy
PS	M	14	White	Daily	Nil	0	0.38	0.00	0.01	1.48	Yes	Mild	Yes	Egg, cow's milk, and kiwi allergy
PS	F	6	Black	<1 wk	Nil	0	2.28	0.06	0.18	0.10	Yes	Severe	No	House dust mite allergy
PS	M	9	Southeast Asian	<1 wk	Nil	0	3.16	0.26	1.79	6.18	N/A	Yes	Yes	Egg, tree nut plus sesame allergy, tree pollen allergy
PS	M	10	White	1 w	Nil	0	3.16	0.26	1.79	6.18	No	N/A	Yes	Egg, tree nut plus sesame allergy, tree pollen allergy
RT-qPCR analysis: Peanut allergy														
PA	M	11	Other-white	4 y	Angioedema with Crunchy nut cornflakes	14	>100	>100	>100	76	No	N/A	No	Tree nut allergy, AR
PA	F	6	White	10 mo	Labial/facial angioedema, quiet	8	N/A	0.05	0.78	0	No	N/A	No	Nil
PA	M	5	White	1 y	Hives/facial angioedema after peanut butter	10	13.1	9.17	6.45	2.1	No	N/A	No	Tree nut allergy, OAS, AR
PA	M	7	White	1 y	Hives, facial swelling after cutaneous contact	11	27.1	5.63	0.12	>100	No	N/A	No	Tree nut, sesame, kiwi allergy, OAS, AR
PA	M	6	Asian	1 y	Facial hives after peanut consumption	10	10.3	1.22	0.9	N/A	Yes	Mild to moderate	No	Cow's milk and egg allergy, AR
PA	M	7	Black, British	4 y	Angioedema and hives	12	31.8	13.3	38	27	Yes	Moderate	Yes	Fish, tree nut, and sesame allergy; AR
PA	M	3	White	2 mo	DBPCFC: >3 hives after 4.35 g of peanut protein	5	2.67	2.94	0.03	0.01	No	N/A	No	Nil else

(Continued)

TABLE E1. *(Continued)*

Peanut allergic status	Sex	Age (y)	Ethnicity	Last known peanut exposure	Allergic reaction	Skin prick test response (mm)	Peanut-specific IgE (kU/L)	Ara h 2	Ara h 1	Ara h 8	Current eczema	Eczema severity	Asthma	Atopic disease
PA	M	6	White	1 y	Facial urticaria and vomiting	14	>100	65.5	>100	0.11	Yes	Moderate	Yes	Tree nut, kiwi, and egg allergy; AR
PA	F	10	Other, white	1 y	Vomiting and generalized hives after chocolate peanuts	12	>100	>100	32	N/A	No	N/A	No	Tree nut and green pea sensitized
PA	M	8	Asian	5 y	Vomiting, wheeze, difficulty breathing	10	56.5	35	6.6	5.2	No	N/A	No	Tree nut and fish allergy, AR
PA	M	15	White	14 y	Cough, vomit, general hives	22	>100	40	68	3.6	No	N/A	No	Tree nut sensitized
PA	M	6	White	5 mo	General urticaria, vomiting, drowsy after Crunchy nut cornflakes	12	>100	>100	25.4	0.02	No	N/A	No	Tree nut sensitized
RT-qPCR analysis: Peanut sensitized but tolerant														
PS	F	2	White	<1 mo	Negative DBPCFC result	4	0.09	0	0	0.01	Yes	Mild	No	Egg allergy
PS	F	1	White	<1 mo	Negative DBPCFC result	4	0.41	0.2	0.01	0.01	Yes	Moderate to severe	No	Egg allergy
PS	F	6	Asian	<1 mo	Negative DBPCFC result	2	2.35	0.1	0.1	0.05	No	N/A	No	Egg and fish allergy
PS	F	3	Southeast Asian	<1 mo	Negative DBPCFC result	3	0.09	0.2	0.01	0.01	Yes	Mild	No	Egg and sesame seed allergy
PS	M	6 mo	Southeast Asian	<1 mo	Negative DBPCFC result	2	0.74	1.2	0.9	N/A	Yes	Mild to moderate	No	Cow's milk and egg allergy, AR
PS	M	7	Asian	<1 mo	Negative DBPCFC result	5	1.86	0.10	0.06	0.06	Yes	Moderate	No	Egg allergy, sensitized to tree nuts
PS	M	2	Southeast Asian	<1 mo	Negative DBPCFC result	5	9.9	0.10	1.40	0.02	No	N/A	No	Cauliflower, fish, shellfish, chickpea, lentil, sesame, egg allergy
PS	M	3	White	<1 mo	Negative DBPCFC result	4	0.83	0.20	0.01	0.01	No	N/A	No	Resolving egg allergy, tree nut allergy
PS	M	4	Black-white	2-3 times/wk	Nil	4	11.2	0.10	0.47	12.5	Yes	Mild to moderate	Yes	Cow's milk, egg, avocado, banana, sesame allergy, AR
PS	F	8	Asian	1-2 times/mo	Nil	3	3.04	0.30	0.30	N/A	No	N/A	No	Egg, sesame seed allergy
PS	M	1	White	<1 mo	Negative DBPCFC result	6	2.29	0.00	0.01	0.01	No	N/A	No	Cow's milk allergy
PS	M	2	White	<1 mo	Negative DBPCFC result	2	0.52	0.00	0.10	0.01	No	N/A	Yes	Tree nut, sesame seed allergy
RT-qPCR analysis: Atopic, non–peanut allergic														
NA	F	12	White	1-2 times/mo	No allergic reaction	0	0.09	0.02	0.00	0.01	No	N/A	No	Recurrent lip angioedema
NA	M	12	White	1-2 times/wk	No allergic reaction	0	0.08	0.01	0.08	0.02	No	N/A	No	House dust mite allergy
NA	M	6	White	1-2 times/mo	No allergic reaction	0	0.01	0.04	0.01	0.01	No	N/A	No	AR, rhinosinusitis
NA	M	9	White	1-2 times/mo	No allergic reaction	0	0.18	0.19	0.01	0.01	Yes	Mild	Yes	Resolving egg allergy, kiwi allergy, AR
NA	M	8	White	1-2 times/wk	No allergic reaction	0	0.01	0.04	0.01	N/A	No	N/A	No	Cow's milk allergy
NA	M	8	Philippines	1-2 times/wk	No allergic reaction	0	0.09	0.00	0.05	0.00	Yes	Moderate	Yes	Grass pollen allergy

AR, Allergic rhinitis; *DBPCFC*, Double-blind, placebo-controlled food challenge; *F*, female; *M*, male; *OAS*, oral allergy syndrome; *N/A*, not available or not applicable (eczema severity).

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TABLE E2. Number of cells sorted for each condition in 3 children with PA and 3 NA children and amount and quality of RNA and cDNA obtained from these samples for microarray analysis

Allergy	Culture condition	Activation	Homing	Cells sorted	RNA (ng)	RIN	cDNA (ng/μL)	cDNA (μg)	260/280	260/230
PA1	P+	$CD69^+$	B7	39,318	5.8	8.2	225.25	6.8	1.98	1.91
PA1	P+	$CD69^-$	B7	244,316	9.0	8.8	253.46	7.6	1.98	1.94
PA1	P+	$CD69^-$	CLA	50,193	4.0	8.8	228.95	6.9	1.97	1.87
PA1	P+	$CD69^+$	CLA	12,161	2.0	8.0	169.28	5.1	1.96	1.78
PA1	P−	$CD69^-$	B7	38,472	5.3	7.9	296.21	8.9	1.97	1.89
PA1	P−	$CD69^-$	CLA	35,134	5.5	8.7	239.73	7.2	1.97	1.85
PA2	P+	$CD69^+$	B7	58,205	4.5	8.6	255.98	7.7	1.94	1.74
PA2	P+	$CD69^-$	B7	584,788	22.1	8.5	265.32	8.0	1.94	1.76
PA2	P+	$CD69^-$	CLA	40,685	2.7	9.1	176.84	5.3	1.95	1.78
PA2	P+	$CD69^+$	CLA	12,678	0.4	N/A*	175.87	5.3	1.96	1.71
PA2	P−	$CD69^-$	B7	44,709	0.7	8.1	230.68	6.9	1.93	1.62
PA2	P−	$CD69^-$	CLA	29,634	1.7	9.3	186.88	5.6	1.97	1.84
PA3	P+	$CD69^+$	B7	35,069	3.9	8.0	185.11	5.6	1.98	1.76
PA3	P+	$CD69^-$	B7	546,030	6.0	N/A*	265.82	8.0	1.95	1.81
PA3	P+	$CD69^-$	CLA	478,926	17.8	9.0	192.68	5.8	1.97	1.88
PA3	P+	$CD69^+$	CLA	55,106	3.3	8.3	138.93	4.2	1.96	1.73
PA3	P−	$CD69^-$	B7	58,439	3.4	8.3	131.97	4.0	1.97	1.83
PA3	P−	$CD69^-$	CLA	148,262	10.1	8.2	151.99	4.6	1.98	1.84
NA1	P+	$CD69^+$	B7	40,271	3.9	8.4	245.45	7.4	1.95	1.76
NA1	P+	$CD69^-$	B7	669,075	3.6	7.9	258.61	7.8	1.95	1.70
NA1	P+	$CD69^-$	CLA	228,356	4.1	8.4	244.64	7.3	1.94	1.69
NA1	P+	$CD69^+$	CLA	17,997	1.3	N/A*	175.56	5.3	1.93	1.55
NA1	P−	$CD69^-$	B7	84,218	2.5	8.2	215.43	6.5	1.96	1.71
NA1	P−	$CD69^-$	CLA	76,554	4.6	8.3	240.61	7.2	1.96	1.77
NA2	P+	$CD69^+$	B7	34,785	8.3	7.2	189.08	5.7	2.00	1.99
NA2	P+	$CD69^-$	B7	374,340	15.9	8.8	265.95	8.0	1.99	1.96
NA2	P+	$CD69^-$	CLA	90,357	8.1	7.3	264.92	7.9	1.98	1.94
NA2	P+	$CD69^+$	CLA	8,505	1.2	7.2	110.82	3.3	1.96	1.81
NA2	P−	$CD69^-$	B7	94,715	8.0	8.6	219.77	6.6	1.97	1.90
NA2	P−	$CD69^-$	CLA	56,690	6.7	6.3	242.87	7.3	1.98	1.91
NA3	P+	$CD69^+$	B7	40,447	7.0	8.5	246.9	7.4	1.91	1.62
NA3	P+	$CD69^-$	B7	759,119	1.9	7.5	248.84	7.5	1.93	1.62
NA3	P+	$CD69^-$	CLA	124,397	5.0	8.4	291.68	8.8	1.95	1.74
NA3	P+	$CD69^+$	CLA	9,854	1.4	N/A*	161.63	4.8	1.91	1.51
NA3	P−	$CD69^-$	B7	73,489	3.6	8.9	290.01	8.7	1.95	1.78
NA3	P−	$CD69^-$	CLA	45,974	2.6	8.7	279.49	8.4	1.95	1.80

N/A, Not available; *P+*, peanut stimulated; *P−*, no peanut stimulation.
*Where results are shown to be not applicable, the Bioanalyzer plots revealed RNA marker and ribosomal peaks at 5s, 18s, and 28s, and good-quality DNA was obtained from these samples.

TABLE E3. Frequency of intracellular IL-9 and IL-5 cytokines inside memory T_H cells cultured in peanut protein (P+) versus medium alone (P−) from 5 donors with PA and 5 donors with PS

Allergic status	Donors with PA						Donors with PS					
Culture	P+			P−			P+			P−		
Donor	$IL9^+$ $IL5^-$	$IL5^+$ $IL9^-$	$IL5^+$ $IL9^+$	$IL9^+$ $IL5^-$	$IL5^+$ $IL9^-$	$IL5^+$ $IL9^+$	$IL9^+$ $IL5^-$	$IL5^+$ $IL9^-$	$IL5^+$ $IL9^+$	$IL9^+$ $IL5^-$	$IL5^+$ $IL9^-$	$IL5^+$ $IL9^+$
1	0.07%	0.21%	0.01%	0.00%	0.06%	0.01%	0.01%	0.07%	0.00%	0.00%	0.04%	0.00%
2	1.40%	1.50%	0.02%	0.06%	0.00%	0.02%	0.02%	0.07%	0.00%	0.00%	0.00%	0.00%
3	1.73%	2.82%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%
4	0.14%	0.29%	0.00%	0.00%	0.02%	0.00%	0.00%	0.24%	0.01%	0.00%	0.22%	0.00%
5	0.22%	0.06%	0.00%	0.04%	0.00%	0.00%	0.00%	0.22%	0.00%	0.01%	0.24%	0.00%

Chapter 8 Discussion

8.1 Summary of principal findings

This is the first study to assess the impact of EPE, measured in household dust, on the development of PS and PA. The findings from this PhD showed that peanut proteins are present in household dust and are widespread throughout the home (publication 1) and that HPC is the single most important factor which is associated with peanut levels in dust in an infant's bed-dust and play-area (publication 2); this is of significant importance as HPC has been shown to increase the risk of PA, particularly when compared against high-risk infants with eczema and/or egg allergy.[104] Whole amino acid sequences of major peanut allergens were identified in dust using the highly specific MS assay, thus confirming the findings of peanut allergen in dust from the Veratox peanut ELISA employed to quantify this exposure. In-vitro work using BAT demonstrated a dose response of CD63+ upregulation from basophils of peanut allergic patients mixed with serial dilutions of peanut dust extracts (publication 2), providing biological plausibility that peanut protein in dust is able to cause allergen-specific activation and thus has the capability to induce PS. Peanut in the environment is more likely to sensitize through the skin than the respiratory tract, as peanut does not easily aerosolise. This was shown in a number of experiments using a personal Air Monitor sampling device; in particular there were undetectable levels of airborne peanut over 22 hours in homes with high levels of peanut protein in dust.

Following work to establish the characteristics of peanut protein in dust, I subsequently employed international cohort studies to determine the impact of peanut antigen in dust in early life on clinically relevant outcomes of PS and PA. Early environmental exposure to peanut in household dust significantly increased the risk of PS and PA in longitudinal studies in children with an impaired skin barrier (defined by carriage of a *FLG* loss-of –function mutation) (MAAS: publication 3) or when compared against egg sensitized children (BAMSE study). In a high-risk study (CoFAR: publication 4) where infants were recruited with either egg or cow's milk allergy or moderate to severe eczema and sensitization to egg or

cow's milk, EPE was a risk factor for PS and likely PA. The effect of EPE on PS was augmented further in children with a history of infantile eczema and even further in infants with a history of severe eczema. These results, taken together suggest that the route of PS may be via the presentation of peanut antigen via an inflamed disrupted skin to antigen presenting cells; this in turn could lead to a Th2 skewing of naïve Th cells in the skin-draining LNs and subsequent development of a peanut allergic response.

In order to further evaluate the hypothesis that PS occurs through the skin, I anticipated that recall responses to peanut in Th cells from peanut allergic children would be predominantly derived from the skin (rather than the gut which is predominantly a tolerising organ) and would be Th2 skewed. I used Th cells from NA children as controls and anticipated showing that recall Th responses to peanut in these children were predominantly derived from the gut (rather than the skin) and would be Th1 skewed or of a Treg phenotype. I therefore recruited peanut allergic and NA children and cultured their PBMCs in peanut then sorted their PBMCs according to their memory Th cell phenotype, activation status and homing markers (skin: CLA+ versus gut: β7+) (publication 5). Differential gene expression between children with PA or NA children was assessed in an explorative gene microarray then confirmed by RT-qPCR. I then compared gene expression in Th cells from peanut allergic children against gene expression in Th cells from peanut sensitized children to assess whether similar results were obtained. There were no differences in CLA+ versus β7+Th cells from peanut allergic children in terms of Th cell counts or Th2/Th1 or Treg cytokine gene expression. This is in contrast to previous research findings which showed higher Th cells proliferative recall responses to peanut antigen in CLA+ versus β7 + Th cells from peanut allergic children and Th2 cytokine predominance in CLA+ memory Th cells cultured in peanut antigen from peanut allergic children.[203] Possible reasons for this discrepancy are discussed in Section 8.11.6.

Using microarray and confirmatory RT-qPCR gene expression analyses I demonstrated that the *IL9* gene (encoding IL9 which is a mast cell activation and proliferation IL) was the most highly differentially expressed gene in peanut allergic children, as compared to both atopic NA individuals and peanut sensitized

individuals (publication 5). IL9 production was also confirmed on a protein level using ICCS in Th cell recall responses to peanut from PA versus PS children. IL9 was produced by different Th cells to those cells producing IL5 which suggests that IL9 producing cells are not Th2 cells but may be produced by the recently described Th9 cell. Although further studies are required to understand the role of IL9 in PA, the findings from this PhD suggest that IL9 may provide a useful biomarker for clinically relevant PA (rather than PS).

8.2 Overview of discussion of results

The primary hypothesis of this PhD was that early EPE, measured by peanut protein levels in household dust, is a risk factor for the development of PS and PA in children with an impaired skin barrier. The epicutaneous route of PS was then further investigated using in-vitro memory responses from skin versus gut derived Th cells in PA, PS and NA children to elucidate where PS may have originated. The work assessing EPE characteristic and its association with PS and PA is epidemiological in nature whereas the in-vitro section is mechanistic; I will therefore separate out these two sections in the discussion for the purpose of reviewing the relevant findings, limitations and future research.

8.3 Relationship between peanut consumption and peanut protein levels in dust

8.3.1 HPC and infant EPE

The premise of this PhD arose due to the findings from the study by Fox et al. (2009) where they showed that HPC during the child's first year of life was a risk factor for PA, especially when compared against atopic controls.[104] The study by Fox et al. (2009) was criticized by the reviewers because an objective measure of EPE had not been used. This led to my establishing the methodology to quantify peanut protein in dust, and in this PhD I showed HPC was highly correlated with peanut-dust levels in an infant's home environment and furthermore that it was the most important variable associated with peanut levels in dust on

multivariate LR analysis. The findings from this PhD therefore provide further support for the findings by Fox et al (2009), that HPC during infancy may be a risk factor for PA in the child via EPE.

8.3.2 HPC of hand-held peanut-containing foods and EPE

Fox et al. (2009) hypothesized that EPE was more likely to lead to PA was through hand transmission from members of the family eating peanut then placing their peanut contaminated hands onto both the child and the environment that the child inhabits. They based this hypothesis on their finding that high household consumption of peanut ***butter*** (which is sticky and easily transferred by hands) was associated with a higher risk of developing PA than household consumption of covered peanuts (e.g. chocolate covered peanut food). In publication 1, I demonstrated that peanut persisted on hands after consumption of whole peanuts for at least three hours which gives plenty of time to contaminate the infants' skin and/or various areas in the home. In order to corroborate whether certain hand-held types of peanut-containing foods were more closely related to peanut-dust levels in the infants' environment, I assessed whether peanut protein levels in the infant's bed and play-area were more closely linked to household consumption of peanut butter and 'hand-held' peanut-foods (whole peanuts) than all types of HPC (Table 14).

Table 14: Spearman rank (r_s) correlations between HPC and peanut levels in dust

	Peanut protein in infant bed-sheet (µg/g)	Peanut protein in infant play-area (µg/g)
All types of HPC over 6 months	r_s=0.71 95% CI: 0.51-0.84 *P*<0.001, n=38	r_s =0.72 95% CI: 0.52-0.84 *P*<0.001, n=38
Household peanut butter and whole peanut consumption over 6 months	r_s =0.72 95% CI: 0.52-0.85 *P*<0.001, n=38	r_s =0.77 95% CI: 0.60-0.87 *P*<0.001, n=38

The correlation between peanut-dust levels in the infant's bed and play-area and household consumption of 'hand-held' peanuts (including peanut butter and whole peanuts) was marginally stronger than the correlation with all forms of HPC, but this did not reach statistical significance (Fisher r-to-z transformation $P>0.05$). However, with an increased sample size, this might have reached statistical significance.

8.3.3 EPE distribution and relation to individual peanut consumption

The highest correlation between peanut protein levels in the home was between the mother and father's bed-sheet ($r_s=0.864$, $P<0.001$); these dust samples were usually obtained from opposite sides of the parental bed and I have shown previously that there is cross-transference between both sides of the parental bed.[312] This high correlation was followed closely by peanut protein levels in the infant bed-sheet and play-area ($r_s=0.862$, $P<0.001$), which were usually in different areas of the home (child's bedroom and living-room respectively). This suggests that the baby, the baby's clothes or the person carrying the baby was transferring peanut to these locations. There was a higher association between peanut levels in the infant bed-dust and the maternal bed-dust rather than paternal bed-dust; this is probably because infants often slept in the maternal bed. Paternal peanut consumption was more highly associated with peanut levels in the infants' bed and play-area than maternal peanut consumption, which is likely due to the finding that fathers tended to have higher peanut consumption than mothers.

8.4 EPE: which route is likely to lead to PS?

Routes of exposure to food antigens appear to be crucial in determining whether food allergy or tolerance develops. Peanut containing dust is bound to be in contact with the child's skin in their bed or play-area (which is where infants spend most of their time) but could also be inhaled or consumed by the infant. Inhalation and epicutaneous exposure have been shown to lead to allergic sensitization in mice (usually only with co-administration of an adjuvant or active skin barrier disruption), whereas high dose oral exposure leads to oral tolerance induction, but low-dose oral exposure may lead to sensitization. Of note, the use of

CT with concomitant oral food antigen exposure usually leads to allergic sensitization regardless of the dose administered, which may be due to barrier impairment in the gastrointestinal tract by CT.

8.4.1 EPE leading to PS via the skin

The hypothesis that EPE results in PS through the skin is supported by the increased susceptibility that children with *FLG* loss-of-function mutations had to EPE, and by the exposure-response observed in children with a history of eczema and severe eczema, as discussed in Section 8.7. Not all children with peanut allergy have a history of overt eczema; these children may have had dry skin and an impaired skin barrier even though they did not have overt eczema; in combination with EPE this could have been the route through which transcutaneous peanut sensitization occurred. Alternatively children could have had a transient abrasion of the skin allowing peanut to enter the epidermis. Finally, an impaired gut barrier, following, for example rotavirus gastroenteritis might also be an alternative route of sensitization.

Filaggrin is predominantly expressed in the skin, and *FLG* loss-of-function mutations lead to an impaired skin barrier (as described in Section 4.1). However, filaggrin is also expressed in other parts of the body, such as the upper respiratory tract and upper gastrointestinal tract which has important ramifications for the potential mechanism of PS. The available literature on the expression of filaggrin (either as mRNA and/or histological staining of the protein) in various parts of the human body is summarized in Table 15. Although filaggrin is not expressed in the lung, the presence of *FLG* loss-of-function mutations modifies the risk of smoking on the development of asthma, which may be through the impact of *FLG* loss-of-function mutation in the nasal mucosa. [398] In summary, on the basis of these findings, although mutations in the gene that codes for filaggrin could potentially predispose to barrier impairment in the upper respiratory and gastrointestinal tract, *FLG* loss-of-function mutations are far more likely to affect the skin where filaggrin protein is highly expressed and has a vital function in the skin barrier integrity and hydration.

Table 15: Filaggrin expression in the body

	Filaggrin mRNA	Filaggrin protein
Epidermis of skin (Presland et al. 2000, Sandilands et al. 2009, Wu et al. 2009) [180;182;240]	Yes	Yes
Oral mucosa (Presland et al. 2000, De Benedetto et al. 2008) [180;181]	Yes	Yes
Upper esophageal mucosa (Blanchard et al. 2010)[179]	Yes	N/A
Proximal, mid and distal esophageal mucosa (De Benedetto et al. 2008) [181]	N/A	No
Stomach (Wu et al. 2009) [182]	Yes	N/A
Small intestine and colon (Wu et al. 2009) [182]	No	N/A
Cornified epithelium of the nasal vestibulum (Weidinger et al. 2008) [277]	N/A	Yes
Epithelium covering inferior nasal turbinate (Weidinger et al. 2008, De Benedetto et al. 2008) [181;277]	No	No
Respiratory and bronchial mucosa (Ying et al. 2006, De Benedetto et al. 2008)[181;274]	No	No

*N/A: not available. If protein detected then need for mRNA obviated

There is observational data in humans which supports the concept of epicutaneous sensitization: in the ALSPAC study children with eczema who had Arachis oil applied onto their skin within the first 6 months of life were 6.8 times more likely to develop PA. Another important argument which supports the concept of epicutaneous sensitization to peanut is the known strong association between tissue specific inflammation of

the skin (eczema presence, severity and duration) and PS. [9] For ethical reasons it would not be possible to demonstrate unequivocally that cutaneous exposure to peanut in children with an impaired skin barrier leads to PS and PA; this would require tape stripping of the skin in infants without eczema followed by topical application of peanut (or peanut containing dust) or application of peanut (or peanut containing dust) onto eczematous skin followed by analysis of the immune response and follow-up of these children with peanut OFCs.

8.4.2 EPE leading to PS via the gastrointestinal tract

During the oral mouthing phase of development between 4-8 months one could argue that the infant is likely to ingest some of peanut in the dust in its environment. In mouse models, low dose oral exposure to peanut was shown to lead to PS and PA (see Section 2.5). One argument against small levels of peanut exposure predisposing to allergic PS was shown in the ALSPAC study by Lack et al.(2003) where infants that breastfed after maternal application of nipple cream containing Arachis oil did not have an increased risk of PA. [49] This may, however, also be because Arachis oil was presented in combination with breast milk which is known to have intrinsic immunoregulatory factors and in mice was shown to protect against PA (see Section 2.3.1).[92]

8.4.3 EPE leading to PS via the respiratory tract

The possibility that sensitization occurs in the respiratory tract via inhalation of peanut containing-dust cannot be excluded; however, an argument against inhalation being the route of exposure is that peanut was very difficult to aerosolise. Peanut protein levels were below the LLQ in a variety of experiments using a personal Air Monitor running at 2L/min (equivalent to an infant's minute respiration) only 1cm away from peanut-rich foods (peanut butter, satay sauce, whole peanuts) and in homes with high levels of peanut in vacuumed dust. Even when peanut was forced into the air by breaking peanut shells directly underneath the Air Monitor sampling head, there was only a transient surge in airborne peanut detected, which disappeared immediately after the deshelling stopped.

Previous studies have found that the allergen concentration from vacuumed dust correlates poorly with airborne allergen concentration; [399-401] this is due to the size and shape of the allergen which affects its aerosolizeability when disturbed in the environment and how long it remains airborne. For example, HDM allergen (Der p 1 and 2) and cockroach allergen (Bla g 1) are mainly associated with particles >10 μm in diameter; [402-404] this means that even after becoming airborne most HDM allergen falls back onto the ground due to gravity. After 15 minutes only 10-20% of HDM allergen remains airborne and several hours later <0.1% remains airborne. [405;406] Similarly, cockroach allergen is only detectable for up to 30 minutes after vigorous dust disturbance. [404] In contrast, more than 20% of cat and dog allergens are less than 5 μm in diameter and these can be measured in the air for days after disturbance of dust in the environment. [407] The nature and size of the inhaled particle as well as the way that it is deposited and cleared from the airway are important determining factors for disease pathogenesis. [408] Particle sizes above 30 μm are non-respirable as they are trapped by the nasal hairs and upper respiratory tract mucous. Particles between 4-10 μm usually reach the trachea, primary and secondary bronchioles, particles up to 4 μm reach the terminal bronchioles and alveoli. [409]

In order to determine the particle size of peanut in dust one could determine in which fraction of the dust the peanut resides. In the online repository of Publication (see Section 7.4.1, Fig E3), I showed that there were similar peanut protein concentrations in the larger structures of vacuumed dust samples (e.g. hair and bed-cotton) as compared to fine dust; this could be explained by peanut being transferred via hands onto the bed-linen or onto hair. Within the fine dust post sieving (less than 300 microns in diameter), I would expect peanut to be found in larger particle sizes (>10 μm) given its lack of sustained aerosolizeability. One problem with separating out particle sizes in dust is humidity: if there is humidity in dust (especially if the dust sample has been frozen and then thawed) then dust particles could stick together and be more difficult to separate. One way to reduce the humidity would be to heat the dust samples prior to particle separation; however, use of such a technique could result in changes to the peanut protein structure. As PS could also occur in the nasal mucosa, even with transient levels of airborne peanut, it would be interesting to measure

peanut protein captured by personal nasal filter devices in a bed with known high levels of peanut in dust. Personal nasal filter devices measure inhaled allergens with a particle sizes of 5 µm, [410] and have been used to measure allergen exposure and the efficacy of protective masks. [411]

8.4.4 Is peanut in dust responsible for PS or just a marker of cutaneous exposure?

Environmental peanut levels in dust reflect only a proxy marker of the actual amount of peanut that the infant is exposed to in their environment. This would be extremely difficult to quantify. Additionally, the question remains whether peanut contained in dust could induce PS or whether is it simply a marker of cutaneous exposure to peanut through household hand to infant body contact, given that peanut persists on hands 3 hours after peanut consumption.[360] Another mechanism of transfer of peanut consumed by other household members onto the skin of the infant and PS is through kissing, given that peanut remains detectable in saliva for at least 3 hours after peanut consumption as shown in Publication 1.

8.5 Peanut protein levels in household dust using ELISA, MS and BAT

This PhD confirmed that peanut protein can be measured in dust through a variety of approaches (ELISA, MS and BAT). Each of these analytical methods provided different information about the peanut antigen found in dust but also had certain limitations.

8.5.1 ELISA

The main advantage of the Veratox polyclonal whole peanut ELISA was that it was able to quantify the concentration of peanut protein in dust using a relatively simple and quick procedure. However, the Veratox peanut ELISA had the following drawbacks:

1) The LLQ of the Veratox peanut ELISA was 100ng/ml which is satisfactory for an ELISA; however, this resulted in a truncated EPE variable in MAAS when a fixed LLQ/2 calculation was assigned to the 37% of dust sample extracts with peanut levels below this value. Therefore two sets of analyses

in MAAS were performed, using all peanut-dust values obtained below the LLQ and using the LLQ/2 calculation. Using these two different peanut dust variables did not make a material difference to the results obtained, thus it is less likely that this introduced error or bias.

2) The results obtained by the Veratox peanut ELISA did not reveal which peanut (Ara h) allergens in dust were present or most prevalent as both capture and detection antibodies in the Veratox ELISA are directed against whole peanut protein (see Section 6.4).

3) Although the Veratox peanut ELISA demonstrated that peanut protein in dust bound to the ELISA capture and detection anti-peanut antibodies, thus showing allergen specific binding, this did not show that peanut in dust could interact with the immune system of a peanut allergic individual.

8.5.2 Mass Spectrometry

MS superseded ELISA analysis in its ability to identify major peanut allergen sequences in dust. The University of Manchester demonstrated the presence of peptides corresponding to the major peanut allergens Ara h 1, 2, 3, 6 and 9, with protein coverage of up to 65.3% for Ara h 1, 57% for Ara h 2, 64.1% for Ara h 3 and 76% for Ara h 9 in an infant play-area (see Section 7.6). MS is highly specific, as peptide sequences are determined from amino-acid sequences within a peptide. Therefore, the MS findings supported the potential for peanut protein in dust to lead to sensitization to major peanut allergens which is more likely to lead to PA. Drawbacks of MS were the labour intensive sample preparation and clean-up, in particular to remove keratins from dust samples that interfered with MS. Given the laborious preparation required for each dust sample it would not have been feasible to analyse thousands of dust samples from the homes of different participants as was performed in this PhD. Additionally MS did not quantify peanut protein concentration in dust (although work is ongoing to try to achieve this) and did not provide evidence that peanut allergens in dust interact with immune cells.

8.5.3 BAT

This brings me to the final assay, BAT: the LLQ of the BAT for dust extracts was 1ng/ml peanut protein which was 100 times lower than the LLQ for the Veratox peanut ELISA. Additionally BAT demonstrated that peanut protein in household dust induced a dose-dependent activation of basophils from children with PA but not in NA children, thus confirming a peanut-specific basophil response. This provides biological plausibility for the ability of environmental peanut levels in dust to interact with immune cells in young children and sensitize the individual to peanut. Drawbacks of BAT were also laborious sample preparation time and that BAT did not quantify peanut protein concentration.

In summary the various methods employed as part of this PhD confirmed the presence of major peanut allergen in dust able to stimulate effector cells of children with PA. In future experiments it would be useful to explore how peanut in dust interacts with Th cells and APC as this would be most relevant in terms of the ability of peanut in dust to cause PS. This could be performed by mixing serial dilutions of peanut-containing dust extracts with the PBMCs of peanut allergic individuals versus NA individuals to determine Th cell activation, interaction between APC and Th cells and Th cell cytokine secretion.

8.6 Factors associated with PS and PA

8.6.1 Overview

The relationship between PS and PA and EPE, infantile eczema, *FLG* loss-of-function mutations and egg sensitization for all three cohorts are displayed in Figure 25 and Figure 26 (p271). In both population based studies (MAAS and BAMSE), EPE was not a risk factor for PS or PA in the whole population, whereas in the high-risk CoFAR study, EPE was associated with both PS and likely PA (trend on univariate, significant on multivariate analysis). The most likely reason for the discrepancy in the effect of EPE in the population versus high-risk cohort is due to atopy and/or skin barrier impairment modifying the individual's response to EPE in the high-risk cohort. Both infantile eczema and *FLG* loss-of filaggrin mutations were associated with PS (MAAS and BAMSE), and PA (MAAS) in the population based studies (discussed in detail in section

8.6.3); however, they were not associated with PS or PA in CoFAR. The lack of association between *FLG* loss-of-function mutations and PS in CoFAR is probably because children with known PA were excluded from inclusion into the CoFAR study; thus the *FLG* mutation rate was very low (14.7%) for this highly atopic cohort. The lack of association between infantile eczema and PS in CoFAR is probably because moderate to severe eczema was one of the inclusion criteria for the study thus 92.5% of children had infantile eczema.

Figure 25: Forest plot of main factors associated with PS on univariate LR analysis.

MAAS: Peanut SPT sensitization at 8 and 11 years, CoFAR: Peanut SPT sensitization at 3-15 months, BAMSE: peanut sIgE sensitization at age 4 and 8 years. EPE: OR per unit increase in EPE. Egg sensitization: Yes vs. No

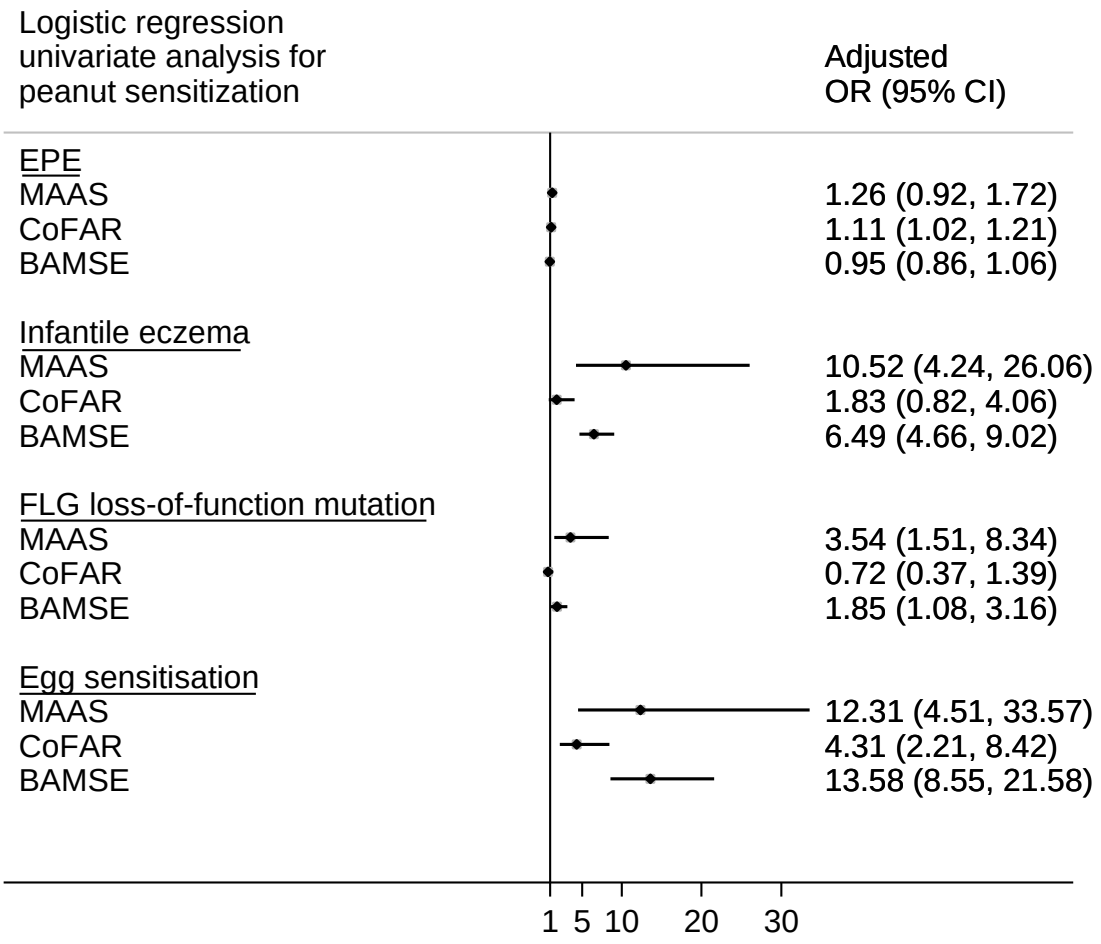
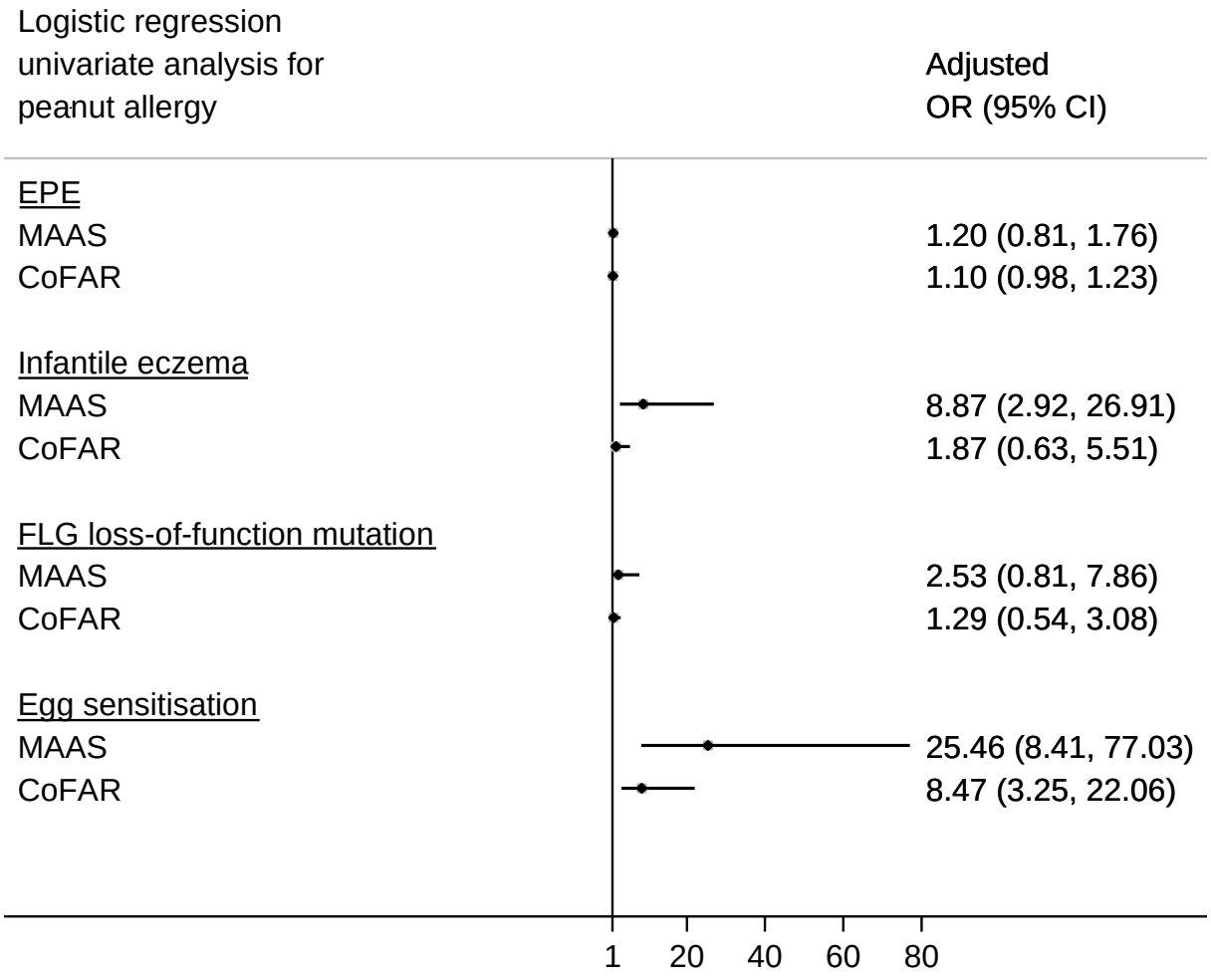


Figure 26: Forest plot of main factors associated with PA on univariate LR analysis.

MAAS: Challenge proven PA at 8 or 11 years, CoFAR: Likely PA (sIgE≥5kU/L) from 3-15 months. EPE: OR per unit increase in EPE. Egg sensitization: Yes vs. No



8.6.2 EPE levels in different cohorts

Although the most likely cause for the discrepancy in the impact of EPE on PS and PA between the population based studies (MAAS and BAMSE) and high-risk studies (CoFAR) is the different atopic status of these population, it should also be noted that peanut-dust levels in the three cohorts were significantly different (Mann-Whitney U $P<0.001$ for each comparison), which may have also influenced the relationship between EPE and PS/PA. Different EPE levels (median [IQR]) between cohorts are shown in Table 16 and Figure 27.

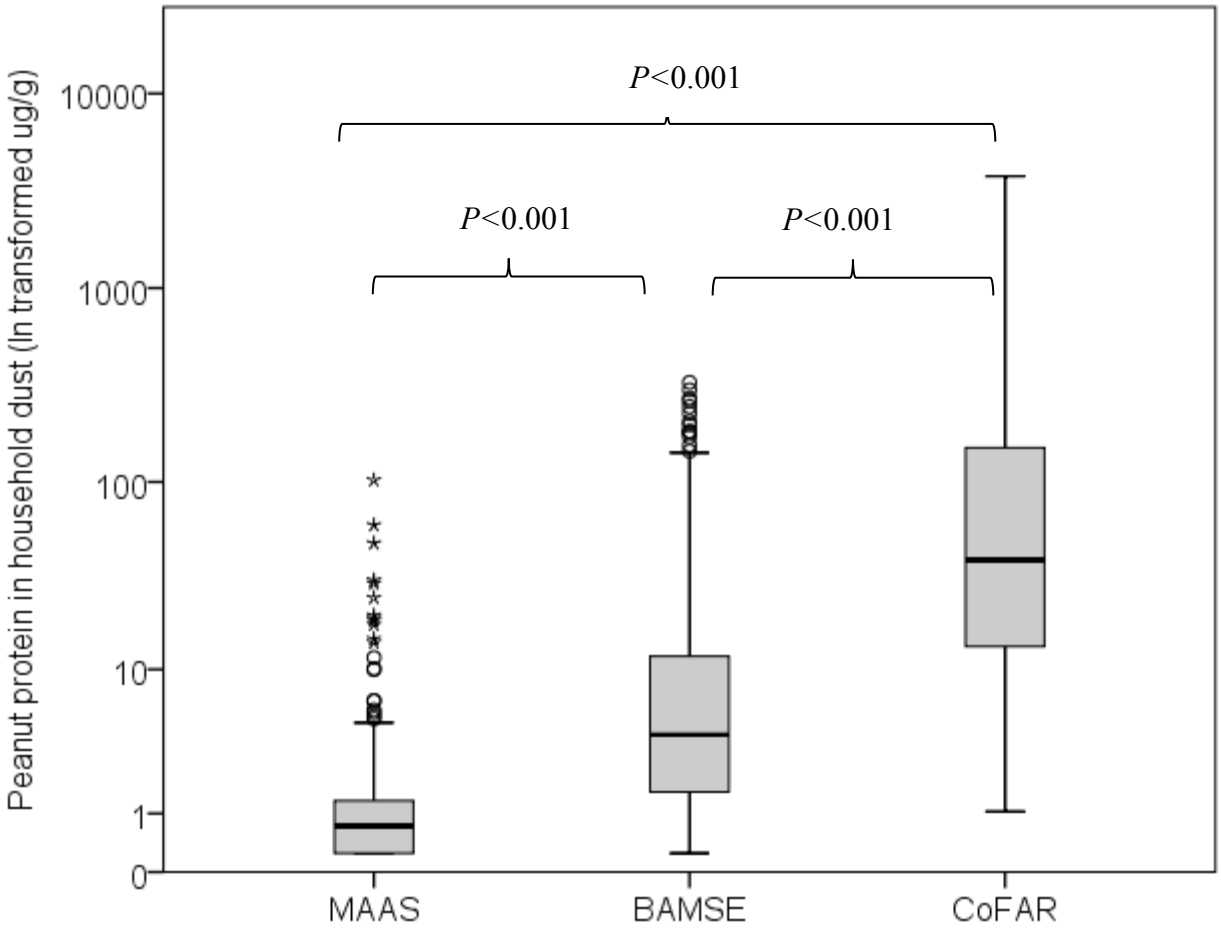
Table 16: Level of peanut protein in dust (µg/g) in three cohort studies.

	Peanut protein in living room dust	Peanut protein in maternal mattress	Peanut protein in infant bed-dust
MAAS (including values below LLQ)	0.73 µg/g [IQR 0.40-1.33]	n/a	n/a
CoFAR	39.1µg/g [IQR 13.4-150.6]	n/a	10.7µg/g IQR [2.5-42.38]
BAMSE	n/a	4.07µg/gram [1.58, 11.86]	n/a

n/a (not analysed)

Figure 27: Comparison of peanut protein in dust (µg/g) in three cohort studies.

MAAS: Living-room dust, BAMSE: maternal mattress dust, CoFAR: living-room floor dust



Participants in the CoFAR (USA) study had the highest levels of peanut in household dust followed by participants in the BAMSE (Sweden) study, then MAAS (UK). It is known that peanut butter is widely consumed in US households; furthermore, 373 out of 421 (86%) families of children recruited to the CoFAR observational study had peanut-containing food in the home during breastfeeding. [98] This is the most likely explanation for the higher levels of peanut protein in dust found in US homes. The difference between peanut levels in dust in BAMSE versus MAAS could be explained by differences in peanut consumption levels in Stockholm versus Manchester respectively; however, no information on HPC was available in these two studies. The difference in peanut levels in dust between these two cohorts could also potentially be explained by different locations of dust samples in these two studies (sofa in MAAS and maternal mattress in BAMSE) as the mattress might provide a larger reservoir of peanut. However, in Publication 1 I showed that there was high within-home distribution of peanut protein in dust, thus one would expect that if peanut levels are high in the maternal mattress they would also be high in the sofa.

Finally, the difference between MAAS and BAMSE peanut levels in dust could be explained by the fact that I was sent peanut dust extracts (liquid extract with dry ice to prevent thawing) from MAAS whereas I was sent dust samples from BAMSE. Peanut protein in extracted dust may be less likely to remain stable over prolonged period of time (these dust samples were collected between1996-1997), than in dust itself.

8.6.3 Infantile eczema and *FLG* loss-of-function mutations as risk factors for PS / PA

In both population based cohorts included in this PhD (MAAS and BAMSE), infantile eczema was a risk factor for PS and PA (where available), over and above the effect of *FLG* loss-of-function mutations. Infantile eczema has been shown to be a stronger risk factor for PS and PA than *FLG* loss-of-function mutations in other studies. In three month old infants enrolled to the EAT (Enquiring About Tolerance) population based study (www.eatstudy.co.uk), infantile eczema increased the risk of PS 4-fold after adjusting for *FLG* loss-of-function mutation and sex. [412] *FLG* loss-of-function mutations did not increase the risk of PS; however, raised TEWL, a phenotypic measure of skin barrier impairment was associated with

a 2.2-fold increase in risk of food sensitization (cow's milk, egg or peanut SPT ≥1mm) after adjusting for *FLG* loss-of-function mutations, infantile eczema and sex. [412] Thus although *FLG* loss-of-function mutations were not associated with food sensitization in the EAT study, a more general measure of skin barrier function was associated with food sensitization at 3 months.

In a recent publication from the Isle of Wight study, Venkataraman et al. (2014) found that *FLG* loss-of-function mutations were likely to be associated with food allergy in later childhood (10 and 18 years) rather than earlier childhood (1, 2 and 4 years). [285] The effect of *FLG* loss-of-function mutation on food allergy was mediated through eczema and food allergic sensitization in the earlier years. *FLG* loss-of-function mutation carriage was associated with early childhood eczema at 1, 2 and 4 years, early childhood eczema was associated with food allergic sensitization at 4, 8 and 10 years, and food allergic sensitization was associated with food allergy at 4, 10 and 18 years. The authors therefore postulated that *FLG* loss-of-function mutations had an indirect effect on food allergy mediated by eczema rather than a direct effect on food allergy.

Entering infantile eczema into the multivariate LR model reduced the association between *FLG* loss-of-function mutations and PS and PA (where analysis available) in MAAS and BAMSE. *FLG* loss-of-function mutations predispose an individual towards developing eczema;[259] however, the inflammatory milieu of eczema (IL4, IL5) also down-regulates the expression of filaggrin protein in the skin.[281] Thus, even without *FLG* loss-of-function mutations, eczema may reduce filaggrin expression and alter skin barrier function through this and other pathways (e.g. the itch scratch cycle). It is therefore difficult to tease out the difference in the effects of *FLG* loss-of-function mutations and eczema as both lead to skin barrier impairment and each independently lead to reduced filaggrin levels in the skin.

The two landmark papers that first described the association between *FLG* loss-of-function mutations and eczema showed that *FLG* loss-of-function mutations were present in 56%[413] and 47%[268] of Irish

Caucasian children with moderate to severe eczema. Studies since then have shown lower associations between *FLG* loss-of-function mutations and eczema ranging from between 14.2% (using R501X only in Germany) to 42% (in England) in adults and children of Northern European descent (n=15, median 22.75% [IQR 18.4-35.0%]).[259] *FLG* loss-of-function mutations vary according to the number of mutations screened, ethnicity, disease ascertainment (population, primary or secondary care) and eczema disease severity. The findings of these studies suggest that around 80% of eczema is not dictated by the individual's *FLG* genotype. There are various other epidermal proteins (Section 4.2), which also contribute to skin barrier function but, in essence, atopic eczema seems to be multifactorial and was a more potent risk factor for PS and PA than *FLG* loss-of-function mutation in population based cohorts.

8.6.4 Egg sensitization as a risk factor for PS and PA

In all three cohorts preceding egg sensitization was the strongest risk factor for PS and PA (see Figure 25, p270 and Figure 26, p271); this remained significant in the majority of multivariate analyses. Although it has long been known anecdotally that egg allergy is a risk factor for PA, as reviewed by Sampson (2002),[27] there had been no original data to prove this until the LEAP study screening data showed that egg sensitization and severe infantile eczema were the strongest determinants of PS.[9] In the MAAS, CoFAR and BAMSE cohorts I found that the inclusion of egg sensitization in the multivariate model strengthened the association between EPE and PS / PA. The influence of egg sensitization in the multivariate model for EPE and PS/PA is not entirely surprising as Fox et al. (2009) found that the impact of HPC (used as an indirect marker of EPE) on the rate of PA in young children was far stronger when comparing peanut allergic cases against egg allergic controls as opposed to comparing peanut allergic cases against population controls.[104] I therefore postulate that egg SPT sensitization reflects the earliest and strongest atopic indicator and that adjusting for this atopic marker increases the positive association between EPE and PS and PA. In this respect it should be noted that most common sensitization in children with eczema is egg (see Figure 7 (p80) and Figure 8 (p80)),[146] and egg allergy is one of the strongest predictors of respiratory allergies.[414]

8.7 Factors that modify the relationship between EPE and PS and PA

There were differences between the three studies with regards to the factors that modified the impact of EPE on PS/PA; PS was modified by *FLG* loss-of-function mutations in MAAS, eczema and eczema severity in the CoFAR study and EPE was only associated with PS in the BAMSE study when compared to the relevant control groups (egg sensitized children or children matched for parental atopy and sex). Whereas one might have expected more homogeneity in the interactions observed in these three studies, given the important differences in demographics, peanut allergic outcomes measured, and atopic risk between the populations it is not surprising that these differences existed. In the sections below I will discuss the differences in the results obtained from the three cohort studies and reasons as to why this may have occurred.

8.7.1 Interaction between *FLG* loss-of-function mutation and EPE on PS and PA

Although there was a very impressive interaction between EPE and *FLG* loss-of-function mutation on PS and PA in MAAS this was not replicated in the CoFAR or BAMSE study. In both CoFAR and BAMSE, *FLG* mutation rates were low which may have in part resulted in the lack of association. In CoFAR the *FLG* mutations rate was 14.9% which is very low given the high rate and severity of eczema in this cohort as previous studies have shown that *FLG* loss-of-function mutations are present in up to 56% of children with moderate to severe eczema. [259;260] The low *FLG* mutation rate in CoFAR can be explained predominantly by the fact that children with confirmed PA prior to study enrolment were excluded from the CoFAR study. Given the known association between *FLG* loss-of-function mutations and PA, this would have excluded a lot of the *FLG* mutation carriers.[286] In the BAMSE study the rate of *FLG* mutation was only 7.14% which is low in comparison with other cohorts (average 9-10%); [257;286] however, eczema rates in BAMSE were not particularly high (17%) in comparison to MAAS (33.7%) and CoFAR (92.5%) thus BAMSE was a relatively non-atopic cohort. In the CoFAR study another explanation for the lack of *FLG* interaction with EPE on PS and PA may be that, as 54% of children already had severe eczema, the skin barrier was already impaired and had reduced filaggrin levels secondary to eczematous inflammation thus the addition of *FLG* loss-of-function mutations did not significantly add to the skin barrier impairment that was already present.

8.7.2 Interaction between a history of eczema and EPE on PS and PA

In a similar fashion, although CoFAR found that a history of eczema augmented the impact of EPE on PS and likely PA, this modifying effect of eczema was not found in the MAAS or BAMSE study. These three cohorts evaluated the period prevalence of infantile eczema in different ways which may have led to different levels of eczema ascertainment. In MAAS, parental report of a history of infantile eczema was assessed using a modified ISAAC questionnaire to apply the UK working party's diagnostic criteria for atopic eczema. The UK working party's diagnostic criteria is derived from the Hanifin and Rajka criteria for eczema,[144;415] and has been validated against physician diagnosed eczema in both hospital and population based settings (as per MAAS). [144;415;416] Diagnostic criteria for the UK working party consist of an itchy skin condition in the last 12 months plus three of more of the following criteria: 1) history of flexural involvement, 2) onset under the age of 2 years, 3) a personal history of other atopic disease (in children aged under 4 years history of atopic disease in a first degree relative may be included), 4) history of a generally dry skin, and/or 5) visible flexural dermatitis as per a photographic protocol.

In the CoFAR study, history and maximum severity of eczema was assessed and graded by a non-validated tool comprising (1) extent of disease (by "rule of 9"), (2) course of disease (by history), and (3) intensity of disease (disturbance of night's sleep by itching), each on a 3-point scale.[417] The "rule of 9" is used to calculate the area of the body's skin affected for SCORAD (scoring AD) assessment, where the head and neck amount to 9%, upper limbs 9% each, lower limb 18% each, anterior trunk 18%, back 18% and genitals 1%.[418] In the BAMSE study infantile eczema was assessed using the following set of criteria; dry skin, itchy rashes for ≥2 weeks and specific localisation (face or arms/legs extension surfaces or arms/legs flexures or wrists/ankles flexures) of rash and/or doctor's diagnosis of eczema up to 1 year of age.[419]

Studies have shown that the UK working party diagnostic criteria for atopic eczema had a high negative predictive value (NPV) for eczema of 97% but a low PPV of 47% when compared against a doctors' diagnosis of atopic eczema; i.e. this suggests that the UK working party diagnostic criteria for atopic eczema

incorrectly diagnoses atopic eczema where it is not present.[144] PPV rises with increasing prevalence of disease, thus the positive pick-up and reported diagnosis of atopic eczema was likely to be higher in a high-risk population such as CoFAR. Given that eczema was more severe in the CoFAR study, individuals with eczema were more likely to have been assessed by a physician and thereby be correctly diagnosed, whereas in the general population (particularly in the non-atopic BAMSE population), the broader spectrum of eczema is likely to have been missed. The interaction between EPE and eczema on PS and PA is likely to require the spectrum of mild to severe atopic eczema in patients, and the lack of pick-up of mild-moderate cases of eczema in BAMSE and MAAS may therefore have resulted in a reduced synergistic interaction with EPE.

Although BAMSE did not show an interaction effect between eczema or *FLG* loss-of-function mutations and EPE on PS, there was a significant impact of EPE on PS when peanut sensitized cases were compared against non-peanut sensitized, egg sensitized controls and when peanut sensitized cases were compared against non-peanut-sensitized high-risk controls matched for sex and parental atopy. Thus this represents a similar picture of EPE having a greater impact when compared against high-risk children. The exposure-response relationship between EPE and PS (when compared against atopic controls) was stronger between EPE and peanut CRD sensitization (Ara h 1,2 or 3≥0.35kU/L) than between EPE and non-clinically relevant PS (peanut sIgE ≥0.35kU/L but negative results for Ara h 1,2 or 3<0.35kU/L) (Figure 24, p230); this supports the concept that EPE increases the risk of clinically relevant PS.

Fox et al.(2009) showed that HPC was more significantly associated with clinical PA when compared against egg allergic controls than when compared against non-atopic controls.[104;143] Egg sensitization was highly associated with PS in BAMSE; thus it may be that egg sensitization shows a predisposition to mount allergic antibodies to other allergens as egg allergy is highly associated with the development of other food allergies, asthma and aeroallergen sensitization.[414;420] Observational studies have shown that in children with severe,

early onset eczema, up to 50% are sensitized to egg;[146] thus egg sensitization may be a marker of eczema severity and thus also an indirect marker of skin barrier impairment.

8.7.3 Summary and clinical implications

In summary, although differences were present in population, study design, characteristics of the population and results obtained a common theme emerges; namely in children with evidence of skin barrier dysfunction e.g. history of infantile eczema or indirect correlates of skin barrier dysfunction e.g. *FLG* loss-of-function mutations or egg sensitization, EPE increases the risk of sensitization to peanut.

The different effect of EPE in the three cohorts assessed highlights the importance of choice of population when designing an interventional study to prevent PA or indeed other food allergies. Should neonatal screening for *FLG* loss-of-function mutations be considered as a screening tool for enrolment or should family history of atopy or eczema be used as an assessment of risk? In the case of PS and PA it seems that it would be better to target eczema rather an *FLG* loss-of-function in an interventional study, given its stronger association with the outcome of interest. There are, however, drawbacks to using infantile eczema as a screening tool for an interventional study as, once eczema is visible, the skin barrier will already be impaired and transcutaneous sensitization is likely to have already occurred, In the LEAP study 20% of infants with eczema already had sIgE to peanut from 4 months of age.[17] If *FLG* mutations were used as a screening tool, one could identify at-risk infants for environmental modifications prior to them developing overt eczema. However, many children without *FLG* mutations develop eczema, whether due to mutations in other skin barrier proteins (Section 4.2) or other inflammatory processes leading to the eczema phenotype thus *FLG* genotyping would miss these children. Additionally, more sophisticated markers of skin barrier impairment could be included in the design of the study: for example in children with *FLG* loss-of-function mutations, increased TEWL (see Section 4.8) has been shown to precede the onset of atopic eczema. [284]

8.8 Limitations of studies assessing EPE

8.8.1 Limitation of study comparing HPC and peanut protein levels in dust

In the semi-quantitative FFQ, peanut consumption in day care or other locations where the child might be exposed to peanut was not included; additionally, participants were only asked to document peanut containing foods consumed in the home. Limitations of this were highlighted by one family where there was very little apparent HPC but surprisingly high levels of peanut in household dust; on further questioning it was revealed that the child's grandmother ate Crunchy Nut Cornflakes every day in the car as she did not want to expose her grandchild (who had confirmed PA) to peanut. This is interesting on two accounts:

1) Peanut consumption may not need to be in the home to be associated with high household peanut levels in dust and further supports the route of hand to environment transfer.

2) The fact that this child was peanut allergic raises the question as to whether the grandmother eating Crunchy Nut Cornflakes was in part responsible for the child's PA.

In future FFQs I would therefore not limit the participant's consumption of peanut-containing foods to those consumed in the home but would also include those consumed outside the home, as peanut foods consumed outside the home may also impact on peanut levels in dust.

8.8.2 Limitation of cohort studies

In the discussion section of publications 3 (MAAS) and 4 (CoFAR study) the limitations of these cohort studies are provided in detail. There are three limitations which affected all cohorts (including also BAMSE) which I will discuss: (1) the lack of information on infant peanut consumption, (2) the lack of available dust for analysis in some participants and (3) the lack of comprehensive PS and PA assessments.

8.8.2.1 Lack of infant peanut consumption

The lack of information on the child's peanut consumption in the first year of life was a significant limitation in all studies. This was due to the retrospective nature of analysis in this PhD and because the cohort studies involved were not designed to answer the questions asked in this PhD. There are two ways in which infant peanut consumption could confound the association between EPE and PS/PA:

1. Infant peanut consumption increases peanut protein levels in household dust, as shown in publication 2. In the cohort studies, peanut levels in dust were used as a measure of exposure to peanut in the environment through other members in the home eating peanut rather than being a reflection of what the infant or young child ate. Given that oral peanut consumption should protect against PS/PA, even in children with high levels of HPC by other family members as shown by Fox et al. (2009),[104] if the child's peanut consumption was contributing to peanut protein levels in household dust this would negate the risk relationship between EPE and PS/PA.

   With regards to the first potential confounding effect, this is not relevant for the MAAS and BAMSE cohort as dust samples analysed for peanut were collected either antenatally or in the first 6 months of life (bar 2 dust samples at 1 year in MAAS) when infants would not have been consuming peanut. However, in the CoFAR study dust was collected at a median age of 9 months [IQR 7-12] when it is possible that children would have started eating peanut containing foods such as peanut butter (especially in a US cohort where peanut butter consumption is very high).

2. Peanut consumption occurring after exposure to peanut in the environment during the first year of life could also protect against PS and PA

   It is possible that despite high EPE around the time of birth, that the introduction of peanut as a weaning food could prevent PS or prevent PS progressing to PA. In order to address this second potential confounder, an RCT would be required where infants who were not eating peanut would be

randomised to eating peanut after dust samples were collected versus continued avoidance of peanut. The Enquiring About Tolerance (EAT) study is a population based RCT assessing whether early introduction of peanut and other allergenic foods (from 3 months of age) with concomitant breastfeeding versus exclusive breastfeeding until 6 months reduces food allergy at 3 years of age. Consumption of peanut after 6 months of age in the control arm has not been forbidden but has also not been actively encouraged. A detailed evaluation of peanut consumption by the child and other household members has been obtained by validated dietary questionnaires. Dust samples have been collected at 3 months of age in the bed-sheet and play-area of infants recruited to the EAT study (www.eatstudy.co.uk). The 1302 children recruited to this study have been followed up and are currently undergoing OFCs to peanut and other allergenic foods. The EAT study design will therefore assess whether EPE at 3 months of age increases the risk of PS and PA at 3 years of age, and whether this effect is modified by the infant consuming peanut from 3 months of age in the intervention arm of the study. These data will be available for analysis towards the end of 2015 once all year 3 EAT study visits have been completed thus is not included in this PhD.

8.8.2.2 Lack of available data for analysis

Dust for peanut protein analysis was only available in a proportion of patients from each cohort. Additionally only data from children with available *FLG* genotyping were included in the final analysis for MAAS and BAMSE (which also therefore excluded non-Caucasians). Excluded data ranged from 54.1% (2211/4089) for BAMSE, to 47.4% (561/1184) for MAAS to 29.9% (359/512) for CoFAR. Although in MAAS and CoFAR there were differences between various population demographics between those participants included versus excluded in the studies, there were no significant differences in the rate of PS or PA (where available), thus these differences are less likely to have influenced the results. Demographics of included versus excluded participants were not compared in BAMSE as the final sample size obtained was large (n=1878), therefore it is more likely that this large number of participants would adjust for any bias incurred through missing data.

8.8.2.3 Lack of comprehensive PS and PA assessments

PS and PA outcomes in the cohorts were not comprehensive except for the MAAS. In MAAS PS status was available from testing with SPT, peanut sIgE and peanut component allergens and PA was also assess by OFC. In the CoFAR study PS was assessed by SPT and sIgE. The CoFAR study has performed peanut component allergen testing and also has assessed PA by OFC at 3 years of age; however, this information was not made available for incorporation into this PhD as the CoFAR group have not yet published these findings. The main limitation in the CoFAR study was therefore that only PS and a 'likely' diagnosis of PA could be assessed using the serological cut-off of sIgE to peanut $\geq 5 kU_A/ml$. The CoFAR group defined this group of children as having likely PA,[98] given that 70-90% of 5-7 year old children have positive diagnostic peanut challenges with this level of peanut sIgE.[18;149;421] Once peanut CRD and OFC based PA outcome data are made available if will be interesting to see whether the association found between EPE and PS and PA remains. Additionally, the 3 year PA outcome data will show whether early EPE is associated with later onset PA, whereas at present only contemporaneous EPE and PS/ likely PA have been assessed. In the BAMSE study, only peanut sIgE and peanut component allergen testing was available as only 2.6% (n=49/1878) of children in BAMSE underwent peanut SPT testing therefore peanut SPT sensitization was not included in the analysis. Furthermore, no children underwent OFCs to assess for clinically confirmed PA.

8.9 Further research for EPE

8.9.1 Impact of dust containing peanut in a mouse model

In order to assess a more causal role of EPE on the development of PS and PA, a mouse model could be employed where neonatal mice with an impaired skin barrier (using the flaky tail mouse or by tape stripping wild-type BALB/c mice) were exposed to peanut protein in dust. Peanut containing dust could be compressed into the bedding of young mice (torn tissue hankies) which they do not usually eat (personal communication Dr Sophie Wavrin, French National Institute for Agricultural Research (INRA), June 2013). Immune responses from spleen and LNs as well as anaphylactic responses to oral peanut gavage could subsequently be assessed in this group of mice versus control to determine whether early EPE could drive Th2 sensitization and /or clinical manifestations of PA.

8.9.2 Randomised controlled study for reduction in EPE

The data obtained in this PhD provides useful information to determine what levels of peanut reduction are required to reduce the risk of PS and PA. Data from publication 2 demonstrated that peanut protein levels in household dust from an infant's home environment were highly correlated with HPC (grams/week) over 6 months. Using the raw data from Publication 2, I calculated the conversion factor for HPC at certain levels of peanut protein in dust in the infant's play-area. Using this conversion factor I was able to assess the impact that a reduction in HPC would provide on EPE. According to this calculation, a reduction in HPC from 20 g/week (ln 3.0 g/week) to 5 g/week (ln 1.6 g/week) would result in a reduction in peanut protein levels in the infant's play-area from 144 µg/g (ln 5.0 µg/g) to 5 µg/g (ln 1.61 µg/g). 20 grams per week of HPC is equivalent to a household of 4 people each eating 1-2 slices of peanut butter on bread (3.75 grams peanut protein) per week. A reduction of HPC to 1g/week would reduce EPE to below the LLQ of the Veratox peanut ELISA (<25 ng/ml peanut protein). Using data from the CoFAR study (Figure 28) I assessed the impact of reducing HPC and peanut protein levels in household dust on the predictive probability of peanut SPT sensitization (Table 17, p286). Reducing from 20grams/week HPC to under 1 gram per week

HPC would reduce the risk of PS in children with atopic eczema (ranging from from mild to severe) from 64% to 39% (a 39% risk reduction). Thus the findings obtained from this PhD could be used towards estimating the effect size in reducing PS with a reduction in HPC and therefore EPE, and this could then provide information towards powering an RCT with the aim to reduce PS by reducing HPC.

There may be a long lag-time between reducing HPC and a concomitant reduction in peanut levels in household dust as per the findings in studies to reduce cat allergen levels in the home once the pet cat has been rehoused.[422] The time-course for the reduction of peanut levels in dust following a reduction in HPC would have important influences on the design of an interventional study i.e. measures to reduce peanut protein levels in household dust such as reducing HPC might need to start before conception which would make patient selection complex.

Figure 28: Mean predictive probability of PS dependant on HPC or EPE in all children, children with a history of eczema or severe eczema.

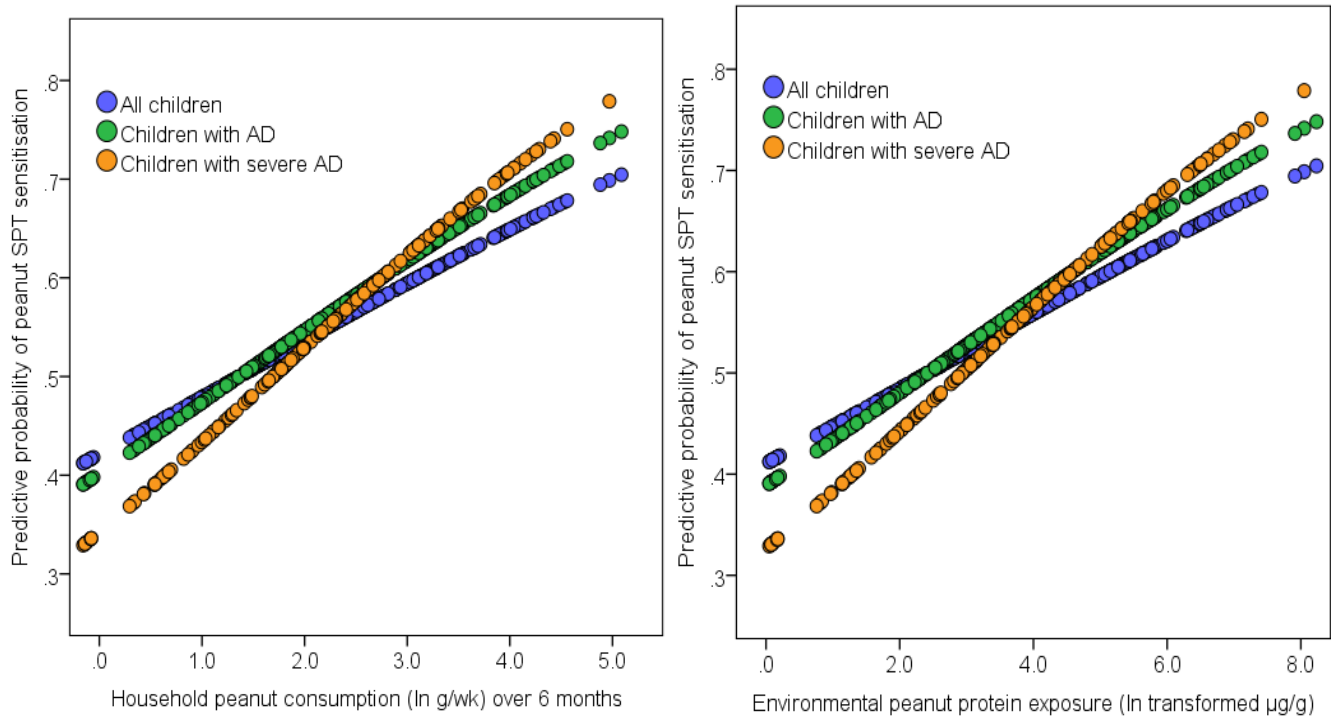


Table 17: Reduction in HPC on EPE and the probability of PS in high-risk infants.

	HPC (6 months)	EPE	Probability of PS
All children	20 g/week (*ln* 3)	150 µg/g (ln 5)	62%
Children with history of infantile eczema	20 g/week (*ln* 3)	150 µg/g (ln 5)	64%
Children with history of severe infantile eczema	20 g/week (*ln* 3)	150 µg/g (ln 5)	67%
All children	10 g/week (*ln* 1.6)	50 µg/g (ln 3.9)	57%
Children with history of infantile eczema	10 g/week (*ln* 1.6)	50 µg/g (ln 3.9)	59%
Children with history of severe infantile eczema	10 g/week (*ln* 1.6)	50 µg/g (ln 3.9)	59%
All children	<1 g/week (*ln* 0)	<LLQ	42%
Children with history of infantile eczema	<1 g/week (*ln* 0)	<LLQ	39%
Children with history of severe infantile eczema	<1 g/week (*ln* 0)	<LLQ	33%

8.9.3 Peanut in dust extinction study

If a RCT were to show that a reduction in EPE (through reduction in HPC) was to reduce the incidence of PS and PA, then, to be able to translate this into public health measures, it would be important to know how quickly peanut protein levels could be removed from the home environment after household members discontinue eating peanut. Findings from publication 1 showed that a significant reduction in peanut protein levels could be achieved by washing soft furnishings (sofa covers had median 1000-fold reduction in peanut protein levels) and bedding (pillows had median 40-fold reduction in peanut protein levels) with a single machine wash at 60 ºC using normal detergent.

It would also be important to know how quickly peanut levels disappear from the home after peanut consumption is discontinued without resorting to intensive cleaning measures. On the basis of currently available data from publication 1, the correlation between peanut in dust and HPC over 1 and 6 months was similar. This may be because eating habits do not usually change (unless there is a specific reason for this such as a new diagnosis of PA in a household member), but also due to peanut protein persisting in the environment due to its resilient nature. In results from my MSc study a single peanut meal led to 48 hours duration of detectable peanut in the participant's bed-dust, however this could have been confounded by removing peanut from the bed surface every time the bed-sheet was vacuumed.

To date there is no study which has assessed the rate of extinction of peanut levels in dust after HPC is discontinued. In the LEAP and LEAP-ON study dust samples have been collected from the child's bed before their peanut OFC at 5 years (LEAP study completion) and 6 years (LEAP-ON study completion). In the LEAP study, infants were randomised to peanut consumption or avoidance for the first 5 years and following this underwent a peanut oral food challenge at 5 years of age to determine PA or tolerance. Children were

subsequently recruited to the LEAP-ON study and asked to avoid all peanut for 1 year and called back for another peanut oral food challenge to determine whether high dose oral peanut consumption in the first 5 years of life led to oral tolerance induction or merely transient desensitization. It is hoped that there will be a significant reduction in the level of peanut protein in the bed-dust of previous LEAP peanut consumers after one year of peanut avoidance. Results of the LEAP-ON study will be available in 2015; they will only show whether peanut levels in dust reduce after one year of peanut avoidance. However, they will not show whether a more short term avoidance of peanut leads to reduction of peanut levels in household dust. Therefore I am currently recruiting 20 adult volunteers into a study where they will be asked to eat peanut regularly for 2 months and then avoid peanut for 6 months (other household members are also asked to avoid eating peanut). Dust samples from their bed will be collected before commencing peanut consumption and will then be obtained every month thereafter until the end of the study (8 months). This will allow a more accurate estimate on how quickly peanut disappears from the home environment.

8.11 Discussion of findings from in-vitro work

The purpose of this mechanistic section of the PhD was to assess the compartmentalization of Th2 recall responses to peanut from skin (CLA+) versus gut (β7) homing Th cells in children with PA; this would have supported the concept that PS occurs through the skin which is the overriding concept for this PhD. Although there was no predominance in Th2 cytokines from CLA+ Th cells in peanut recall responses, we postulated that, whilst the original Th2 gene expression profile is limited to the skin-homing CLA+ Th cells, over time Th2 responses became dominant in all lymphocyte homing compartments. Although the findings from this mechanistic work were not what had been expected, they did reveal a potential novel biomarker for PA, IL9, which furthermore ties in with the concept of epicutaneous sensitization (see Section 3.9). The gene with the highest differential expression between PA and PS was not one of the usual Th2 signature genes (e.g. *IL4, IL5* or *IL13*) but the more recently described *IL9* gene (publication 5). IL9 induces mast cell proliferation and production of IL4 induced IgE thus may have an important role in the pathogenesis, severity and persistence of PA. Although, *IL9* was also expressed in both skin and gut-homing memory Th cells in children with PA, the role of IL9 in the skin provides an argument for its contribution in epicutaneous PS as discussed in the introduction (Section 3.9) and in the discussion below (8.11.5).

8.11.1 IL9 as the best differentiator of PA in children

IL9 was shown to be the best differentiator of PA in children using Affimetrix gene microarrays analysed by ANOVA and Random Forest classifiers and using RT-qPCR (publication 5). Although *IL9* had the highest differential gene expression when recall responses from PBMCs from children with PA were compared against NA children and peanut sensitized children, *IL9* was not actually the most highly expressed gene in peanut allergic children (RT-qPCR analysis). *IL5,* a classical Th2 cytokine, was more highly expressed in peanut allergic children than *IL9* (25.4-fold for *IL9* versus 61.7-fold for *IL5)*; however, *IL5* was also more highly

expressed in peanut sensitized and atopic NA children than *IL9* (see Table 18). Thus *IL9* expression in PA had a lower false positive rate and a higher specificity than *IL5* which is why *IL9* was the highest differentially expressed gene for PA.

Table 18: Differential gene expression of *IL9* and *IL5* in children with PA, PS and NA

Gene	Peanut allergic children RQ (AU)	Peanut sensitized children RQ (AU)	Atopic non-peanut allergic children RQ (AU)
IL9	25.4 IQR [4.09-65.33]	1.37 IQR [1.21-3.88]	1.09 IQR [0.96-2.10]
IL5	61.73 IQR [4.29-167.87]	3.40 IQR [1.14-14.29]	2.86 IQR[1.80-4.97]

*AU: Arbitrary units

The lower specificity of *IL5* may be because *IL5* is stimulated more non-specifically in both PS and atopic NA children, thus has a higher background level, making it less useful in ruling in PA. *IL9* was able to classify PA using the Random Forest classifier with 100% accuracy, even when selecting only 10, then 5, then 2 genes, which is quite remarkable. Although we did not proceed further to evaluate *IL9* as a predictive tool in the diagnosis of PA, this would be interesting research and further mechanistic assessments of IL9's role in PA are warranted. There is a need for novel biomarkers that will improve diagnostic testing for PA so as to be able to avoid the risk of undertaking a diagnostic OFC, as well as the inconvenience to the family and cost to the healthcare system. There is already a patent on file by Jordana M and Waserman S (2011) for a diagnostic classification algorithm to diagnose PA which incorporates the age of the individual, SPT size, total IgE and the cytokines IL9, IFNγ, IL10, IL13 and IL5.[(423)] Additionally, biomarkers could be used to monitor efficacy of immunomodulatory treatment for PA (such as peanut desensitization) by providing surrogate endpoints.

8.11.2 IL9 and Th9 cells

IL9 is secreted by a variety of cells, including mast cells and innate lymphoid cells,[424] as well as Th cells (Th2, Th9 and inducible T regulatory cells[iTregs]).[425] Until recently IL9 was described as a Th2 cytokine as it was thought to be produced by Th2 cells and was involved in various Th2 responses; however, ICCS of cytokines in naïve Th cells from mice cultured in Th9 conditions (TGFβ and IL4) showed that these cells produced IL9 (and IL10) but did not produce IL4, IL5 or IL13, suggesting a distinct lineage of Th cells.[426] In publication 5 we also found evidence that IL9 was being produced by cells that did not produce IL5; this suggests that the cells producing IL9 in response to peanut antigen were not Th2 cells. I would have liked to further characterise the lineage of IL9 producing Th cells in order to strengthen the argument that IL9 is produced by the newly discovered Th9 cell; however, this was difficult due to the lack of confirmatory characteristics of the Th9 cell.

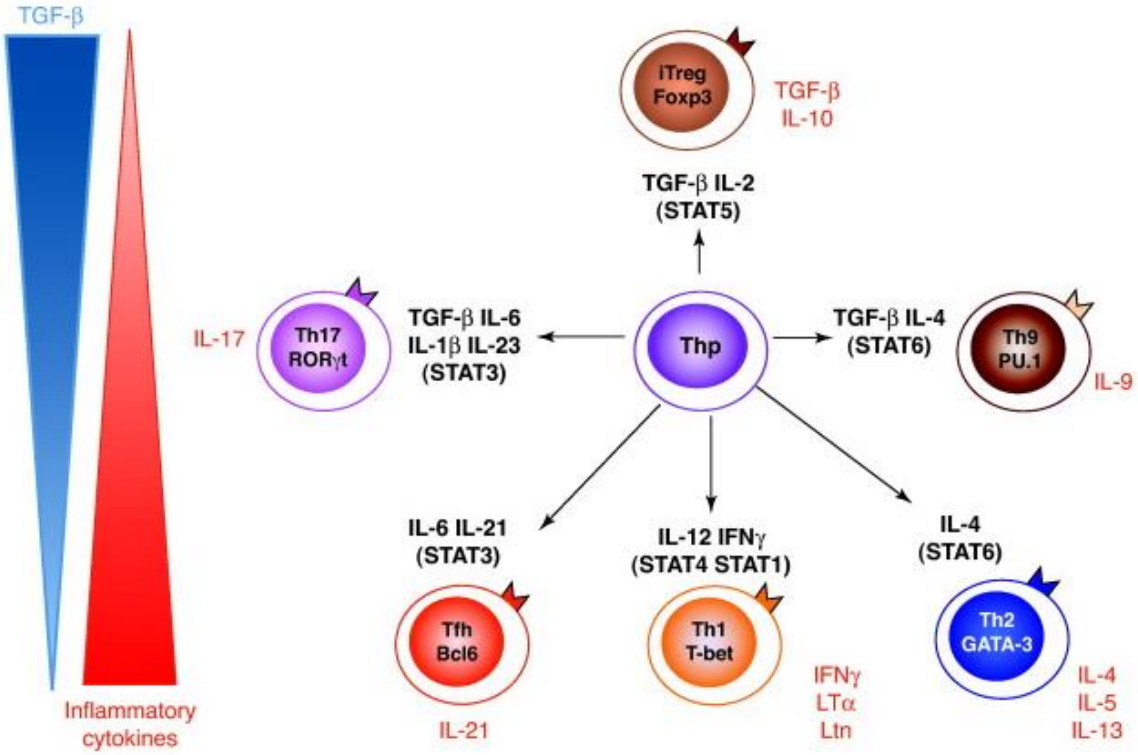
There are generally two ways to identify Th cell phenotypes: (1) by their immune function and cytokine secretion and (2) by their lineage specific transcription factors. Murine data have demonstrated that Th9 cells produce IL10;[427] however, the evidence for Th9 production of IL10 in humans is lacking. [425;428] Th9 cells produce IL9 but, as described above, so do Th2 and iTregs, [425] thus it may be more prudent to define Th9 cells by the cytokines that they do not secrete (Th2: IL4, IL5 and IL13 and iTreg: IL10, TGF-β). Th cells are often defined by their lineage specific transcription factors; for example Tbet for Th1 cells,[429] GATA3 for Th2 cells[430] and RORγT for Th17 cells.[431] These transcription factors, which determine the T-cell phenotype, are stimulated in naïve Th cells by cytokines in the surrounding milieu via the T cell receptor complex and co-stimulatory molecules (see Section 1.6 and Figure 29).

The development of IL9 secreting Th cells is promoted by the cytokines IL4 and TGFβ; if only IL4 signalling occurs then Th2 cells are created, whereas if only TGFβ signalling occurs then inducible T regulatory cells are

produced (via induction of Foxp3).[432] The lineage specific transcription factors for IL9 secreting Th cells in murine models are a mixture of transcription factors downstream of IL4 (STAT6,[433] GATA3, and IRF4[434]) and TGFβ (PU.1).[433;435]

Figure 29: Development of Th effector subsets from Th precursors (Thp).

Cytokines and STAT proteins required for the differentiation into each Th phenotype. A sliding scale TGF-β or inflammatory cytokines is shown to display the variable concentration of cytokines in the microenvironment that primes the differentiation of each Th subset. Reprinted from Perumal NB et al. (2011) with permission from Elsevier.[436]



8.11.3 Lineage transcription factors for Th9 in mice

PU.1 is a transcription factor which binds to the PU-box, a purine rich sequence found near the promoters of target genes and regulates their expression in coordination with other transcription factors. [435] In murine models, TGFβ switches off GATA3 (the master transcriptional regulator of Th2 cells) with a resulting switch in Th cytokine secretion from IL4, IL5 and IL13 to IL9.[426] Murine models have also shown that TGFβ prevents expression of the transcription factor Tbet in favour of PU.1; [437] PU.1 then induces an open chromatin formation at the *IL9* locus, which allows for binding of transcription factor IRF4 and transcription of the *IL9* gene. [437]

8.11.4 Lineage transcription factors for Th9 in humans

Less data exist regarding lineage specific transcription factors for IL9-producing T-cells in humans. When Th9 cultures from human PBMCs were transfected with PU.1-specific small interfering RNAs (siRNAs) there was decreased IL9 production.[438;439] In atopic human subjects, PU.1 was only found in a proportion of IL9 producing Th cells (median 52%; range 5%-90% of Th9 cells). The authors postulated that whilst PU.1 might be necessary for the differentiation of naïve Th cells into Th9 cells, once the Th cell was polarized this transcription factor was no longer expressed. [440] In the study by Cortelazzi et al. (2013) staining for PU.1 was present in 23.4% of total lymphocytes in the epidermis of individuals with atopic eczema versus 5.8% of total lymphocytes in the epidermis of individuals with contact dermatitis; some of the epidermal PU.1 + cells co-expressed CD4, thus could have represented Th9 cells. IL9 staining was also higher in the skin of patients with atopic eczema than contact dermatitis; however, the authors did not state whether cells that stained positive for IL9 cells also stained positive for PU.1.[226] In the Asthma, Allergy and Respiratory Science Department at King's College London researchers were unable to detect the PU.1 transcription factor in human IL9-producing

Th cells (personal communication: Professor David Cousins, King's College London, October 2012), thus I did not evaluate PU.1 as a lineage specific transcription factor for the Th9 cell phenotype.

8.11.5 Biological relevance of IL9 for PS and PA

8.11.5.1 IL9 and its role in epicutaneous sensitization and clinical reactivity

The biological relevance of IL9 in epicutaneous sensitization is likely to be related to the mast cell function of IL9 which includes mast cell activation, release of inflammatory mast cell mediators (e.g. histamine) and expression of mast cell protease, pro-allergic cytokines (IL4, IL5, IL6, IL9, IL13) and FcεRIα receptors.[236;441;442] IL9 additionally potentiates IL4 induced IgE production by B cells.{Dugas, 1993 964 /id} The interplay between IL9, mast cells, Langerhans cells and TSLP in the process of epicutaneous sensitization is discussed in Section 3.9.4.1.[233] The role of IL9 on mast cell migration and proliferation in the skin and gut may also play a role in clinical reactivity following epicutaneous peanut sensitization as discussed in Section 3.9.4.2. Mast cell IgE cross-linking of the high affinity FCεRI receptor by specific antigens results in the release of pro-inflammatory mediators such as histamine and prostaglandins resulting in the immediate effects of an allergic response and recruitment of other inflammatory cells (see Section 1.6).

8.11.5.2 IL9 and its role in oral sensitization and clinical reactivity

In mouse models *IL9* has been shown to be a crucial factor for anaphylaxis following oral antigen exposure.[443;444] *IL9* deficient mice failed to develop experimental oral antigen–induced intestinal anaphylaxis (acute diarrhoea, intestinal mastocytosis and mast cell activation) after being sensitized with IP OVA with aluminium adjuvant. Following sensitization *IL9* deficient mice had reduced intestinal mast cells and serum mMCP-1 levels in the gut, but mice with *IL9* overexpression in the enterocytes of the intestine (using a transgenic

intestine-specific promotor gene)[445] developed OVA specific IgG1 and total IgE, intestinal *IL4* (mRNA and protein), intestinal mast cell expansion, leakage and anaphylaxis (diarrhoea and increase in mMCP-1) following oral exposure to OVA.[443;446] In the mice with intestinal *IL9* overexpression intestinal anaphylaxis occurred after repeated oral doses of peanut without the need for prior IP OVA sensitization, whereas in wild-type BALB/c mice prior IP OVA sensitization was required with aluminium adjuvant to mount Th2 responses or symptoms upon rechallenge. These findings suggest that in mice with IL9 overexpression in the intestine, there is a phase of sensitization that occurs through the gut which then leads to intestinal anaphylaxis.

In humans, intestinal mast cell expansion has also been reported in subjects with food allergy; [447] this may play a role in increasing intestinal permeability and transcellular passage of large amounts of antigen across the intestinal epithelium leading to food sensitization and subsequently food allergic reactions. [446] The mast cell stabilizer cromolyn has been used to prevent intestinal permeability and mast cell activation in mice which constitutively overexpress IL9 in the enterocytes of the gastrointestinal tract. In the study by Forbes et al. (2008) pretreatment with cromolyn prevented orally induced antigen sensitization (OVA sIgE) and anaphylaxis.[443] These findings suggest that mast cell mediated intestinal activation and degranulation may be an important factor for specific antigen sensitization and subsequent anaphylaxis through oral exposure.

8.11.5.3 Mechanism why IL9 differentiate between PS and PA

The importance of IL9 for mast cell for mast cell proliferation in the gut may explain why this cytokine differentiates between PS and PA. PS occurs via both Th2 and Th9 pathways; however, lack of IL9 means that there will be lower densities of mast cells in the skin and gut; this in turn may mean that the threshold for clinical reactivity is not reached.

8.11.6 Homing markers of Th2 and Th9 cells in peanut allergic children

Th9 cells (CD4+IL9+IL13-IFNγ-) have been shown to circulate in the blood of allergic patients, but not in non-allergic patients, and a significant population of these Th cells have CLA+ homing markers.[448] Thus it is surprising that in PMBCs from peanut allergic patients we did not find a CLA+ predominance in IL9 secreting Th cells and Th2 cells; however, given the murine literature that epicutaneous sensitization leads to migration of mast cell and mast cell expansion in the gut, it may be that, that whilst the original Th2 and Th9 gene expression profile is limited to the skin-homing CLA+ Th cells, over time Th2 and Th9 responses became dominant in all lymphocyte homing compartments. This could be as a consequence of migration of mast cells from the skin to both skin and mesenteric LNs following mechanical injury to the skin; once the mast cells are in these LNs the MIP-1β recruits Th cells into both skin and mesenteric LNs.

8.12 Limitations of mechanistic work

8.12.1 Further characterization of IL9 and its role in PA

The main limitation of the mechanistic work was the need to further evaluate *IL9* as a biomarker for PA. By increasing the sample size of participants and employing statistical models it may have been feasible to evaluate *IL9* as a predictor for correctly diagnosing PA and to define PPVs and NPVs for optimum *IL9* cut-offs. Additionally, evaluation of downstream signalling pathways for IL9 in children with PA would have been very useful to understand the role of *IL9* in PA.

8.12.2 Further characterization of IL9 secreting Th cells

Although the findings from this PhD suggested that IL9 was secreted by Th cells which were not Th2 cells (due to expression of IL9 and IL5 by different CD4+CD45RO+ cells on ICCS), this did not prove that these cells

were Th9 cells. Further work still needs to be done to identify lineage specific transcription factors for Th9 cells in humans in order to identify whether IL9 secreting Th cells are indeed Th9 cells.

8.12.3 PBMC culture conditions

One limitation in the in-vitro work performed was that several patients' blood was cultured in RPMI with autologous serum before the decision was made to switch to the culture free AIM-V medium to increase the amount of cells and RNA obtained for microarray analysis. Initially the low RNA obtained was thought to be solely due to the technique of RNA extraction. Thus several patient blood samples that had been cultured in RPMI could not be used in the final analysis. Another limitation was that PBMCs were cultured for only 18 hours rather than over a period to time to assess kinetics of *IL9* expression. In previous microarray studies using recall responses to HDM in atopic versus non atopic controls, *IL9* had the highest differential expression between 24-48 hours rather than at 12-24 hours.[79] Xie et al. (2012) found that *IL9* from PBMCs of peanut allergic versus non atopic controls started to increase predominantly after 2 days of culture up to a maximum fold increase at 5 days.[449] Thus culturing PBMCs for at least 48 hours may have led to even higher differential expression of IL9 in the microarray and RT-qPCR experiments used in this PhD.

8.13 Further mechanistic research

8.13.1 Kinetics of *IL9* expression

IL9 differential gene expression in Th cells from peanut allergic children was found after 18 hours peanut culture, whereas Bosco et al. (2006) found *IL9* upregulated in recall response from PBMCs of HDM allergic individuals at 48 hours.[79] Kinetic experiments using microarray gene expression analysis following PBMCs cultured in peanut for 18, 36 and 54 hours would evaluate whether *IL9* continued to be the predominant gene expressed or whether *IL9* would be superseded by other genes.

8.13.2 Longitudinal studies to assess Th homing markers as PS progresses to PA.

Given the lack of predominance of CLA+ Th cells in peanut recall responses in peanut allergic children, I postulated that the CLA+ Th cells might predominate initially during PS and then spread to other Th cells subsets (i.e. include β7+ Th cells) once PA is established. Longitudinal analysis following up immune responses in children at high-risk of developing PA would elucidate whether there is migration from CLA+ Th cell to both CLA+ and β7+ Th cell predominance in recall responses to peanut as children progress from PS to PA.

8.13.3 IL9 as a biomarker for PA prediction and prognosis

It would also be interesting to conduct a longitudinal study assessing *IL9* expression in peanut sensitized children to determine whether memory Th cells from peanut sensitized children who go on to develop PA have higher *IL9* expression in the presence of peanut than memory Th cells from PS children that do not go on to develop PA. This would provide evidence in support of IL9 being used a predictive biomarker for PA. Assessment of *IL9* as a biomarker of anaphylaxis in humans would also be valuable given its central role in anaphylaxis in mouse models (see Section **Error! Reference source not found.**).

8.13.4 Anti-IL9 antibody therapeutics

The obvious direction for any research on biomarkers of disease is to evaluate whether a monoclonal antibody directed against this biomarker could reduce the severity or cure the disease. Given the success of anti-IL9 antibodies in mice on the prevention and reduction in severity of food anaphylaxis, there may be a role for IL9 antagonists in the prevention of PS and PA in humans. Although no studies to date have evaluated humanized anti-IL9 antibodies for the prevention or treatment of PA, there have been studies assessing anti-IL9 monoclonal antibodies in the treatment of asthma. In a small RCT (n=34) primarily conducted to assess the

safety of a humanized anti-IL9 antibody (MEDI-528), there were positive effects on asthma symptom control, reduction in exacerbations in adults and reduced exercise induced bronchoconstriction.[450] Unfortunately, in a larger RCT (n=327), addition of an IL9 antagonist was not associated with any improvement in asthma symptoms scores, exacerbation rate or forced expiratory volume in one second (FEV1) in adults with severe asthma.[451] The authors concluded that given the heterogeneous nature of asthma, in particular severe uncontrolled asthma, that the identification of subgroups of patients would be key in delivering an optimal response to anti-IL9. However, disappointingly when they assessed subgroups with atopic asthma, peripheral blood eosinophilia and patients taking moderate to high doses of inhaled corticosteroids, the researchers did not find a response to anti-IL9.

The efficacy of biologicals directed against specific cytokines or transcription factors (using monoclonal antibodies) is often disappointing, which may be due to the network of signals which are responsible for a particular clinical outcome and redundancy within immunological systems. It may be, therefore, that combinations of biologicals (including for example anti-IL9 and anti-IL5 monoclonal antibodies) need to be used to achieve the outcome of interest. However, biologicals have drawbacks due to the requirement for subcutaneous or intravenous injection and the risk of adverse effect given that nature reuses signalling pathways that are effective (such as IL9 and IL5) throughout the body.

8.14 Summary and conclusions

This PhD is the first study to evaluate the role of environmental exposure to a food antigen on the development of food sensitization and allergy. Although Fox et al. (2009) showed that HPC was a risk factor for PA, this was only an indirect measure of EPE, whereas now this PhD confirms that HPC does indeed lead to higher levels of EPE and therefore provides the 'missing link' in the study by Fox et al. (2009).[104] The findings in this PhD reveal previously unknown characteristics of peanut antigen in dust, such as the ability of the peanut allergens in dust to elicit antigen specific responses in immune effector cells of peanut allergic children, and the presence of major peanut allergens in dust. It would be interesting to assess whether other food allergens maintain their biological activity and major allergens once distributed in the environment as dust.

This PhD is also the first study to evaluate the impact of EPE on PS and PA in cohort studies and to determine factors that modify this such as atopy and markers of skin barrier impairment. The finding that a *FLG* loss-of-function mutation and a history of eczema interacts with EPE to increase the risk of PS and PA provides an exciting avenue to identify high-risk children from birth (using genetic markers) or early infancy (in children with eczema or raised TEWL) and target them with environmental modifications. On the basis of the publications arising from this PhD to date, this approach has been cited as an avenue of interest in a recent review by Leung et al. (2014): [452] '…*environmental peanut might drive sensitization to peanut allergy in patients with atopic dermatitis (AD), particularly in those with FLG mutations, which is a clinically relevant example of the importance that environmental exposure in house dust might contribute to allergen sensitization in patients with AD*.' (p. 771)… '*These articles suggest the possibility that controlling environmental peanut levels in the household might reduce peanut allergen sensitization'* (p. 774).

Additionally in the study by Tordesillas et al. (2014) evidence that epicutaneous application of peanut antigen was able to induce PS and PA in mice without the need for skin abrasion or an adjuvant led these authors to postulate '*Our results with peanut allergen support the hypothesis that environmental exposure to peanut is an important risk factor for sensitization*.' (p. 2). In this PhD, I demonstrated that HPC is the factor most highly associated with peanut levels in dust in an infant's bed and play-area (where infants are most likely to be exposed in their environment). These findings therefore also provide the strategy by which EPE could be reduced in interventional studies. It is, however, important to consider that children may be exposed to peanut outside the home, such as in nursery or at their grandparent's home.

Finally, this PhD supports the role of *IL9* as a potential biomarker for PA, which has only been previously shown in one study in adults by Xie et al. (2012). [(449)] My PhD supports the findings from the Xie et al. (2012) manuscript but also extends them by assessing children, and by comparing PA versus PS (rather than just PA versus NA). In this PhD *IL9* was the most highly differentially expressed gene in PA compared both to atopic NA children and PS children. A biomarker that was able to differentiate between PA and PS would be useful due to the limitations of current SPT and sIgE testing which often requires OFC to differentiate between PA versus tolerance in PS individuals. Further research is required to evaluate *IL9* in predictive models to assess sensitivity, specificity and reliability of *IL9* in PA diagnosis and prognosis (by natural resolution or to monitor outcome in peanut desensitization studies). The interplay between Th9 and Th2 subsets may clarify whether the success of preventative therapeutic approaches aimed at PA resolution could be evaluated on the basis of IL9 secretion and/or Th9 suppression in peanut-specific in vitro responses. Future research into the role of Th9 cells in the development of PS and PA via epicutaneous sensitization is warranted in both murine and human studies, given the possible interplay between EPE leading to epicutaneous PS via TSLP and Th9 cells.

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Chapter 10 Appendices

10.1 Appendix 1: Environmental peanut protein sampling methodology

Wipe sampling methodology

'Benchkote' filter paper (Whatman Ltd, Maidstone, UK) measuring 4x4cm was moistened with PBS to wipe different surfaces for peanut protein as shown in Figure 30. The Benchkote wipes were decided upon because alternative wipes were not appropriate; Cellulose wipes became fragmented and glass fibre filter wipes were thought to potentially release glass fibres which might be harmful for infants when sampling near their bed. A different pair of gloves was used to collect each wipe sample. Wipes were weighed before and after sampling to calculate results in µg peanut protein/gram filter.

Figure 30: Wipe sampling

Dust sampling methodology

Philips cylinder vacuum FC8262 (1600 Watts) was connected to a Dustream adaptor and collector (Indoor Biotechnologies Inc., Warminster, UK) with a nylon collection filter (pore-size 40µm) for dust samples collected for Publication 1,2 and 4 (Figure 31 and Figure 32). For vacuuming indoor allergens in dust, motor power wattage of over 1000 Watts is recommended (personal communication Dr Jill Warner, Imperial College London, January 2009). Although motor power is not directly related to suction power, the vacuum needs

enough motor power to have enough suction power to sample sufficient quantities of dust, and should be used at full power. The Indoor Biotechnology Dustream Collector kit has been shown to be compatible with most Commercial U.S. vacuum cleaners (n=48). [453] The dust collector was able to fit 9 vacuums tested without the adaptor, 18 vacuums with the adaptor and 21 vacuums with the adaptor reversed. In this study they also showed that this method of dust collection resulted collection of a mean of 326 mg of dust from carpets and 254 mg of dust from beds and mattresses.

Figure 31: Dust collection apparatus

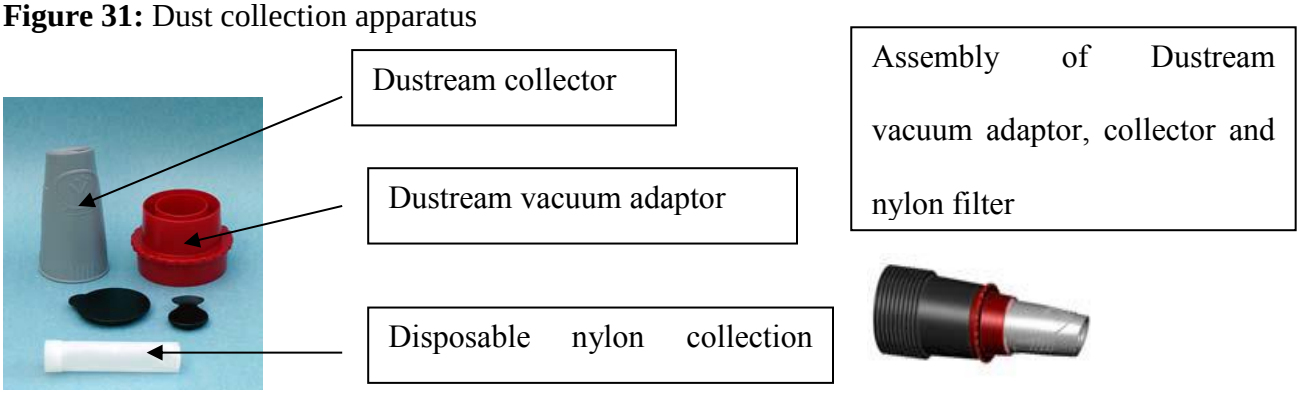


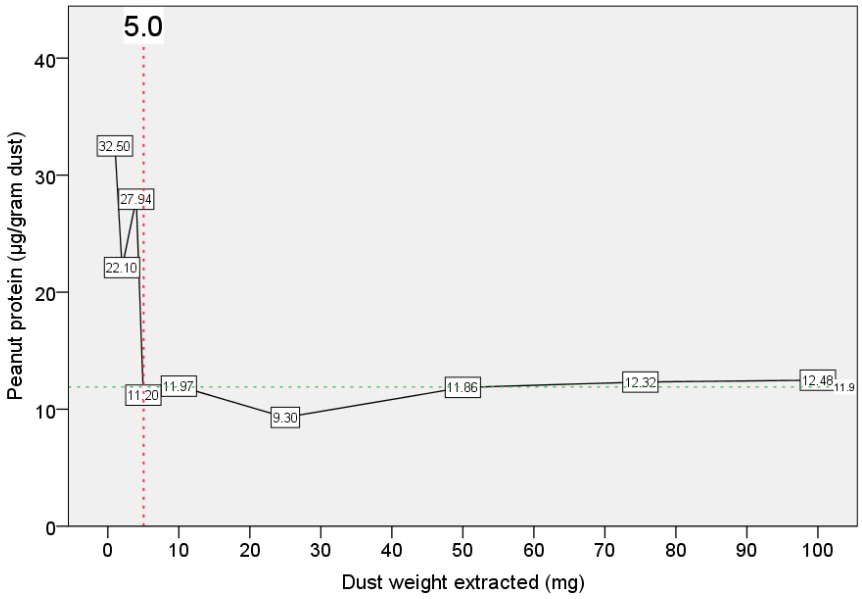
Figure 32: Vacuuming bed

10.2 Appendix 2: Dust processing

Weight of dust validation work

To assess the minimum weight of dust that could be used for peanut protein analysis, one dust sample with 11.9 µg/g peanut protein was separated out into dust weights of 100mg, 75mg, 50mg, 25mg, 10mg, 5mg, 4mg, 2mg and 1mg. Peanut protein was quantified using the Veratox peanut ELISA kit against whole peanut protein (see Section 6.4). Results obtained for peanut protein (ng/ml) were extrapolated to µg/gram of dust taking into consideration the initial weight of dust extracted. For example 5mg of dust was multiplied by 10 in order to compare with the results obtained for a 50 mg dust sample. Dust sample weights extracted down to 5mg gave similar peanut protein levels to dust samples weighing 10mg, 25mg, 50mg, 75mg and 100mg; however, dust samples weighing 4mg, 2mg or 1mg significantly overestimated (Figure 33). For this reason, all dust samples less than 5mg were excluded from analysis.

Figure 33: Weight of dust validation experiment



10.3 Appendix 3: ELISA Background, methodology and validation

Background of ELISA methodology

The ELISA is common antibody-based laboratory technique that is used to measure the concentration of antigen or antibody in solution. There are three common ELISA formats: direct assays, indirect assays and sandwich assays.

1) Direct assays have an antigen or antigen-specific antibody directly immobilized to a multiwell plate; the antigen or antigen-specific antibody are detected by an antibody or antigen (respectively) which is directly conjugated to an enzyme.

2) Indirect assays also have primary antigen-specific antibodies immobilised to the multiwell plate; subsequently, secondary enzyme-linked antibodies are bound to the primary antibodies. The secondary antibody is usually an anti-species antibody (for example anti-rabbit IgG) and is often polyclonal thus increases the sensitivity of the assay as more than one secondary antibody can bind each primary antibody.

3) Sandwich assays comprise matched antibody pairs that are specific for non-overlapping different sections (epitopes) of the antigen of interest. The primary 'capture' antibody is immobilized to the multiwall plate, then the antigen is added to each well and a secondary antibody directed against the antigen of interest measures the concentration of the antigen. This type of assay has a higher specificity as the capture and detection antibody bind two different epitopes of the antigen of interest.

a. In a *direct* sandwich ELISA the capture antibody is conjugated to an enzyme
b. In an *indirect* sandwich ELISA the detection antibody is conjugated to an enzyme and the capture antibody remains unlabelled.

Veratox ELISA kit background

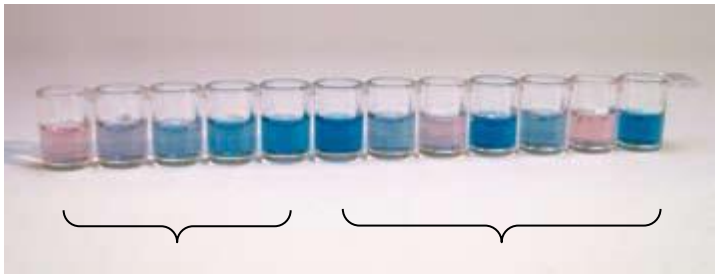
The Veratox ELISA is a polyclonal indirect sandwich ELISA. The Veratox ELISA comprises polyclonal IgG 'detection' (that line the bottom of the ELISA well) and 'capture' antibodies (within the conjugate antibody ELISA solution) against whole peanut protein. This means that it detects multiple different peanut allergens *and* multiple different epitopes within those allergens. This results in a very sensitive assay. The Veratox ELISA is a commercially available ELISA; Neogen (Europe) use the following methodology to produce the components of the ELISA. The detection and capture antibodies are synthesized using rabbits immunized with a blend of 32 raw and roasted peanuts. The rabbits are then bled and the antibodies obtained are screened for activity and purified before use. The Veratox ELISA has been validated for sensitivity, specificity, reliability and efficacy of extraction for determining peanut protein contamination in food. [358;454] In Section 5.7 I discussed the validation previously performed on ELISA kits to measure peanut protein in food in terms of accuracy and reliability. As part of my MSc and in the online repository of Publication 1 the Veratox polyclonal peanut ELISA (Neogen Corporation, Europe) was compared against two other peanut ELISAs and assessed performance characteristics of the Veratox peanut ELISA. Following this validation step the Veratox polyclonal peanut ELISA (Neogen Corporation, Europe) was used to quantify peanut protein levels in dust and wipes.

In this PhD, each 24-well ELISA run was able to analyse 6 extracted dust samples performed in duplicate (n=12) and five peanut standards also performed in duplicate (n=10). Additionally one positive control (250ng/ml) from the independent peanut standard and one negative control (plain extraction solution) were also analysed. First 150 µl of standards and samples were individually pipetted into the red transfer wells. A multi-channel pipette was used to transfer 100µl of each sample from the transfer wells to the corresponding antibody coated well. Following a ten-minute incubation period antibody coated wells were emptied into the sink and washed five times with wash buffer. The wells were then tapped onto a flat surface with paper towels to

remove the excess wash buffer and were visually inspected to ensure no air bubbles remained. The polyclonal anti-peanut IgG conjugated horse-radish peroxidase (100 µl) was subsequently added to each well and incubated for ten minutes. Wash steps were performed as previously described. The substrate TMB was then added to each well (100 µl) and incubated for ten minutes. The ELISA wells changed from pink to blue dependent on the amount of TMB cleaved by the enzyme horse-radish peroxidase, which in turn was dependent on the amount of peanut bound to the antibody-coated ELISA well (see Figure 34). Sulphuric acid (100µl) was added to the solution to halt the horse-radish peroxidase enzymatic breakdown of TMB and the colour change was analysed on the spectrophotometer within ten minutes.

Figure 34: Veratox peanut ELISA colour change

The first 5 wells are peanut standards (0, 100, 200, 400 and 1000 ng/ml whole peanut) provided by the Veratox peanut ELISA kit. Subsequent 7 wells are individual samples.



Standards Unknowns

Optical density (OD) measurements were made on a Precision EMax microplate reader (Molecular Devices Inc. USA) at 650 nm wavelength. Results were compared against the standard curve run with each plate. Log-log analysis of the standard curve and samples was selected as recommended by the Veratox peanut ELISA kit. To accept the OD values obtained, the correlation coefficient of standard curve (ranging from 0ng/ml to 1000ng/ml) had to be ≥0.98, or the ELISA was repeated. Results were converted from ng/ml whole peanut into

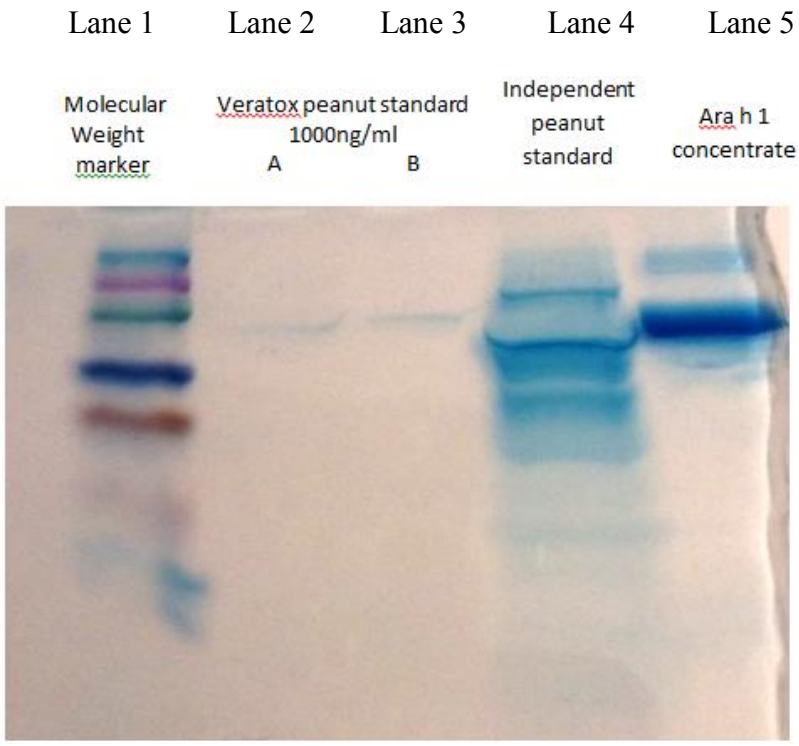
peanut protein by dividing the results by four as recommended by the manufacturer. Results were converted from ng/ml to μg peanut protein/gram dust in a manner proportional to the amount of extract solution used for each sample. Dust samples weighing 50-100 mg after sieving were extracted in proportional amounts of extraction solution between 1-2 ml respectively. Thus to obtain results of peanut protein per gram dust, the results in ng per ml were multiplied by 20 to convert from 50 mg to 1gram. To convert peanut protein results from nanograms to micrograms the results were divided by 1000. Dust samples less than 50 mg were all extracted in 1ml of extraction solution as it was not possible to use less extraction solution or there would be insufficient volume to perform the ELISA analysis. In order to account for the fact that a dust sample weighing 5mg through to 50 mg would be extracted in 1ml of extraction solution, peanut protein results (μg/g) were multiplied up according to the weight of dust extracted i.e. 5 mg was multiplied by 10 to become 50 mg.

Protein band corresponding to Ara h 1 in Veratox ELISA standard

The Veratox ELISA has been shown to detect peanut allergens Ara h 1 and 2 using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Densitometry by other investigators. [(455)] SDS-PAGE is the most common type of gel electrophoresis which employs a polyacrylamide gel and buffers loaded with Sodium Dodecyl Sulphate (SDS). The SDS-PAGE maintains proteins in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structures (such as disulphide bonds) and thus allows separation of proteins by their molecular weight. These proteins become negatively charged as they are covered with SDS and when a current is run through the polyacrylamide gel they migrate towards the positively charged electrode. Small proteins migrate faster through the polyacrylamide gel mesh than larger sized proteins. Thus the proteins are separated according to size, and a molecular weight which runs contemporaneously to the sampled proteins is used to determine the size of the protein.

To confirm the presence of major peanut allergen in the independent peanut standard and the Veratox peanut ELISA standards an SDS-PAGE was run on the independent peanut standard (Lane 4: which was used in various validation experiments and as an independent positive control in the Veratox ELISA analysis) and on the Veratox 1000 ng/ml standard (Lane 2 and 3 duplicate) which was used for the standard curve analysis. The standards used in the Veratox peanut ELISA were the same blend of raw and roasted peanuts used to sensitize the rabbits and create the detection and capture antibodies. A molecular weight marker Coomassie Brilliant Blue staining was run in Lane 1 and the monoclonal Ara h 1 standard from the Indoor Biotechnologies ELISA was run in Lane 5. Figure 35 shows the presence of a protein around 70 kDa in size in the independent peanut standard (Lane 4) and Veratox ELISA peanut standard (Lanes 2 and 3 in duplicate), which corresponded with a protein band in the independent peanut extract and monoclonal Ara h 1 concentrate. Anti-Ara h 1 antibodies were not used to confirm that the protein band was Ara h 1(as per Western Blot) which is a limitation.

Figure 35: SDS-PAGE to evaluate Ara h 1 content in the maximum peanut standard



Independent positive peanut standard and negative standard

All ELISAs suffer from batch to batch variability because different batches of peanuts are used to create the standards for the ELISA. To ensure consistency of results for each cohort, an independent positive standard, created from one single batch of aliquoted lyophilised defatted peanut protein, was used throughout all dust analyses. An independent peanut standard was provided by ALK Abello (Hørsholm, Denmark) and created as described in the online repository of Publication 1. This peanut extract was used as an independent positive standard (at 250ng/ml) with each ELISA plate that was run in addition to the standards provided by the Veratox ELISA kit. An independent negative peanut standard which consisted of plain extraction solution was also employed so as to ensure that the extraction solution used to extract peanut protein from dust had not already been contaminated with peanut protein. For each ELISA these independent positive and negative standards were assessed and if they were within allotted range acceptable for the ELISA (calculated as one standard deviation from the mean) then the results for that ELISA were included in the analysis, otherwise the ELISA was repeated. Additionally, every time a new Veratox peanut ELISA kit batch was received from Neogen Europe, we ran all the Veratox peanut standards (0-1000ng/ml) and compared them to the ODs obtained for the independent peanut standard (0-1000ng/ml).

Analysis of extracted dust samples above the upper limit of the standard curve

All samples above the upper limit of the standard curve (1000ng/ml) were diluted so that results were interpreted on the basis of OD results obtained along the linear part of the standard curve. Samples were therefore diluted 2-fold, 5-fold, 10-fold. 20-fold then up to a maximum of 30-fold in a step-wise approach so that the results obtained ranged between 0-1000ng/ml. Results obtained in ng/ml were then multiplied up depending of the dilution factor used.

Analysis of extracted samples below the lower limit of quantitation (LLQ)

There are several ways in which results below the LLQ are usually dealt with: either using imputation or by diving the LLQ by two or the square root of two.[392] I chose to divide peanut protein levels below the LLQ by 2 as this has been shown to be more applicable to skewed data (all of the EPE data were skewed before log transformation). [392] All results below the LLQ of the Veratox peanut ELISA (100 ng/ml whole peanut) were therefore assumed to be 50 ng/ml; however, in the case of the MAAS study, given that 37% of samples were below the LLQ and thus would be censored, it was felt by the reviewers at the JACI that it would be more appropriate to include the actual EPE values obtained for values below the LLQ.

10.4 Appendix 4: Semi-quantitative food frequency questionnaire

To assess the relationship between environmental peanut protein levels in household dust and surfaces with peanut consumption in the home, a semi-quantitative peanut FFQ was employed. The FFQ used in this study was previously compiled using paediatric dietitians' peanut avoidance dietary sheets from a tertiary allergy centre and from the Anaphylaxis Campaign (a charitable organization that offers support to the families of children with allergies). [456] The peanut-containing foods compiled were categorized into a variety of food groups including spreads, snack, cereal bars, chocolate bars/sweets and miscellaneous foods, which included many savoury foods from different ethnic backgrounds (e.g. peanut soup, satay sauce). The FFQ did not include peanut oils as these have not been shown to lead to sensitization or allergy (see Section 2.6), or foods containing 'trace' amounts of peanut as these would have not contributed significantly to overall peanut consumption. Peanut containing foods were mixed in with other types of foods, including foods typically associated with food allergy (cow's milk, sesame, wheat, soya and fish) so as to prevent too much emphasis on peanut and thus potential recall bias. Peanut containing foods were favoured towards the beginning of the FFQ as evidence suggests that accuracy may reduce towards the end of FFQs due to participant completion fatigue.[457]

To calculate how many grams of peanut protein were in each serving of food in the FFQ, both the peanut content of the food and a standard portion size needed to be determined (see Table 19). People have difficulty estimating portion sizes in all forms of dietary assessments,[458] including when relating their consumption to pre-defined reference portion sizes as is the case in FFQs.[459] Therefore, for pre-packaged food such as chocolate bars the portion size was defined in terms of this standard unit, however, for other foods standard portion sizes were obtained from the Ministry of Agriculture,[460] and converted into household measures such as a slice, bowl, cup or handful. The actual peanut protein content in each product on the FFQ was obtained from the manufacturer directly. The frequency of peanut consumption was assessed over 1 day, 7 days, 4 weeks and 6 months, by asking participants how many times they had eaten

the food and what the typical amount of the food was (in terms of household measurements). Using this information average weekly peanut protein consumption over 1 week, 1 month and 6 months could be calculated. HPC was the sum of peanut protein consumed by all household members. Moderate to high peanut protein consumption was defined as ≥10 grams/week as previously described. [28]

The gold standard in dietary questionnaire assessments is the 7 day food diary; however, this is time-consuming and laborious for the family as all foods consumed needs to be documented. The peanut FFQ employed in this PhD was validated against a 7-day food diary for maternal recall of her own diet and of her infants' diet over six months; thus was relevant to the study design.[461] In the study by Sofianou et al.[461] mother and infant peanut protein consumption was divided into three groups of none/low, moderate and high peanut consumers. Although there was considerable variation at the individual level between the two tools, when diary responses were grouped into small numbers (5 and 7) of mother and child 'units', the FFQ responses were very close to the diet monitored by the 7-day food diaries. [461] This was the case not just for peanut but also for other foods that were also assessed by this dietary questionnaire.

Table 19: Peanut containing foods in FFQ and peanut protein content

Food	% Peanut composition (26% of peanut is protein)	Peanut protein/serving (g)
Spreads:		
Peanut butter (Sunpat)	95%	3.75g/spreading (15g) on bread slice
Snickers spread	20%	0.8g/spreading (15g) on bread slice
Snacks:		
Bamba peanut snacks	49%	3g per packet (25g)
Whole peanuts	100%	2.6g per handful (10g)
Cereal bars:		

Snickers flapjack (Mars)	12%	1.1g per bar (34g)
Tracker Roasted nut bar (Mars)	16 %	1.1g per bar (26g)
Eat natural- fruit and nut bars	11%	1.4g per bar (50g)
Breakfast cereals:		
Crunchy Nut Cornflakes	7%	0.6g per bowl (30g serving)
Crunchy Nut Red	5 %	0.4g per bowl (30 g serving)
Crunchy Nut Nutty	6%	0.6g per bowl (40g serving)
Crunchy Nut Clusters	5%	0.5g per bowl (40g serving)
Honey Nut Branflakes (Tesco)	5%	0.5g per bowl (4og serving)
Weetabix Minis: honey nut crisp	7%	0.7g per bowl (36g serving)
Chocolate bars/sweets:		
Snickers (Mars)	17%	2.9g per bar (64.5g)
Peanut M&Ms	22%	2.6g per 45g pack
Cadbury's Star Bar	20%	2.8g per bar (53g)
Cadbury's Fuse	7% of 49g bar	3.43g per bar
Cadbury's Picnic	10%	1.2g per bar (48g)
Reese's Peanut Butter Cups	29%	1.3g per cup (3 cups in 51g packet)
Reese's nutrageous bar	28% peanuts 23.5%peanut butter	7.7g.per bar (60g)
Miscellaneous:		
Satay sauce		25g peanut in typical serving
Snickers ice cream (Mars)	12%	1.5g per bar (48g)
Peanut soup (one bowl)		54g peanut in typical serving
West African dish		27g (range 8.5 - 45g)
Thai dish		7g (range 7 - 10g)
Indonesian dish		20g (range 14 - 27g)

Figure 36: Semi-quantitative peanut FFQ

Dietary History

Dear Parent/carer, the following questions relate to the types of food that you and your family have eaten in the past six months. The questions may ask about foods which are unfamiliar to you, this is because they may only be sold in Israel. Even if you or your family have never eaten the food in question, **it is important to answer all questions** leaving no blank answers. Thank-you for participating in this research project. Please hand in this questionnaire to the dietician in clinic or the researcher visiting your home after completion.

1) Your Name			
2) Today's Date	DAY	MONTH	YEAR
3) Date of Birth of child	DAY	MONTH	YEAR
4) What is your main occupation?			
5) How are you related to the child in clinic?			
6) What is your child's ethnicity?			

QUESTIONS ABOUT YOUR INFANT AND HIS/HER RELATIVES

6) Does anybody living in your home have an immediate allergic reaction to a **FOOD?** *i.e. soon after eating the food (within 60 minutes) one or more of the following occur: itch, wheals/hives, flushing, face swelling, throat tightness, vomiting, diarrhoea, stomach pain, wheeze, blue lips.*

(Please tick answer)	Peanut	Other Nuts* (see below)	Sesame	Hens' Egg	Cow's milk	Soya
a) Mother						
b) Father						
c) Child visiting clinic today						
d) Sibling 1 Enter Age [ ]						
e) Sibling 2 Enter Age [ ]						
f) Sibling 3 Enter Age [ ]						
g) Other household members (please specify)						

7) *If you answered YES to 'other nuts,' please circle the responsible nut:

Cashew nut / Walnut / Hazelnut / Pine nut / Brazil nut/Pistachios / Macadamia nuts / Almonds / Pecan

Food Frequency Questionnaire Main carer

The following questions will ask you to remember what types and how much food has been eaten recently **in the home** by **yourself**. It is important to differentiate how much is eaten in the home and eaten outside so please only enter what is eaten in the home.

NB, if the food is unfamiliar to you or has never been eaten by YOU then simply FILL IN 0, but **please answer all questions.**

Please read these examples of how best to answer the following questions

Examples: If you ate 1 snickers bar on 2 occasions in a given time at home then you would fill in:

Snickers 1 bar 2 times

If you ate 2 slices of bread & butter on 4 occasions in a given time at home then you would fill in:

Butter on bread 2 slices 4 times

N.B. a sandwich of 2 slices of bread filled with butter would only count as 1 slice of butter.

	Please try and remember how many times YOU ate the following foods in the HOME	TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
1	Peanut butter on bread						Slices
2	Snickers spread on bread						Slices
3	Butter on bread						Slices
4	Plain bread						Slices
5	Margarine on bread						Slices
6	Jam						Spoons
7	Fruit loaf						Slices
8	Cheddar slices						Slices
9	Salami						Slices
10	Crackers						Crackers
11	Whole peanuts						Handfuls
12	Cherries						Handfuls
13	Bamba peanut snack						Packets
14	Shoosh peanut snack						Packets

Please try and remember how many times YOU ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
15	Peanut M&Ms						Packets
16	Crisps						Packets
17	Crunchy Nut Cornflakes						Bowls
18	Crunchy Nut Nutty						Bowls
19	Crunchy Nut Clusters						Bowls
20	Crunchy Nut Red						Bowls
21	Weetabix Minis: Honey and Nut crisp						Bowls
22	Honey and Nut Branflakes (Tesco)						Bowls
23	Cornflakes						Bowls
24	Branflakes						Bowls
25	Custard						Bowls
26	Pasta						Bowls
27	Tuna flakes						Spoons
28	Muesli						Bowls
29	Snickers Flapjack (Mars)						Bars
30	Trackers roasted nut bar						Bars
31	Eat natural – fruit /nut bar						Bars
32	Reese's Nutrageneous Bar						Bars
33	Cadbury's Star Bar						Bars
34	Cadbury's Fuse						Bars
35	Snickers (Mars)						Bars
36	Cadbury's Picnic						Bars
37	Peanut kitkat						Bars
38	Snickers ice cream						Bars
39	Bounty bars						Bars
40	Milk chocolate						Bars
41	Reese's Peanut Butter Cups						Cups

Please try and remember how many times YOU ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
42	Chocolate muffins						Muffins
43	Coffee cups						Cups
44	Tea						Cups
45	Milk						Cups
46	Satay sauce						Servings
47	Peanut soup						Servings
48	Thai dish						Servings
49	West African dish						Servings
50	Indonesian dish						Servings
51	Curry sauce						Servings
52	Soya sauce						Servings
53	Sweet and sour sauce						Servings
54	Natural yoghurt						Pots
55	Fromage frais						Pots
56	Chips						Portions of 10
57	Mushrooms						Spoons
58	Apples						No. of fruits
59	Oranges						No. of fruits
60	Pears						No. of fruits

INFANT Food Frequency Questionnaire

The following questions will ask you to remember what types and how much food has been eaten recently **IN THE HOME** by **YOUR INFANT**. It is important to differentiate how much is eaten in the home and eaten outside so please only enter what is eaten in the home.

	Please try and remember how many times YOUR INFANT ate the following foods in the HOME	TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
1	Peanut butter on bread						Slices
2	Snickers spread on bread						Slices
3	Butter on bread						Slices
4	Plain bread						Slices
5	Margarine on bread						Slices
6	Jam						Spoons
7	Fruit loaf						Slices
8	Cheddar slices						Slices
9	Salami						Slices
10	Crackers						Crackers
11	Whole peanuts						Handfuls
12	Cherries						Handfuls
13	Bamba peanut snack						Packets
14	Shoosh peanut snack						Packets
15	Peanut M&Ms						Packets
16	Crisps						Packets
17	Crunchy Nut Cornflakes						Bowls
18	Crunchy Nut Nutty						Bowls
19	Crunchy Nut Clusters						Bowls
20	Crunchy Nut Red						Bowls
21	Weetabix Minis: Honey and Nut crisp						Bowls
22	Honey and Nut Branflakes (Tesco)						Bowls
23	Cornflakes						Bowls

Please try and remember how many times YOUR INFANT ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
24	Branflakes						Bowls
25	Custard bowls						Bowls
26	Pasta						Bowls
27	Tuna flakes						Spoons
28	Muesli						Bowls
29	Snickers Flapjack (Mars)						Bars
30	Trackers roasted nut bar						Bars
31	Eat natural – fruit /nut bar						Bars
32	Reese's Nutrageneous Bar						Bars
33	Cadbury's Star Bar						Bars
34	Cadbury's Fuse						Bars
35	Snickers (Mars)						Bars
36	Cadbury's Picnic						Bars
37	Peanut kitkat						Bars
38	Snickers ice cream						Bars
39	Bounty bars						Bars
40	Milk chocolate						Bars
41	Reese's Peanut Butter Cups						Cups
42	Chocolate muffins						Muffins
43	Coffee cups						Cups
44	Tea						Cups
45	Milk						Cups
46	Satay sauce						Servings
47	Peanut soup						Servings
48	Thai dish						Servings
49	West African dish						Servings
50	Indonesian dish						Servings

Please try and remember how many times YOUR INFANT ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
51	Curry sauce						Servings
52	Soya sauce						Servings
53	Sweet and sour sauce						Servings
54	Natural yoghurt						Pots
55	Fromage frais						Pots
56	Chips						Portions of 10
57	Mushrooms						Spoons
58	Apples						No. of fruits
59	Oranges						No. of fruits
60	Pears						No. of fruits

PARTNER Food Frequency Questionnaire

The following questions will ask you to remember what types and how much food has been eaten recently **IN THE HOME** by **YOUR PARTNER**. It is important to differentiate how much is eaten in the home and eaten outside so please only enter what is eaten in the home.

Please try and remember how many times YOUR PARTNER ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
1	Peanut butter on bread						Slices
2	Snickers spread on bread						Slices
3	Butter on bread						Slices
4	Plain bread						Slices
5	Margarine on bread						Slices
6	Jam						Spoons
7	Fruit loaf						Slices
8	Cheddar slices						Slices

Please try and remember how many times YOUR PARTNER ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
9	Salami						Slices
10	Crackers						Crackers
11	Whole peanuts						Handfuls
12	Cherries						Handfuls
13	Bamba peanut snack						Packets
14	Shoosh peanut snack						Packets
15	Peanut M&Ms						Packets
16	Crisps						Packets
17	Crunchy Nut Cornflakes						Bowls
18	Crunchy Nut Nutty						Bowls
19	Crunchy Nut Clusters						Bowls
20	Crunchy Nut Red						Bowls
21	Weetabix Minis: Honey and Nut crisp						Bowls
22	Honey and Nut Branflakes (Tesco)						Bowls
23	Cornflakes						Bowls
24	Branflakes						Bowls
25	Custard bowls						Bowls
26	Pasta						Bowls
27	Tuna flakes						Spoons
28	Muesli						Bowls
29	Snickers Flapjack (Mars)						Bars
30	Trackers roasted nut bar						Bars
31	Eat natural – fruit /nut bar						Bars
32	Reese's Nutrageneous Bar						Bars
33	Cadbury's Star Bar						Bars
34	Cadbury's Fuse						Bars
35	Snickers (Mars)						Bars

Please try and remember how many times YOUR PARTNER ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
36	Cadbury's Picnic						Bars
37	Peanut kitkat						Bars
38	Snickers ice cream						Bars
39	Bounty bars						Bars
40	Milk chocolate						Bars
41	Reese's Peanut Butter Cups						Cups
42	Chocolate muffins						Muffins
43	Coffee cups						Cups
44	Tea						Cups
45	Milk						Cups
46	Satay sauce						Servings
47	Peanut soup						Servings
48	Thai dish						Servings
49	West African dish						Servings
50	Indonesian dish						Servings
51	Curry sauce						Servings
52	Soya sauce						Servings
53	Sweet and sour sauce						Servings
54	Natural yoghurt						Pots
55	Fromage frais						Pots
56	Chips						Portions of 10
57	Mushrooms						Spoons
58	Apples						No. of fruits
59	Oranges						No. of fruits
60	Pears						No. of fruits

INFANT'S SIBLING Food Frequency Questionnaire

The following questions will ask you to remember what types and how much food has been eaten recently **IN THE HOME** by **YOUR INFANT'S SIBLING**. It is important to differentiate how much is eaten in the home and eaten outside so please only enter what is eaten in the home.

	Please try and remember how many times YOUR INFANT'S SIBLING ate the following foods in the HOME	TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
1	Peanut butter on bread						Slices
2	Snickers spread on bread						Slices
3	Butter on bread						Slices
4	Plain bread						Slices
5	Margarine on bread						Slices
6	Jam						Spoons
7	Fruit loaf						Slices
8	Cheddar slices						Slices
9	Salami						Slices
10	Crackers						Crackers
11	Whole peanuts						Handfuls
12	Cherries						Handfuls
13	Bamba peanut snack						Packets
14	Shoosh peanut snack						Packets
15	Peanut M&Ms						Packets
16	Crisps						Packets
17	Crunchy Nut Cornflakes						Bowls
18	Crunchy Nut Nutty						Bowls
19	Crunchy Nut Clusters						Bowls
20	Crunchy Nut Red						Bowls
21	Weetabix Minis: Honey and Nut crisp						Bowls
22	Honey and Nut Branflakes (Tesco)						Bowls
23	Cornflakes						Bowls

Please try and remember how many times YOUR INFANT'S SIBLING ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
24	Branflakes						Bowls
25	Custard bowls						Bowls
26	Pasta						Bowls
27	Tuna flakes						Spoons
28	Muesli						Bowls
29	Snickers Flapjack (Mars)						Bars
30	Trackers roasted nut bar						Bars
31	Eat natural – fruit /nut bar						Bars
32	Reese's Nutrageneous Bar						Bars
33	Cadbury's Star Bar						Bars
34	Cadbury's Fuse						Bars
35	Snickers (Mars)						Bars
36	Cadbury's Picnic						Bars
37	Peanut kitkat						Bars
38	Snickers ice cream						Bars
39	Bounty bars						Bars
40	Milk chocolate						Bars
41	Reese's Peanut Butter Cups						Cups
42	Chocolate muffins						Muffins
43	Coffee cups						Cups
44	Tea						Cups
45	Milk						Cups
46	Satay sauce						Servings
47	Peanut soup						Servings
48	Thai dish						Servings
49	West African dish						Servings
50	Indonesian dish						Servings

Please try and remember how many times YOUR INFANT'S SIBLING ate the following foods in the HOME		TIMES EATEN TODAY	TIMES EATEN PAST 7 DAYS	TIMES EATEN IN PAST 4 WEEKS	TIMES EATEN IN PAST 6 MONTHS	TYPICAL AMOUNT EATEN EACH TIME (Write how many slices/handfuls/ bowls/packets)	
51	Curry sauce						Servings
52	Soya sauce						Servings
53	Sweet and sour sauce						Servings
54	Natural yoghurt						Pots
55	Fromage frais						Pots
56	Chips						Portions of 10
57	Mushrooms						Spoons
58	Apples						No. of fruits
59	Oranges						No. of fruits
60	Pears						No. of fruits

At home do you usually eat with hands or cutlery?

Comments?

Please hand in this questionnaire to the researcher visiting your home after completing it so that they can go through it with you

Thank-you for participating in this research study

10.5 Appendix 5: Participant questionnaire

A participant questionnaire was created in order to be able to account for factors independently affecting peanut levels in dust and for factors that might create discrepancies between the dietary questionnaire and environmental peanut levels in dust and wipes. The number of families living in the home was recorded, as I sometimes visited homes with more than one family living together who shared the same kitchen. In such situations, although there was shared EPE, the main carer of one family was not able to document how many peanut-containing foods members of the other family were eating. In our preliminary work I also ascertained that sometimes pets were fed peanut. In one particular home the family owned a pet parrot who ate peanuts, thus although the family was a low peanut consuming family there were still detectable levels of peanut in their home.

The accuracy of the peanut data were at risk of compromise by whether, as requested, the family had left the bed-sheets in place for at least 5 days prior to vacuuming. For example a clean bed-sheet would be less likely to have peanut levels in dust which reflect peanut levels in dust in the rest of the home. Age of the parental and infant mattress was captured as this has been shown to affect the concentration of inhalant allergens (i.e. the older the mattress the more HDM was found).[462-464] As maternal mattresses are usually older than infant mattresses, information was collected on whether the infant slept in the mother's bed or in their own cot. Use of an 'anti-allergy' mattress cover was also recorded as this has been shown to reduce indoor allergen exposure (in particular HDM) in previous studies. [299;301] The material of the parent and infant eating table was assessed as previously I found that the type of table surface influenced persistence of peanut protein despite cleaning with detergent (peanut persisted more on wood followed by laminate followed by granite surfaces). [465] The questionnaire used for household and demographic factors is displayed in Table 20.

Table 20: Participant questionnaire on household and demographic factors

Flat or house		Number of bedrooms:
All household members sampled?		Reason
Number of siblings of infant		Ages:
Number of 'other' household members		Day/ Day and night time household members
Number of families in home		Kitchen Use Yes/No
Bed-linen left for >/=5 days?		
Infant sleeps in maternal bed or cot		
Location of infant's cot		
Age of infant's mattress		Type of Mattress
Mattress cover?		Type of Mattress cover
Age of parent mattress		Type of Mattress
Mattress cover?		Type of Mattress cover
Location of infants' play area		
Play area description (plastic/carpet/quilt)		
Pets		Type
Infant eating table used		Material
Parent eating table used		Material
SFFQ performed by		

10.6 Appendix 6: Recruitment and study procedures for publication 1 and 2

Recruitment and consent process

Details of the participant recruitment process are provided in Figure 37. Participants were recruited from a tertiary allergy clinic. During the clinic consultation they were asked whether they would be interesting in participating in this study and, if so, provided with a participant information sheet (**Figure 38**, p367). During their appointment they were given time to read through the information leaflet (whilst waiting for allergy testing or dietetic review) and had the opportunity to discuss the study with the researcher (myself). If they agreed to participate then written consent was obtained in clinic and a copy was given to the parent. If parents wanted more time to consider the study then they were called 2-3 days later and a home visit was arranged at this point. In this scenario the consent form was signed in the parent's home and a copy of the consent form was sent in the post following the home visit. Families were asked to avoid washing their bed-sheets and vacuuming their infant's play-area for 5 days prior to the home visit.

Home visit

For safety purposes and to abide with the Lone worker Policy, the date, time and location of the home visit were recorded either with Professor Lack's secretary or a member of the laboratory team. If the family had not yet given written informed consent, this was obtained prior to any environmental sampling or questionnaire completion. Once the consent form was signed the family were asked to complete the FFQ and household factor questionnaire whilst dust and wipe samples were collected. Wipe samples were obtained from the parent table, infant high-chair table, tap, dishwasher-handle, fridge-handle and infant cot-rail. Dust samples were obtained from the beds of all household members and from the infant's play area. Parents usually slept in the same bed, thus dust samples were taken from each side of the bed. The infant's play-area was designated as the place where the infant spent most of their day such as the Moses basket, play-mat/quilt or a specified area of the living room carpet/floor. The infant's bed-sheet was vacuumed for 1

minute within a $0.5m^2$ surface area. Other bed-sheets and the infant's play-area were vacuumed for 2 minutes within a $1m^2$ surface area. After environmental samples were collected, the FFQ was reviewed with the family to ensure that it had been filled out correctly.

Figure 37: Participant Flow diagram

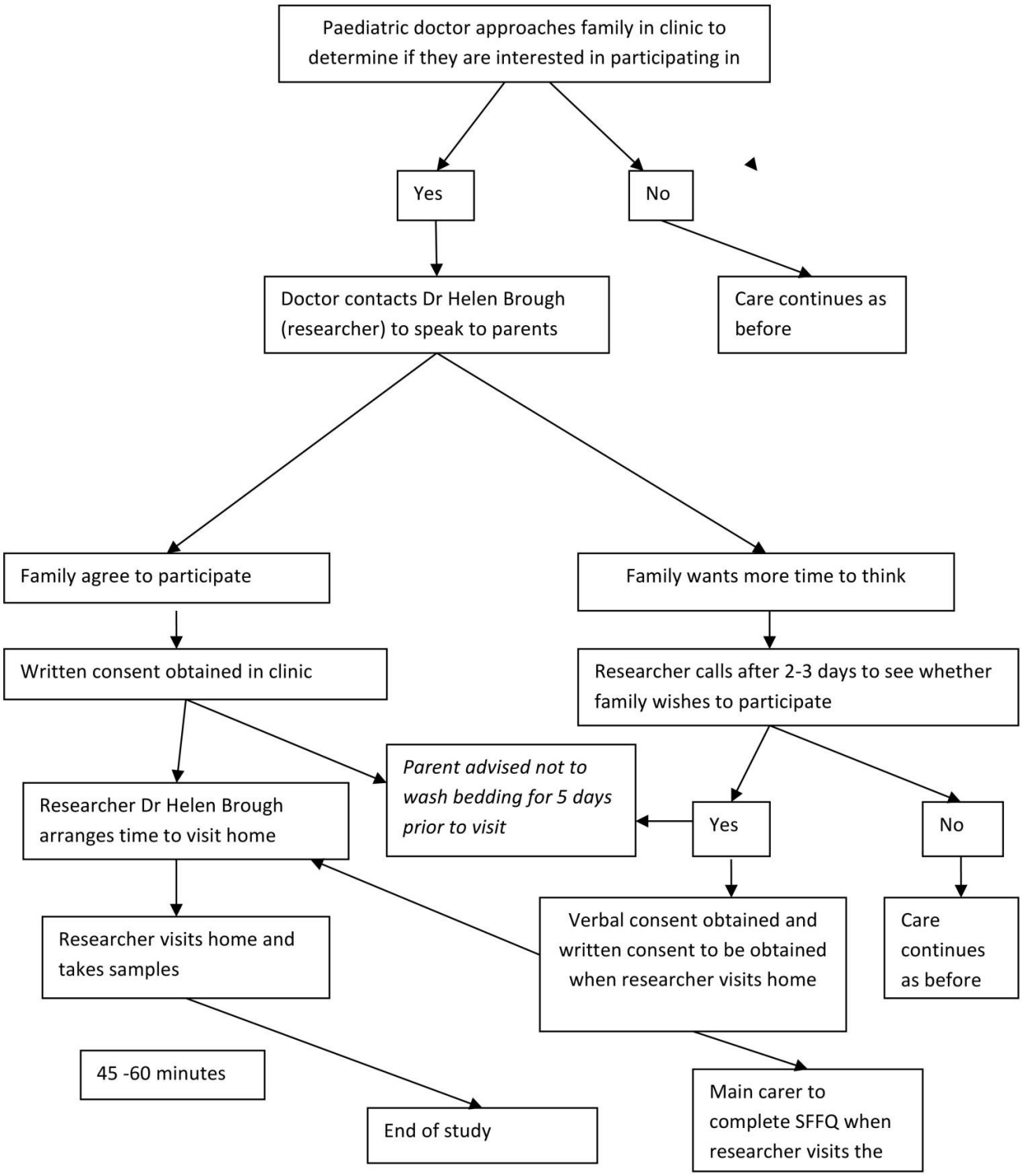


Figure 38: PIS for HPC and EPE study

Participant information sheet

Study title: Measuring food proteins in the home environment

We would be grateful if you could read through this information sheet so that you understand why the research is being done and what it will involve.

Section 1 tells you the purpose of the study and what will happen if you take part.

Section 2 gives more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. You can contact Dr Helen Brough (contacts details below) if you have any questions. Please take time to decide whether you wish to take part and discuss with your family whether they agree to participating as the study involves the researcher visiting your home.

SECTION 1

1) What is the aim of the study?

The main aim of our study is to see if there is a link between what a family eats and the levels of food proteins (elements from certain foods) found in their home environment. For the purposes of this study the home environment will consist of areas in the kitchen such as the table, fridge, cupboard handles, bin lids, dishwasher handles and in the bedroom areas such cot-sides, bed-sheets and pillow-cases of family members. We will also measure dust from the floor play-area of your infant.

2) Why is the study being done?

Food allergy is increasing in children. We do not know why and different theories have been put forward. One theory is that infants (children < 12 months) with eczema may be getting 'sensitized' though the skin. Becoming 'sensitized' is a process where the immune system is exposed to a certain protein (food or otherwise) and becomes predisposed to having an allergic response to it. The first step towards this kind of study needs to show that the type of food eaten in the home relates to the type of food proteins that can be found in an infant's home environment.

We will be trying to answer the following questions:

1) Does the type and amount of food consumed by household members relate to the type and amount of food proteins found in the general home environment?

2) Does the type and amount of food consumed by household members relate to the type and amount of food proteins found in an infant's home environment?

3) Why have I been chosen?

You have been chosen because you have a child aged <12 months.

4) Do I have to take part in the study?

No. The doctor/dietician seeing you in clinic will ask you whether you are interested in participating in the study. If you are interested, then either a member of the research team will come and speak to you or the clinician will ask you whether they can pass on your contact details to the researcher. You will then be sent this information by email or post and the researcher will contact you by phone or email 2-3 days after receiving the information sheet. You will be able to discuss the study with the researcher and she will arrange a time and date convenient for you to visit. Written consent will then be obtained when she visits your home. If you wish for more time to consider participating in the study then we will arrange a time to contact you 2-3 days later.

Even if you take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time or a decision not to take part will not affect the standard of care that you or your family may receive in the future. If you decide to withdraw your data later on then the samples taken from your home can be destroyed.

5) What do I have to do?

The study will involve taking wipe samples from areas in the kitchen and dust samples from each household member's bed-sheet and pillow so you need to make sure that you and other household members are happy for the researcher to do this before consenting.

Once you agree to the study, the researcher will ask you to do the following things:

1) You will need to think of a time when it would be convenient for you to have the researcher visit your home for approximately 45-60 minutes depending on how many household members you have at some point within the next month.

2) We will ask you not to wash the bed-sheet and pillow-case of all family members for 5 days leading up to when the researcher visits your home.

3) Fill out a diet history questionnaire for all household members during the home visit (10 -15 minutes depending on number of household members)

4) We will ask you to not to clean your home more than usual before the researcher visits your home but to leave it as it would usually be so she can get a more realistic representation of what food proteins are usually present in the home.

6) What will happen to me if I decide to take part?

The research assistant will visit your home at a time which is convenient to you. She will take samples from the kitchen surfaces and each bed sheet and pillow in the home using wipes or a vacuum. She may also collect two air samples using an air monitor device which will be collected from the infant's bedroom and the parent's bedroom. This will take approximately 45-60 minutes depending on the number of people living in the house. We will also record the type of table surfaces/cot sides and where the infant sleeps/plays as well as other information that will influence the amount of food proteins that will be measured.

7) What are the risks or inconvenience?

Risks: The researcher will need to plug the vacuum into the electricity supply in your home. As there is a lead to the vacuum there is a potential risk that a child may trip over the lead. We will therefore ask you to keep children away from the bedrooms whilst the researcher is doing the vacuuming of the bed-sheets.

Inconvenience: The main inconvenience for you and your family will be allowing the researcher to visit your home, allowing her the time to collect samples (approx. 45-60 minutes), and being asked not to wash your bed-sheets for 5 days prior to the visit.

8) What are the potential benefits?

The study may not help you directly, but the information that is obtained might help future studies to look into what might be causing certain food allergies.

9) What happens when the research study stops?

When the research study finishes, the results may be published in a scientific journal specific to Allergy. Your care will continue as before. Further details are given in Section 2.

10) What if there is a problem?

Further details are given in Section 2.

11) Will my taking part be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. Publications and presentations from this study will not contain any identifying details. Further details given in Section 2.

12) Contact details:

Chief Investigator: Professor G. Lack, Professor of Paediatric Allergy and Immunology

Address: Paediatric Allergy, Evelina Children's Hospital, St. Thomas' Hospital, London, SE1 7EH

Telephone: 02071889730 Email: Gideon.lack@kcl.ac.uk

Co-collaborator: Dr Helen Brough, Clinical Lecturer Paediatric Allergy

Address: Evelina Children's Hospital Allergy department, Westminster Rd, SE1 7EH

Telephone: 02071889730 after 31/08/08 Email: Helen.brough@kcl.ac.uk

This completes Section 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue *to read the additional information in Section 2 before making any decision.*

Participant information sheet continued

SECTION 2

13) Who will have access to the case/research records?

All information which is collected during the course of the research will be kept strictly confidential. Personal information such as addresses and contact details will only be available to the research team directly involved with participant recruitment and home visits. All data will subsequently be labelled anonymously so that they can only be identified by these researchers. Data will be kept in a password protected file on a securely kept computer. The data will be stored securely in a computer hard-drive for fifteen years and as a hard copy for fifteen years and then will be destroyed.

15) Who do I speak to if problems arise?

Complaints: If you have a concern about any aspect of this study, ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through St, Thomas' Patient Advice and Liaison service (02071888801) if you are recruited from Evelina Children's Hospital, St. Thomas' Hospital.

Harm: In the unlikely event that you or your child is harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the indemnity sponsor, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

What will happen to the samples that are taken?

The wipes and dust samples will be analyzed at King's College Laboratory for the Division of Asthma, Allergy and Lung Biology, Guy's Laboratory, Guy's Hospital. We will ask for your consent to allow us to measure for other food proteins and inhalant allergens (such as house dust mite) from the samples taken in future studies.

What will happen to the results of the research study?

We hope to publish the results of this study and disseminate findings to clinicians practicing allergy. No personal details will be mentioned in publication or presentations of the data. We will ask for your consent to allow us to measure for other food proteins from the samples taken in future studies.

Who has approved the study?

The project has been approved by the Research and Development Departments at Northwick Park Hospital and Guy's and St. Thomas' Hospital and by Brent Research Ethics Committee prior to recruitment.

Who is organizing and funding the study?

The study is being organized by the Chief Investigator, Professor Gideon Lack, Professor of Allergy and Immunology and Dr Helen Brough, Specialist paediatric Registrar. Funding will be provided from internal funds by Professor Gideon Lack's research department based at King's College London.

The participant will be given a copy of the information sheet and the signed consent form to keep

10.7 Appendix 7: How to depict correlation plots for Publication 2

A review of the British Medical Journal, The Lancet and the New England Journal of Medicine during 1997 found that correlation scatterplots were often incorrectly represented. [466] Porter et al. (1999) found eight errors (detailed below) which they deemed to be important either because they considered them to be major errors or due to the frequency with which they occurred.

1. Failure to state in the text the number of cases used in a correlation coefficient.
2. Citing a correlation coefficient without a 95% confidence interval
3. The inappropriate use of a regression line through a scatterplot of a correlation
4. Failure to justify the inclusion of outliers
5. Use of correlation when numbers were very small
6. Use of Pearson correlation in non-parametric data
7. Progressive increase or decrease in spread of dots around the regression line
8. Attaching importance to a significant *P*-value with a low correlation coefficient.

I adopted these principles when constructing the scatterplots published in Publication 1 and 2; for example, the highest peanut consumers in the correlation plots in Publication 2 (where both parents and infants were consuming peanuts) were removed and the correlation coefficients were repeated to assess the impact of removing these high values.

10.8 Appendix 8: Peanut sampling on humans and environment

Participant information sheet - Pilot study 2

Study title: Household consumption of peanut related to environmental peanut protein in the home

You will be invited to take part in a research study. We would be grateful if you could read through this information sheet so that you understand why the research is being done and what it will involve.

Section 1 tells you the purpose of the study and what will happen if you take part.

Section 2 gives more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. You can contact Dr Helen Brough (contacts details below) if you have any questions.

SECTION 1

1) What is the aim of the study?

The aim of this Pilot study is to determine whether there is a change in the amount of peanut protein on hands, saliva and sweat that can be detected after eating a peanut containing food (peanut butter sandwich).

2) Why is the study being done?

Food allergy is increasing. We do not know why and different theories have been put forward. One theory is that we may be getting 'sensitized' though the skin. Becoming 'sensitized' is a process where the immune system is exposed to a certain protein (food or otherwise) and becomes predisposed to having an allergic response to it. We know that peanut can be found in the home environment after peanut consumption but do not know how it gets there. We will be trying to answer the following question: Is there a significant change in the amount of peanut protein detectable in hand wipes, saliva, sweat and blood after eating a peanut containing meal ?

3) Do I have to take part in the study?

No. If you consent to participating then we will ask you to sign a consent form. If you wish for more time to consider participating in the study then we will arrange a time to contact you 2-3 days later.

Even if you take part, you are still free to withdraw at any time and without giving a reason. If you decide to withdraw your data later on then the samples taken can be destroyed.

4) What do I have to do?

Once you agree to the study, the researcher will ask you to do the following things:

1. We will ask to eat 1-50grams of peanut that we will provide for you
2. We will ask you to provide a sweat sample (using the sweat testing method), a saliva sample, a wipe from your hand and/or a 15 ml blood sample before and after peanut butter consumption.

5) What will happen to me if I decide to take part?

The researcher will ask you to eat 1-50grams of peanut. Before this she will take a sweat sample, saliva and/or hand wipe and blood sample. Another sample will be taken 1-3 hours later.The blood sample will be taken by a medical doctor experienced in taken blood.

6) What are the risks or inconvenience?

The blood sample may lead to a small amount of discomfort and bleeding at the site of taking blood.

7) What are the potential benefits?

The study may not help you directly, but the information that is obtained might help future studies to look into what might be causing certain food allergies.

8) What happens when the research study stops?

When the research study finishes, the results may be published in a scientific journal specific to Allergy. Your care will continue as before. Further details are given in Section 2.

9) What if there is a problem? Further details are given in Section 2.

11) Will my taking part be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. Publications and presentations from this study will not contain any identifying details. Further details given in Section 2.

12) Contact details:

Chief Investigator: Professor G. Lack, Professor of Paediatric Allergy and Immunology

Address: Paediatric Allergy, Evelina Children's Hospital, St. Thomas' Hospital, London, SE1 7EH
Telephone: 02071889730 Email: Gideon.lack@kcl.ac.uk

Co-collaborator: Dr Helen Brough, Clinical Lecturer in Paediatric Allergy
Address: Evelina Children's Hospital Allergy department, Westminster Rd, SE1 7EH
Telephone: 02071889730 after 31/08/08 Email: helen.brough@gstt.nhs.uk

This completes Section 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue *to read the additional information in Section 2 before making any decision.*

Participant information sheet continued

SECTION 2

13) Who will have access to the case/research records?

All information which is collected during the course of the research will be kept strictly confidential. All data will subsequently be labeled anonymously so that they can only be identified by these researchers. Data will be kept in a password protected file on a securely kept computer. The data will be stored securely in a computer hard-drive for 15 years and as a hard copy for 15 years and then be destroyed.

15) Who do I speak to if problems arise?

Complaints: If you have a concern about any aspect of this study, ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through St, Thomas' Patient Advice and Liaison service (02071888801).

Harm: In the unlikely event that are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the indemnity sponsor, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

What will happen to the samples that are taken?

The wipes and dust samples will be analyzed at King's College Laboratory for the Division of Asthma, Allergy and Lung Biology, Guy's Laboratory, Guy's Hospital.

What will happen to the results of the research study?

We hope to publish the results of this study and disseminate findings to clinicians practicing allergy. No personal details will be mentioned in publication or presentations of the data. If you wish to know the results of the samples taken then you can contact Dr Brough and she will inform you of the results.

Who has approved the study?

The project has been approved by the Research and Development Departments at Guy's and St. Thomas' Hospital and by Brent Research Ethics Committee prior to recruitment.

Who is organizing and funding the study?

The study is being organized by the Chief Investigator, Professor Gideon Lack, Professor of Allergy and Immunology and Dr Helen Brough, Clinical Lecturer in Paediatric Allergy. Funding will be provided from internal funds by Professor Gideon Lack's research department based at King's College London.

The participant will be given a copy of the information sheet and the signed consent form to keep

10.9 Appendix 9: Tryptic digest SOP

10.9.1 KCL SOP: Trypsin digest for Ara h 1 to use on Mass Spectrometer

1. 50 µL of (2 mg/ml Ara h 1 protein stock in H_2O) was prepared.
2. Serial dilutions were prepared:
    - 100µg/ml (50 µl of 2000µg/ml solution)
    - 10 µg/ml (add 450µl water to 50 µl protein and take 50 µl of this)
    - 1 µg/ml (add 450µl water to 50 µl protein and take 50 µl of this)
    - 500 ng/ml (add 50µl water to 50 µl protein and take 50 µl of this)
    - 250 ng/ml (add 75µl water to 50 µl protein and take 50 µl of this)
    - 50 ng/ml (add 50µl water to 50 µl protein and take 50 µl of this)
    - 10 ng/ml (add 40µl water take 10 µl of this)
3. 50 µl of tetrafluoroethylene (TFE) was added to the protein solution to denature the protein and the solution was vortexed (protein concentrations may vary but the volume of 50 µl remains the same).
4. The addition of 11 µl of 150mM Dithiothreitol (DTT) to give 15mM DTT was then carried out and the solution was heated to 55^{o}C for 45 min. This breaks up disulphide bonds. To make up DTT (154g/L=1M) need 0.23g (flakes) in 10ml deionised water (make up fresh every time)
5. The samples were cooled to room temperature (~5min) and then 12 µl of 550mM iodoacetamide (IAM) was added to give 55mM IAM. IAM stabilises the mixture whilst preventing any new disulphide bonds by binding to the thiol group of the cysteine residue. To make IAM 550mM need 0.501g of stock (184.96g/L=1M) in 5ml deionised water (make up fresh every time) The solution must then be incubated in the dark at room temperature for 30 minutes.
6. The sample was diluted by adding 880 µl of 50mM Tris + 2mM CaCl2 pH 8.0 (to achieve 5% TFE concentration). For Promega sequencing grade calcium is not required. TRIS available is at pH 10.5 thus this needs to be acidified using HCL to a pH 8 to help activate the trypsin.

7. Trypsin (Promea sequencing grade) was then diluted with TRIS (1:500) in order to have a dilute sample enough to pipette down to 10ng of Ara h 1. If 10 µg=20µl of original solution then add 12 µl to 2988µl of TRIS to make up 1:500 fold dilution. Final concentration of 1:50 enzyme:protein was made and the solution was incubated at 37^{o}C overnight.
8. Following overnight incubation, 10 µl of formic acid (to get 1% v/v total) was added to stop the digestion. Formic acid 0.25M was multiplied up by 32 (0.25x32=8M which is 100%)
9. The sample was cleaned up using solid phase extraction. A C18 column (Varian bond elut) was placed in a vacuum box and primed first by the addition of 2 ml of methanol (add 2 x 1 ml) and then 2ml of H2O (add 2 x 1 ml).
10. 20µL of the peptide mixture was then injected onto the RP-LC column and analysed using LC-MS/MS equipped with an electrospray source (LTQ, Thermo Scientific, Loughborough, UK). [467]

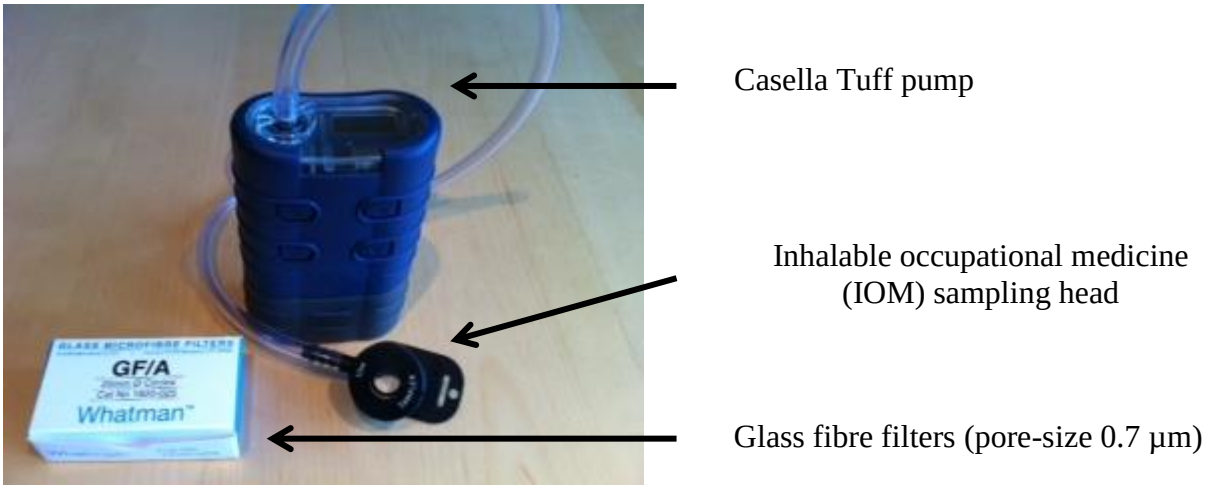
10.9.2 University of Manchester Trypsin digest protocol

1. Chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless indicated otherwise.
2. Extracted dust samples were sequentially reduced, alkylated and digested using an in solution protocol designed to reduce volumes of reagents and therefore maximise protein concentration in the final sample as follows.
3. To 5 µl sample 10 µl of 250 mM ammonium bicarbonate and 4 µl of 50 mM dithiothreitol (DTT) were added prior to heating at 80^{o}C for 10 min.
4. Samples were then incubated at room temp for a further 20 min before the addition of 4.5 µl of 150 mM iodoacetamide.
5. Alkylation was performed at room temp for 30 min in the dark.
6. Samples were then digested by the addition of 2.5 µl of 0.1 mg/ml trypsin with incubation at 37^{o}C for 3 hours with subsequent addition of 2.5 µl of 0.1 mg/ml trypsin before incubation at 31^{o}C overnight.

10.10 Appendix 10: Airborne peanut experiments

The Casella Tuff personal air sampling monitor (Figure 39) to measure airborne peanut was recommended by Professor Frank J Kelly PhD, Director of the Analytical & Environmental Sciences Division at King's College London University who has extensive experience in environmental air sampling methods. The Casella Tuff personal air sampling monitor has been used in previous studies to measure other airborne indoor allergens (HDM allergen in the homes of asthmatic children), [401] and is easily portable, has controllable airflow control and the pump has a long (22 hour) battery life; it could therefore be left running for a long periods of time, which was particularly useful when the air sampling monitor was left overnight in different homes in the attempt to detect airborne peanut.

Figure 39: Casella Tuff personal air monitor and Whatman glass-fibre filters



The pump was run at 2 litres (L) of air per minute as recommended by the manufacturer. This is equivalent to an infant's respiratory minute volume: tidal volume (5 ml/kg) x respiratory rate (40 breaths per minute) using an estimated infant weight of 10 kg. Whatman glass-fibre filters with a pore-size of 0.7 µm were inserted into the IOM sampling head. These glass fibre filters were extracted using the Veratox peanut ELISA kit extraction solution in the same way that surface wipes were extracted (see Publication 1 online

repository). To convert from the results obtained in the Veratox ELISA read-out to µg/m^3 the following calculations and assumptions were used:

1. Veratox ELISA results for whole peanut divided by four to obtain peanut protein, thus for example 1000ng/ml whole peanut equates to 250ng/ml peanut protein.
2. Glass-fibre filter is extracted in 2ml extraction solution thus multiply result by 2 to obtain results in ng/ml, thus 250ng/ml would become 500ng/ml
3. To convert from ng/ml to µg/m^3 the following information was utilized:
    a. Casella pump runs at 2 litres (L)/min
    b. For the majority of experiments the Casella pump was run over 10 minutes which equates to 20 L of air suctioned
    c. There are 1000 L of air in 1m^3 of air thus 20 L of air = 0.02 $m^3$of air
    d. Therefore results for a 10 minute Casella pump run were divided by 0.02 to give value in µg/m^3, thus 500 ng/ml would equate to 25 µg/m^3
    e. Thus overall 1000ng/ml whole peanut equated to 25µg peanut protein per m^3

The Veratox polyclonal peanut ELISA LLQ was 100 ng/ml (equivalent to 2.5 µg/m^3).

The following experiments performed to detect airborne peanut are depicted in Figure 40.

The sampling head was held 1 cm (n=3) and 1 m (n=3) above a peanut butter jar/dry roasted peanut bag for 22 hours and above a simmering pan of satay sauce (Amoy, UK) (10.8 grams peanut) for 10 minutes.

1. Whilst eating peanut butter or dry roasted peanuts, the sampling head was pinned to the researcher's clothes, on the dining-room table, breathed on for 10 minutes or placed overnight on the bedside table (n=3).

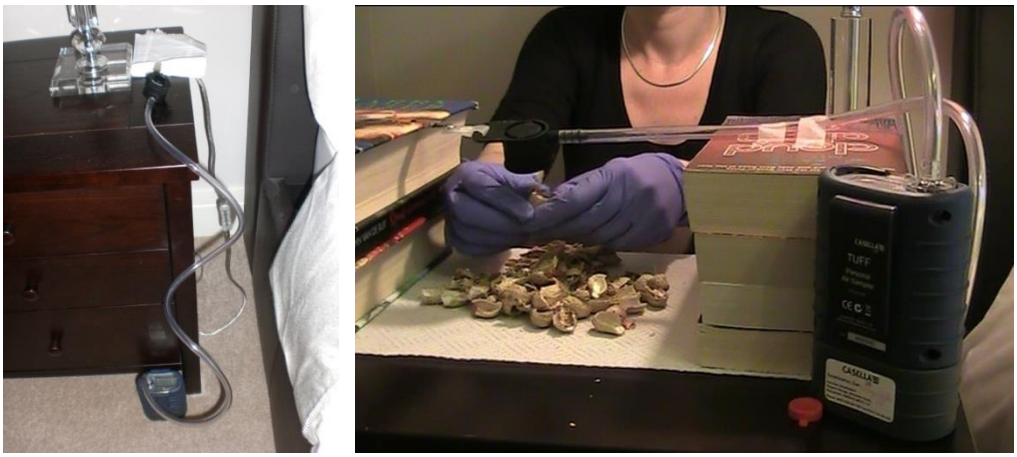
2. The IOM was run for 22 hours in homes with high peanut protein levels in dust (n=5, median peanut protein 163.8 µg/gm, range 51.2-365.2 µg/gm).

3. The sampling head was held 1 cm and 1 meter above peanuts being deshelled. New glass fibre filters were run in the IOM for 10 minutes before, during, immediately after, 30 minutes and 1 hour after deshelling peanuts (n=6).

Figure 40: Airborne peanut experiments with IOM: over peanut butter jar (a), simmering satay sauce (b), KP peanut bag (c), bed-side table overnight (d) 1cm above shelling peanuts (e)

(a) (b) (c)

(d) (e)



10.11 Appendix 11: MAAS PA definition and dust collection methodology

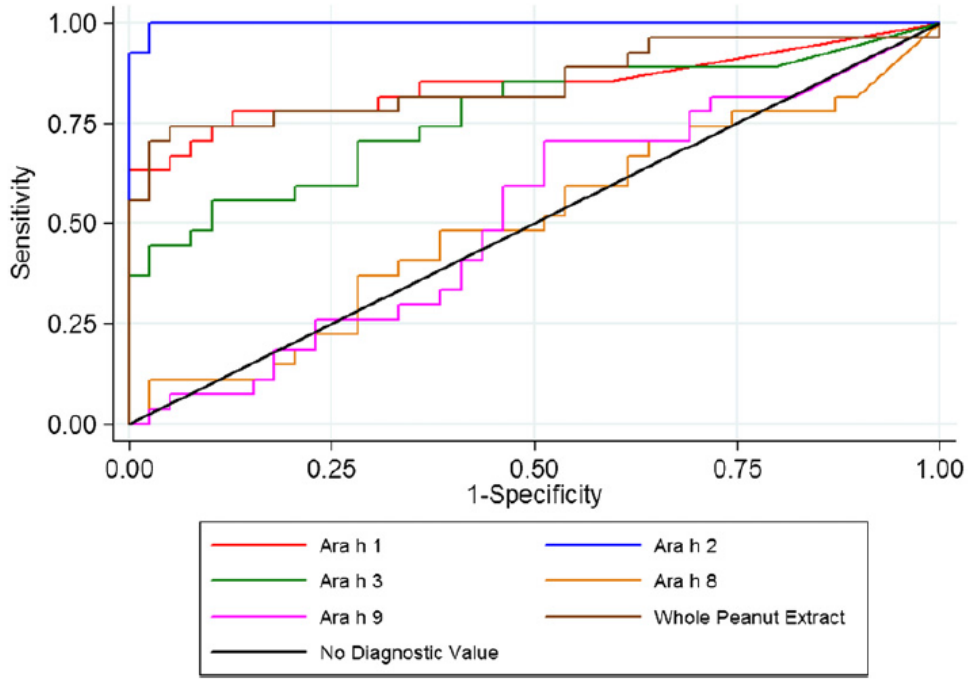
10.11.1 Details of PA definition in MAAS

All children with evidence of PS at age 8 years (peanut SPT ≥3mm or sIgE ≥0.2kU$_A$/L) were offered an OFC to peanut. Open OFCs were applied amongst children who had a history of tolerating peanut on consumption; all other children underwent a DBPCFC.[468] OFCs were considered positive after development of at least two objective signs indicating an allergic reaction; these included flushing, pruritus, urticaria, angioedema, abdominal tenderness with increased bowel sounds, vomiting, diarrhoea, sneezing, rhinorrhoea, cough, hoarse voice, stridor, wheeze, >20% fall in FEV_1, >30% drop in blood pressure or loss of consciousness. [468] Children with a convincing history of an immediate hypersensitivity reaction upon exposure to peanut, combined with: (1) peanut sIgE ≥ 15kU$_A$/L[149] and/or SPT ≥ 8mm[18] (age 8 years) were considered peanut allergic and did not undergo OFC. Previously this cohort showed that using a cut-off of peanut sIgE ≥ 15kU$_A$/L had excellent specificity of 96.2% thus correctly classifying peanut allergic children, however a relatively poor sensitivity of 55.2% thus falsely classifying children with peanut sIgE < 15kU$_A$/L as peanut tolerant. Thus using this 15kU$_A$/L cut-off to not challenge children to peanut would have resulted in 3.8% of children being incorrectly classified as peanut allergic when they were tolerant; however, children with peanut sIgE <15kU/L did undergo OFC thus this decision was not affected by the low specificity of peanut sIgE using a cut-off of 15kU$_A$/L.

Children with a convincing history of an immediate hypersensitivity reaction upon exposure to peanut and SPT ≥ 3mm who refused consent for OFC were either excluded from analysis or considered peanut allergic based on Ara h 2 ≥ 0.35 ISU at subsequent follow up at age 11 years.[468] Previous work by Nicolaou et al. [468] showed that Ara h 2 was the most important component allergen to accurately discriminate between PA and peanut tolerant subjects (see Figure 41). Using an ImmunoCAP cut-off of Ara h 2 ≥ 0.35kU/L correctly diagnosed 97.5% of children as peanut allergic versus peanut tolerant. Sensitivity was 100% thus correctly

diagnosing all children with PA, but specificity was 96.1% thus resulting in 2 children receiving a false positive PA diagnosis.

Figure 41: ROC curve for PA versus tolerance using whole peanut versus component allergens. Reprinted from Nicolaou et al.(2010) with permission from Elsevier.[468]



10.11.2 MAAS dust samples collection procedure

Dust samples were collected predominantly at 36 weeks gestation from a $1m^2$ area of the lounge-sofa as previously described.[463] If no dust sample was available antenatally from the lounge-sofa then dust samples from 6 or 12 months were analysed for peanut protein (where available). The sampling head was loaded with a mesh filter, to remove particles >300 µm diameter (such as carpet fluff and gravel) allowing a sample of fine dust to be collected onto a 5 µm pore size vinyl filter (Plastok Associates Ltd, Wirral, UK). Immediately after collection, the dust sample was transferred into pre-weighed petri dishes and coded in the Manchester Immunity and Inflammation Laboratory. Dust processing and extraction was already performed by the Manchester group before the samples were sent.

MAAS dust collection procedure:

The sampling head was loaded with a mesh filter, to remove particles >300mcm diameter (such as carpet fluff and gravel) allowing a sample of fine dust to be collected onto a 5 mcm pore size vinyl filter (Plastok Associates Ltd, Wirral, UK). Immediately after collection, the dust sample was transferred into pre-weighed petri dishes and coded. On return to the laboratory the filled petri dish was weighed to calculate the mass of fine dust collected and stored at -4 ^{o}C until extraction. After each sample collection the sampling head was cleaned using an Alcowipe to prevent contamination of subsequent samples.

MAAS dust extraction procedure:

A 100 mg* aliquot of the dust was then extracted by rotation with 2 ml borate-buffered saline with 0.1% Tween 20 pH 8.0**, at room temperature for 2 hours before being centrifuged for 20 minutes at 2500 rpm at 4oC. The supernatant was stored at -20 ^{o}C until analysed for allergen content.

* For samples weighing <50mg, 1ml of borate-buffered saline (0.1% Tween 20, pH 8.0) was added for extraction. For samples weighing 50mg-100mg, a proportional amount of borate-buffered saline was added, e.g. for a sample weighing 75mg, 1.5ml of buffer would be added.

** Borate-buffered saline: A concentrated stock solution was made with 360g NaCl, 12.2g Boric acid and 19.5ml NaOH (1N). This stock solution was made up to 2 Litre with distilled water. A working solution was made by diluting the above stock solution by 20-fold with distilled water. 1/1000 (0.1%) volume of Tween 20 was added.

10.12 Appendix 12: *FLG* loss-of-function mutations and genotyping in MAAS and CoFAR

10.12.1 FLG loss-of-function mutations

Six *FLG* loss-of-function mutations (R501X, 2282del4, S3247X, R2447X, 3673delC and 3702delG) associated with eczema in individuals of European Caucasian descent were assessed. The mutations for these *FLG* polymorphisms are described as follows:

R501X:

- ARG501TER' arg501-to-stop (R501X) mutation arises from a G (guanine) to A (adenine) point mutation (DNA) (or C (cytosine) to T (thymine) transition on probe) near the start of repeat 1 in exon 3 of *FLG* gene.
- In wild type *FLG* this sequence (CGA in RNA) codes for the amino acid arginine however individuals with R501X mutations the UGA RNA sequence codes of a premature stop codon.
- Minor allele frequency (MAF) for this mutation (G (guanine) to A (adenine) point mutation) is 5%.

2282del4:

- Not a SNP but a 4 base pair (bp) deletion
- 4-BP deletion of CAGT acts as a stop mutation
- The 2282del4 mutation leads to a premature termination codon 107 bp downstream and, like R501X, stops protein translation within the first filaggrin repeat.

R2447X:

- R2447X mutation arising from a C-to-T transition (same mutation as R501X) near the start of repeat 8 in exon 3 of the *FLG* gene.
- WT: CGA (RNA) codes for Arginine amino acid
- MUT: TGA (probe) or UGA (RNA) codes for nonsense (premature stop codon)

S3247X:

- S3247X mutation arising from a transition from G to T (DNA) (C to A on probe) near the start of repeat 9 in exon 3 of the *FLG* gene
- WT: UCA (RNA) codes for serine amino acid
- MUT: UAA codes for nonsense (premature stop codon)

3673delC:

- Deletion of cytosine base in *FLG* repeat 3 in exon 3
- Codes for nonsense (premature stop codon)

3702delG:

- Deletion of guanine base in *FLG* repeat 3 in exon 3
- Codes for nonsense (premature stop codon)

10.12.2 DNA Extraction

DNA was extracted from PBMCs using a standard DNA extraction kit. The amount of DNA was normalised following extraction to ensure consistency between patients prior to *FLG* genotyping. This was achieved by diluting DNA with water to achieve a 100ng/µl solution.

10.12.3 Real-time quantitative polymerase chain reaction

FLG genotyping employs the use of real-time quantitative polymerase chain reactions (RT-qPCR) thus I will explain this assay in more detail. PCR is a method whereby a specific region of DNA can be targeted for amplification, eliminating the need for large quantities of DNA. The PCR Mastermix contains DNA extracted from PBMCs (normalised for each patient), *FLG* primer sets, deoxynucleoside triphosphates (dNTP), Taq DNA polymerase, Probe sets, Magnesium Chloride (MgCl), and PCR buffer.

1. dNTPs are molecules which contain a nucleoside (A, C, G, and T, which stand for adenine, cytosine, guanine, and thymine) bound to three phosphates and are the building blocks of DNA.

2. Primers are short oligonucleotides, which are complementary to specific regions of the DNA template to be amplified. These have been designed specifically for each *FLG* polymorphism of interest and are described in **Error! Reference source not found.**.[(268)] There is a forward primer which allows DNA polymerase to add dNTPs to the unzipped DNA strand in a 5' to 3' direction.

There is a second reverse primer which allows DNA polymerase to add dNTPs to the unzipped DNA strand in a 3' to 5' direction. The primers do not bind to the target DNA sequence; this is identified using specific probes for the wild-type or mutant allele.

3. Probe sets are short oligonucleotides, designed to hybridize to the DNA sequence of interest, synthesised between the forward 5' and reverse 3' primers. These probes contain a fluorescent reporter molecule (such as 6-carboxyfluorescein (FAM) or VIC®) linked covalently to the terminal base nucleotide at the 5' end of the probe and a quencher molecule (such as MGB-NFQ (non-fluorescent quencher) or tetramethylrhodamine (TAMRA)) linked covalently to the terminal nucleotide at the 3' end of the probe. Probes cannot be extended by Taq DNA polymerase as they lack a free hydroxyl group. These have been designed specifically for each *FLG* wild-type or mutant allele and are described in 6.15.[(268)]

4. DNA polymerases are enzymes, which catalyse the synthesis of DNA strands from dNTPs using an existing strand of DNA as the sequence template. *Taq* DNA polymerase from *Thermus aquaticus* is a thermostable DNA polymerase which means that the enzyme is able to withstand denaturation temperatures of 98 ºC and only one aliquot needs to be added at the start of the PCR process (rather than after each cycle).

Additionally Taq DNA polymerase has 5'-3' exonuclease activity; this disassembles the probe bound to the DNA sequence of interest that is in its way whilst it is building the second strand of DNA. This exonuclease activity is how the fluorescent reporter probe method works (described below).

5. Magnesium ions (Mg^{2+}) are an essential cofactor for PCR as Mg^{2+} forms a soluble complex with dNTPs, which is essential for incorporation of the subunits. Mg^{2+} also stimulates the DNA polymerase activity.

6. Buffer is used to ensure a suitable pH for the PCR reaction to take place.

Table 21: Primers and probes for *FLG* mutation screening.

Point mutations highlighted in bold for R501X, R2447X and S3247X.

Mutation	Primer pairs	Probes
R501X	Forward 5'CAC TGG AGG AAG ACA AGG ATC G 3' Reverse 5' CCC TCT TGG GAC GCT GAA 3'	Wild type 5' 6-FAM-CA**C** GAG ACA GCT C-MGB-NFQ 3' Mutant 5' VIC-CA**T** GAG ACA GCT CC-MGB-NFQ 3'
2282del4	Forward-1 5' TCC CGC CAC CAG CTC C 3' Forward-2 5' CCA CTG ACA GTG AGG GAC ATT CA 3' Reverse 5' GGT GGC TCT GCT GAT GGT GA 3'	Not used for sizing of a fluorescent-labelled PCR fragment
R2447X	Forward-1 5' ACG TGG CCG GTC AGC A Forward-2 5' AGC ACT GGA GGA AGA CAA GGA T 3' Reverse 5' CCT GAC CCT CTT GGG ACG T 3'	Wild type 5' 6-FAM-CA**C** GAG ACA GCT C-MGB-NFQ 3' Mutant 5' VIC-CA**T** GAG ACA GCT CC-MGB-NFQ 3'
S3247X	Forward 5' CCA GAA ACC ATC GTG GAT CTG 3' Reverse 5' TGC CTG ATT GTC TGG AGC G 3'	Wild type 5' 6-FAM-CAG T**C**A AGG CAC GG-MGB-NFQ 3' Mutant 5' VIC-AGC AGT A**A**A GGC ACG-MGBNFQ 3'

10.12.4 Background of RT-qPCR technique

The first step of RT-qPCR is where the two DNA chains of the double helix are 'unzipped' by disrupting the hydrogen bonds at 98 ºC (denaturation step). The temperature in the thermocycler then reduces to between 50-65 ºC (annealing step). At this stage primers hybridise to DNA sequences at each end of the target DNA sequence. Taq DNA polymerase catalyses the synthesis of DNA strands from these primers adding complementary dNTPs to the existing strand of DNA to reform a double stranded DNA chain (extension step). The intervening DNA is thus synthesised by DNA polymerase reactions in opposite directions. As a result, two double stranded DNA copies are produced of the target DNA. Thus, for each PCR cycle the DNA sequence of interest is amplified 2 fold.

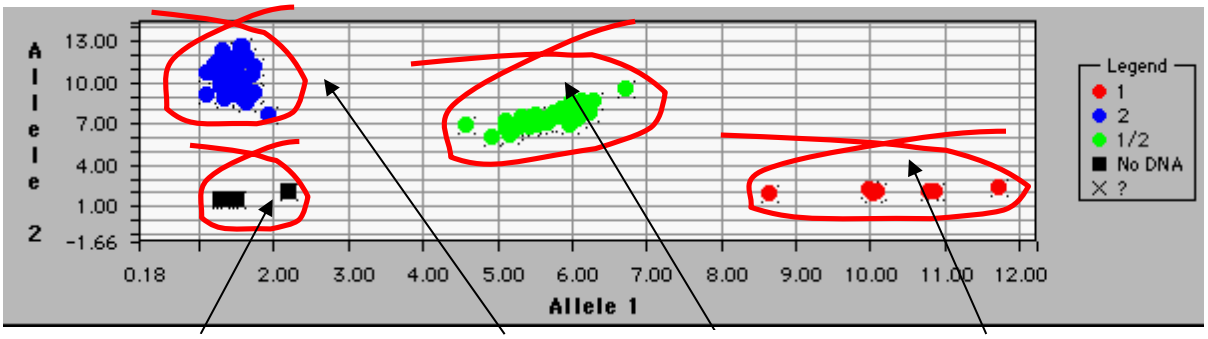
Real-time quantitative PCR uses a fluorescent reporter probe method and allows for quantification of the amplified DNA sequences (or amplicons) as they are generated during the PCR cycle. The fluorescent reporter probe method detects only DNA sequences contained within the probe, thus allows for specific detection of the DNA sequence required, even in the presence of non-specific DNA amplification. There is a fluorescent reporter molecule at the 5'end of the probe and a quencher molecule at the 3' end of the probe. While the probe is intact, the proximity of the quencher dye rapidly absorbs any light emitted by the reporter dye by a process called fluorescence resonance energy transfer (FRET).

The probe anneals downstream from one of the primer sites and is cleaved by the exonuclease activity of the Taq DNA polymerase as the forward or reverse primer is extended. The nucleotide carrying the reporter molecule (fluorescent marker) and the nucleotide carrying the quencher molecule are thereby separated from one another and the fluorescent marker can now emit detectable light when stimulated by a laser. Thus, as the number of amplicons doubles in each PCR cycle so the amount of fluorescent energy also doubles; this allows for real-time quantification of DNA.

10.12.5 RT-qPCR system employed for *FLG* genotyping

Genotyping for R501X, S3247X and R2447X *FLG* loss-of-function mutations was performed using a Taqman based allelic discrimination assay (Applied Biosystems, Life Technologies, Cheshire, UK). [260] The ABI PRISM® 7700 Sequence Detection System is a fully integrated RT-qPCR system which includes a built-in thermal cycler, a laser to induce fluorescence, charge-coupled device detector, real-time sequence detection software, and TaqMan® reagents for the fluorogenic 5' nuclease assay. Primer and probe sets were ordered from ABI (Warrington, UK). The *FLG* wild-type probes were labelled with FAM reporter dyes and the *FLG* mutant probes were labelled with VIC® reporter dyes in order to be able to differentiate these alleles. Applied Biosystems Universal PCR Master mix, reaction volumes of 10 ul and 30ng of DNA were used. Primers were used at 500 nM and probes at 300 nM and 1.5mM of Magnesium Chloride (MgCl) was already added to the PCR Mastermix. The cycling reaction was as follows: 95 ºC for 10 minutes, then 40 cycles of 92 ºC for 15 seconds and 60 ºC for 1 min. Using the plate type 'Allelic Discrimination' the ABI PRISM® 7700 Sequence Detection System, the different alleles are identified by their respective fluorescent marker (**Figure 42**).

Figure 42: Taqman based allelic discrimination assay



Water (no amplification) Major allele homozygote Heterozygote Minor allele homozygote

10.12.6 Sizing of a fluorescent-labelled PCR fragment

Mutation 2282del4 was genotyped by sizing of a fluorescent-labelled PCR fragment on a 3100 or 3730 DNA sequencer (Applied Biosystems). The primers were ordered from Metabion, Planegg-Martinsreid, Germany. For this technique two primers were used and no probes. The forward primer was labelled with the fluorescent dye FAM and the reverse primer was not labelled. PCRs were performed in 10µl reaction volumes in 384 well plates and contained 40ng genomic DNA per reaction. The PCR Mastermix was made up of the following; Water (5.75ul), 10 X buffer (1ul), 2282del4 Forward 2 primer (0.1ul), 2282del4 Reverse 1 primer (0.1ul), dNTPs (1ul), Taq DNA polymerase (0.05ul). PCR reactions were amplified by heating to 95 ºC for 15 minutes (initializing step), then 40 cycles of 95 ºC for 20 seconds (denaturation step), 61 ºC for 30 seconds (annealing step) and 72 ºC for 30 seconds (extension step) then 72 ºC for 10 minutes. The product was pooled with 3 other fragments – diluted 1:4, and 1ul of the diluted and pooled products were added to 5ul of formamide and 0.2ul ROX size standards. The product was then run on the ABI 3100. Data were analysed and the fragments were sized using the Genescan software. Genotypes were assigned based on size; the smaller mutant fragment was 192bp and the larger wild-type fragment was 196bp.

10.12.7 GeneScan analysis

FLG loss-of-function mutations 3673delC and 3702delG were assessed by GeneScan analysis of fluorescently labelled polymerase chain reaction (PCR) products. Initially screening was performed by sizing of fluorescent labelled PCR products through ‘Genescan fragment analysis’ (GeneMapper 4.1, Applied Biosystems), as the 3673delC and 3702delG has one less base than the wild-type genes. The heterozygous 3673delC mutation is 223bp versus wild-type which is 224bp (Figure 43). Following screening, DNA sequencing was performed to assess where the deletion had occurred. The guanine deletion in one allele led to the two alleles becoming out of sync thus overlapping base pairs highlighted where this deletion had occurred (Figure 44).

Figure 43: Genescan fragment analysis of *FLG* 3673delC.

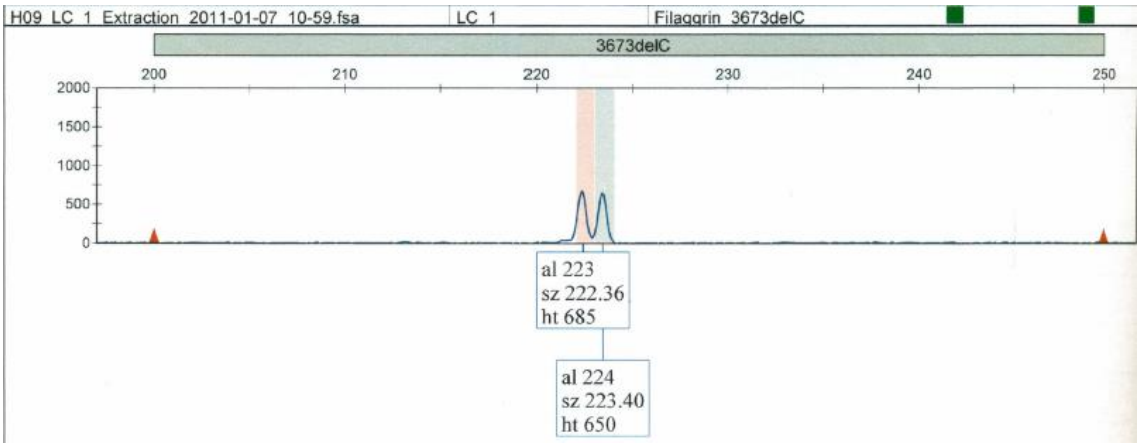
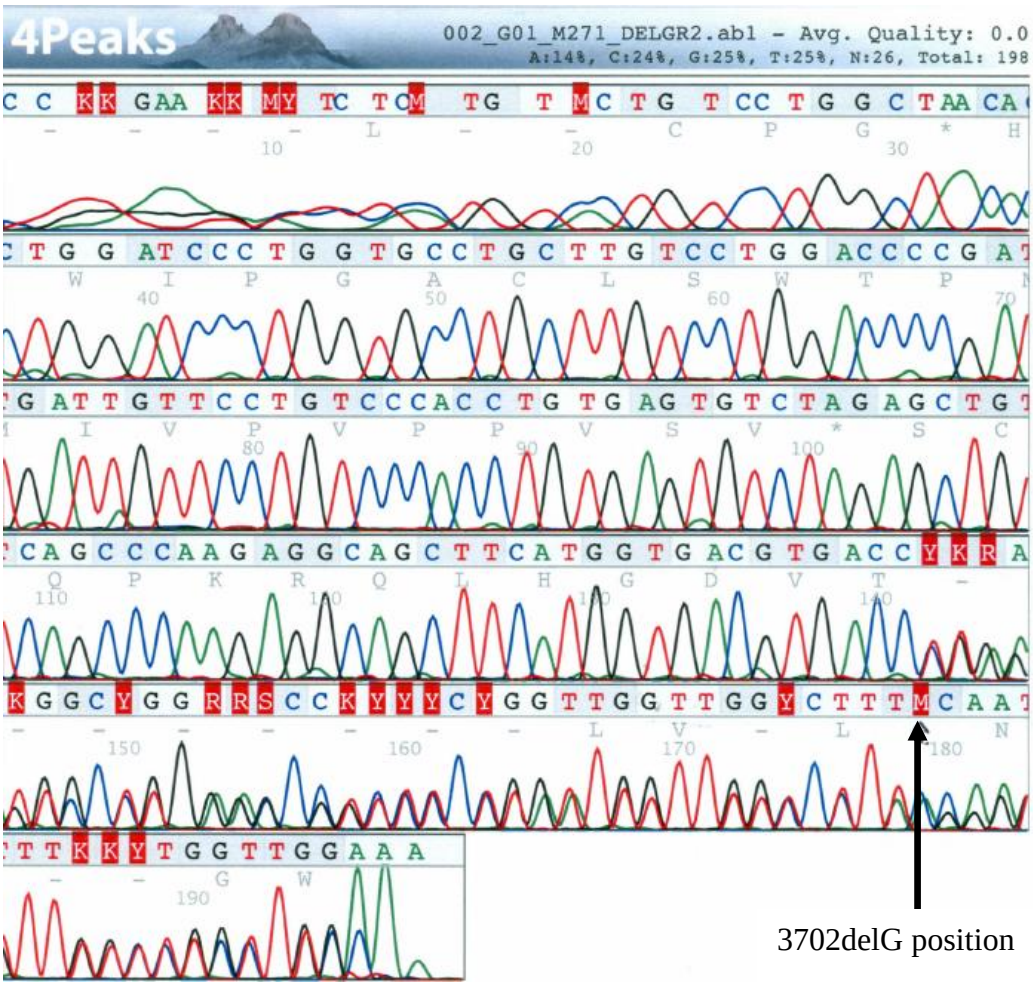


Figure 44: DNA sequencing of *FLG* heterozygous mutation 3702delG



10.13 Appendix 13: Statistical analysis for MAAS and CoFAR

10.13.1 Comparison of demographics and clinical characteristics

In all cohorts, demographics and clinical characteristics of participants (see Section 6.14) were compared between the group of participants that were included in the LR analysis i.e. those with available dust for analysis and *FLG* genotyping (and in the case of the CoFAR study infantile eczema) versus demographics and clinical characteristics from excluded participants. This was performed to assess whether the included group was biased towards more atopy, PS or PA as this could affect the results obtained. Demographics and clinical characteristics on information were compared using Chi-squared test for proportions (such as peanut SPT and sIgE data), Student's t-test for normally distributed data (such as age at assessment of child) and Mann-Whitney U for non-normally distributed data (such as peanut protein levels in dust).

10.13.2 Logistic regression (LR) analysis

Theory of the LR model

LR can be used to predict a categorical dependent/ outcome variable on the basis of continuous and /or categorical independent or predictor variables to determine the effect size on the outcome variable in terms of odds ratios.[469] The LR function f (z) is defined by the equation in $f(z) = 1/ (1-e^{-z})$; where z (logit) equates to α_0 + the sum of (β*x); α_0 is the constant of the equation (intercept) and β is the regression coefficient of the predictor variable x. In a multivariate LR model there can be several predictor variables (e.g. *FLG* genotype, atopic eczema and parental atopy) which are added together. In the LR equation the risk of the outcome variable (z) always falls between 0 and 1 regardless of its estimate which is useful for predicting the risk of an individual getting a disease from 0-100% risk. [470]

Application of the LR model

LR analysis was used to determine risk factors for PS and PA. PS/PA was used as the dependent (outcome) variable and the independent (predictor) variables included *FLG* genotype, atopic eczema, egg sensitization and other predictors of PS/PA. Log transformation was used for peanut protein levels in dust in order to reduce the extent of skewing of this variable; however, as the independent variable in a LR analysis, normal distribution is not mandatory. Univariate followed by multivariate LR analysis was performed to adjust for variables which showed a trend towards an association with PS or PA (see Section 6.14). Highly related predictor variables such as presence of eczema and severity of eczema were not included in the same LR model, as the model would become unstable. Where *FLG* mutations were entered into the multivariate statistical model, participants with non-Caucasian or missing ethnicity data were excluded from the analysis as the *FLG* loss-of-function mutations assessed have only been associated with eczema in Caucasian European populations. [257;277] In LR models where the outcome variable was a very small proportion of the total number of participants (such as PA), a penalized LR model was used to adjust for unbalanced data.[471] The number of covariates in the multiple logistic regression model were reduced following review by the JACI so that only those covariates with the highest clinical relevance were included in the final model. Additionally goodness of fit analyses for regression models were used to ensure that the addition of each covariate improved the fit of the model.

Longitudinal LR analyses

In MAAS analysis of PS was repeated at different time-points which allowed for longitudinal LR analysis. A penalized Generalized Estimating Equations (GEE) via quasi-least squares approach was used with an exchangeable working correlation matrix to account for repeated measures within individuals who were assessed at different ages.[472] GEE represents an extension of the generalized linear model (GLM) to allow for analysis of repeated measurements or other correlated observations such as clustered data. [473] In this

study, PS assessment at two time-points meant that there was clustering of data at these two time points, however genetic and lifestyle factors which would stay consistent over time would be a source of within-person correlation over time. The working correlation matrix represents the within-subject dependencies which can have one of the following structures: independent (no correlation between repeated measures), autoregressive (where correlation diminishes exponentially with time), exchangeable (assumes a constant time dependency), M-dependent (correlation does not change with time until it drops to zero at time M) and unstructured (correlations are estimated without constraints). [474]An exchangeable working correlation matrix was chosen for the GEE analyses as it was assumed that the within person correlation would not change with time. GEE has the advantage that it provides consistent regression parameter estimates regardless of the choice of working correlation structure. GEE was used for PS status only and not PA as PA was thought to be a more permanent state (i.e. you are either peanut allergic or not) whereas PS could be more variable over time.

10.13.3 Relationship between EPE and skin barrier function

Wald's test was used in the LR model to assess the interaction of EPE and *FLG* genotype or eczema on PS/PA. Statistical analysis involved a logistic model to investigate the relationship between the probability of PS/PA and EPE and markers of atopy or skin barrier impairment in each study. The hypothesis was assessed by testing the significance of the interaction slope using Wald's test (two-sided with $P<0.05$) in the LR model: log (Probability sensitization / (1-Prob Sensitization)) = $a + b_{EXP}E + b_{FIL}M + b_{INT}ExM$.
Where $_{EXP}$ = EPE, $_{FIL}$= *FLG* genotype and $_{INT}$ = Interaction

The measure of EPE in this model was the log of the peanut concentrations in the dust samples and M was an indicator variable with value 1 for *FLG* loss-of-function mutation or eczema and 0 for wild-type *FLG* genotype or no eczema. In MAAS the additive risk of EPE in children with *FLG* loss-of-function mutations

and with *FLG* wild-type was also assessed using the equation: $e^{((\beta\text{-coefficient } FLG * \text{peanut exposure interaction}) - (\beta\text{-coefficient peanut exposure}))}$.

In CoFAR, the relationship between EPE and eczema on PS/PA was also assessed in a stratified analysis comparing all children, children with a history of eczema and children with a history of severe eczema. The predictive probability of PS and PA was calculated from the univariate and multivariate regression model in order to create the predictive probability plots in both MAAS and CoFAR studies.

Threshold levels for environmental peanut protein in dust

In MAAS threshold levels for peanut protein in dust for PS and PA were calculated using the intersection between *FLG* wild-type versus mutation in the multivariate regression model. To evaluate the reproducibility of the thresholds obtained and uncertainty around these thresholds bias corrected bootstrap cross-validation was performed with 1000 replications to determine 95% CI for threshold peanut protein in dust levels. Bootstrapping is the preferred method for internal validation where data for model development and evaluation are both random samples from the same underlying population.[475] Bootstrapping is performed by the bootstrap sample being drawn from the same number of patients (*n*) as in the original sample, but some are excluded, others included once, twice, etc. (called replacement). The model is developed in the bootstrap sample, and then validated in the original sample; thus it is called internal validation. To obtain stable results, the procedure is usually repeated 1000 times. The bias corrected bootstrap confidence intervals were used rather than percentile confidence intervals because the distribution of the model was not normal; therefore bias correction adjusted for this.

10.14 Appendix 14: Optimisation of techniques for mechanistic assays in Publication 5

10.14.1 Selection of culture medium

Initial peanut cultures were performed using RPMI 1640 culture medium (Sigma-Aldrich Company LLC) with 5% autologous serum. The serum was created by spinning down plasma at 3000 rpm for 5 minutes at room temperature followed by passage through a sterile filter to remove all debris. Two problems were encountered after performing cultures with RPMI 1640 with 5% autologous serum: on some occasions clotting occurred in the culture well and there was insufficient RNA yield for microarray hybridisation. I thought that clotting might be trapping cells in the culture wells that were required for FACS sorting, thus a serum free medium was considered which has been shown to be effective in human T cell culture.[476;477] Advantages of using a serum free medium were the lack of coagulation factors, likely increased yield of cells following culture, and a standardized culture method, given the variability of autologous serum from each individual. Drawbacks were increasing the artificiality of the experimental system by removing serum factors (including IgE which could lead to facilitated antigen presentation) and the possibility of higher background Th cell activation due to properties within the serum free medium; however, given that gene microarray would differentiate between gene expression from peanut stimulated versus unstimulated cultures it was thought that this would counteract any non-specific stimulation related to the culture medium.

Thus, the serum free medium AIM-V® (Adoptive Immunotherapy Media) containing 50ug/ml Streptomycin sulfate and 10ug/ml Gentamycin sulfate was used in addition to 2-Mercaptoethanol (2-ME) (both from Invitrogen Life Technologies, Thermo Scientific Inc, UK), with a stock solution of 0.04mM 2-ME per 1ml of AIM-V® medium. AIM-V® is a proprietor medium based on Dulbecco's modified Eagle medium and Ham's F-12 nutrient mix, supplemented with Tween-80, human albumin and transferrin, linoleic and palmitic acid.[478] 2-ME reduces disulphide bonds and scavenges hydroxyl radicals thus acting as a

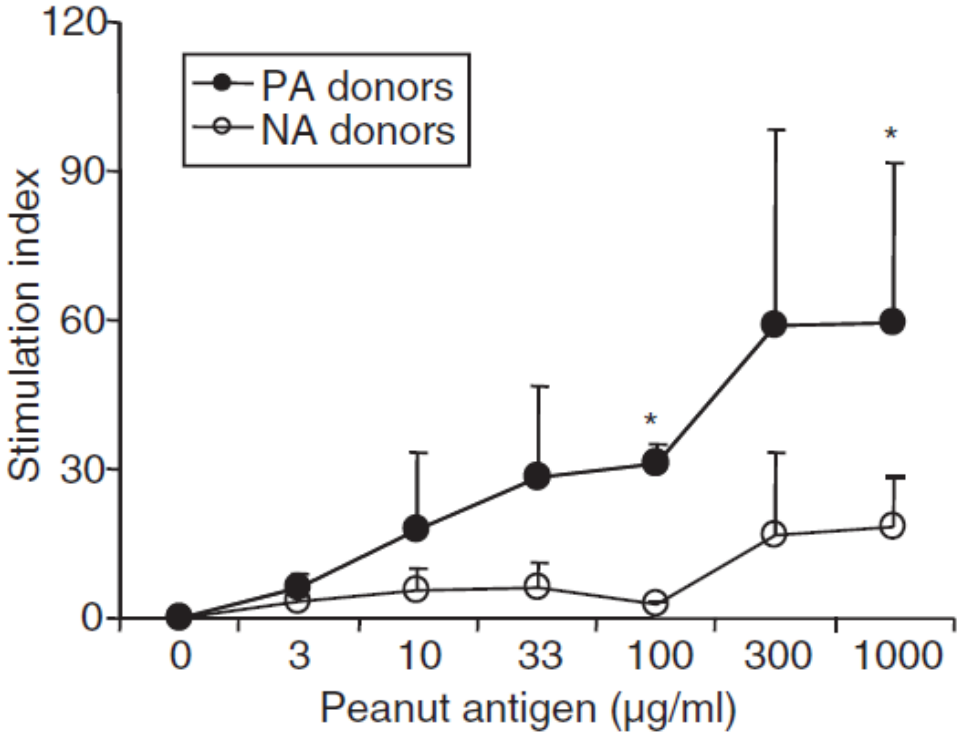
biological antioxidant and is therefore used in cell culture. [479] No further clotting was found in the culture wells and the number of cells sorted became sufficient for further analysis.

10.14.2 Peanut protein concentration for stimulating cultures

Cultures were stimulated in 200µg/ml whole defatted peanut protein extract (ALK Abello) or serum-free medium for 18 hours to detect early allergen-specific response as previously described.[79] A ratio of 90% peanut stimulated versus 10% non-stimulated was used in order to ensure that there were enough peanut stimulated cells for RNA extraction. The optimum concentration of peanut extract was previous established by titration experiments by culturing PBMCs in different peanut concentrations to determine the levels of PBMC proliferation in peanut allergic versus NA individuals; [480] there were good PBMC proliferative responses at day 5 in doses above 100µg/ml peanut antigen (see Figure 45). Rather than choosing the maximum concentration of peanut antigen (300-1000 ug/ml) to obtain proliferative PBMC responses, it was important to consider the optimum peanut antigen concentration where there was a difference in proliferation between memory T helper cells from PA versus NA donors, but not too high so as to get non-specific responses from naïve T cells or cytotoxic T cells. In addition, at higher peanut antigen concentrations B cells could also be stimulated through peanut agglutinins. Previous studies have also evaluated mRNA expression of IL4, IL13, IL9 and IFN-γ following PBMC culture in 50µg/ml, 100µg/ml or 200µg/ml peanut protein; similar results were found for all three concentrations at 3 hours, 1 day, 2, 3, 4, 5 and 6 days culture. [449] Therefore 200µg/ml peanut protein was chosen as the concentration for peanut cultures in order to get a proliferative response from both from PA and NA children.

Figure 45: Proliferation of PBMCs from children with PA versus using different concentrations of peanut antigen in PBMC culture

Reprinted from Turcanu et al. (2010) with permission from John Wiley and Sons.[480]



10.14.3 Obtaining sufficient RNA for microarray

I initially had difficulty obtaining sufficient numbers of FACS sorted cells to obtain RNA that was enough good quality for microarray analysis. One option, which has previously been performed, would have been to pool RNA samples;[79] however, I had concerns that it would not be possible to detect an outlier sample and thus decided to review ways to optimise the amount of RNA that could be extracted from the sorted cells. I anticipated that the number of peanut specific Th cells following FACS sorting would be low for peanut allergic children and even lower for NA children. Previous studies have shown that the frequency of peanut-specific circulating CD4+ Th cells is 0.61% for peanut allergic individuals and 0.08% in NA individuals;[480] peanut specific cells were identified by the number of divisions cells underwent in the presence of peanut antigen using carboxyfluorescein succinimidyl ester (CFSE) cell staining which halves with each cell

division. The Qiagen RNA extraction kit guidance advises that mammalian cells contain 10-30pg of total RNA; [481] however, our laboratory team advised that FACS sorted T cells typically contain 1 pg of total RNA (personal communication Prof David Cousins, Sept 2012); of this 1-5% of total RNA is mRNA.

10.14.3.1 Pilot experiment to determine RNA yield

A pilot experiment was performed to assess the quality and quantity of RNA that could be extracted per PBMC. Increasing numbers of PBMCs were extracted using the miRNeasy Mini kit (Qiagen, UK) and were assessed for integrity using the Agilent RNA 6000 Pico Kit. A second elution was performed for the 500,000 cell sample by adding another 30 µl of RNAse free-water to the column to determine whether it was worth increasing the volume of eluate and thus decreasing its concentration. There was both increased quantity and increased RNA quality (RIN) obtained dependant on the number of cells from which the RNA was extracted (Table 22). On the basis of this pilot experiment I obtained a median 0.78 pg total RNA per PBMC (range 0.28-1.65pg/cell). There was an additional 20% RNA to be gained from repeated elution on the miRNeasy Mini spin column; thus it was worthwhile performing this step and then using a vacuum concentrator to reduce the total volume of eluate thereby increasing the concentration.

Table 22: Quantity of RNA and RIN dependent on number of PBMCs assessed.

Number of cells	Total RNA quantity (pg/µl)	Total RNA quantity (ng)	RNA per cell	RIN
5,000	275	8.25	1.65	1.0
10,000	288	8.64	0.86	2.2
20,000	317	9.51	0.48	4.2
40,000	605	18.15	0.45	8.1
80,000	754	22.62	0.28	6.9
125,000	3243	97.29	0.78	8.5
500,000	14250	427.5	0.86	9.1
2nd elution 500,000	2918	87.54	N/A	8.6

10.14.4 RNA integrity evaluation

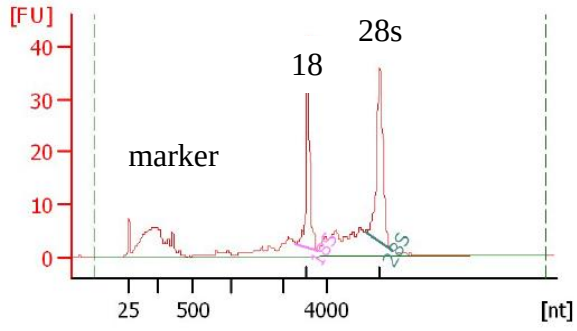
Following methodological optimisation a higher yield of RNA was obtained. The quality and quantity of total RNA was examined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Wokingham, UK) using the Agilent RNA 6000 Pico Kit (Agilent Technologies, UK) which has a range of detection from 50-5000pg/µl total RNA. Analysis was performed according to the manufacturer. Integrity of RNA was assessed using the RNA Integrity Number (RIN) and Bioanalyzer plots for marker and ribosomal peaks, [482] an example of which is displayed in Figure 46. The amount and quality of RNA and cDNA obtained from the FACS sorted cells are displayed in the online repository of publication 5 (see Section 7.10).

Figure 46: Agilent RNA 6000 Pico Kit Bioanalyzer for 2nd peanut allergic child (PA2).

RIN, marker and ribosomal peaks (*18S* and 28s) displayed. nt=nucleotides. FU=fluorescence units.

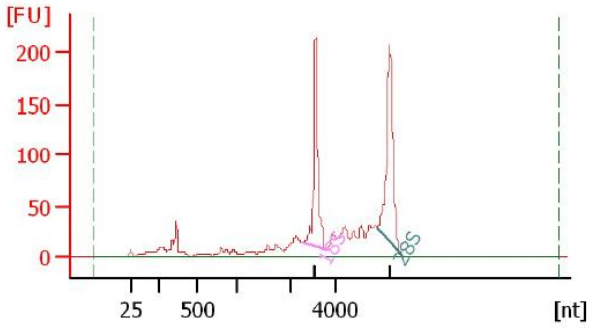
PA2 P+ CD69+B7+

RIN: 8.6



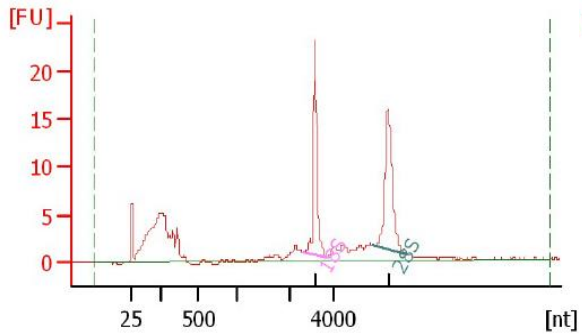
PA2 P+ CD69-B7+

RIN: 8.5



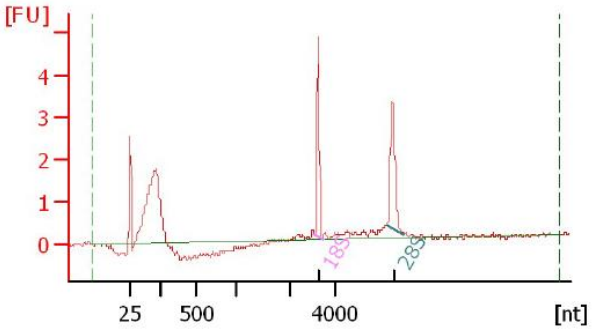
PA2 P+ CD69-CLA+

RIN: 9.1



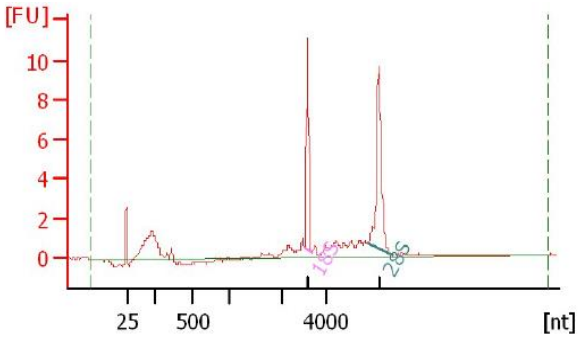
PA2 P+ CD69+CLA+

RIN: N/A



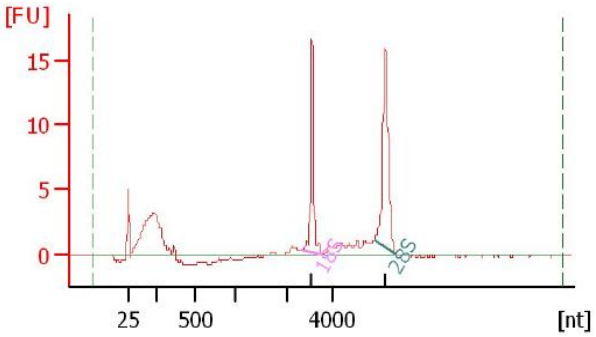
PA2 P+ CD69-B7+

RIN: 8.1



PA2 P+ CD69-CLA+

RIN: 9.3



10.15 Appendix 15: RT-qPCR analysis

To confirm the microarray findings, RT-qPCR was performed on newly recruited individuals from a tertiary paediatric allergy clinic. RNA was extracted from peanut-stimulated PBMC obtained from an independent group of 12 PA and 18 peanut tolerant donors (of which 12 were peanut sensitized). Methods of the PBMC culture and RT-qPCR are described in the online repository of Publication 5. Target gene expression in a given sample was quantified by assessing how many PCR cycles were required before the fluorescent marker for a specific gene crossed the cycle threshold (Ct); this was then compared against the endogenous control *18S* ribosomal RNA (ΔCt) then against the peanut unstimulated control (ΔΔCt) and converted to relative quantitation (RQ) which equates to $2^{-(\Delta\Delta Ct)}$.[483] Genes with no amplification were assigned a Ct value of 40 as previously described;[484] this would be the Ct value obtained if there was only one copy of the DNA sequence of interest in the starting sample.

Figure 47 displays an RT-qPCR amplification plot of a range of genes expressed in PBMCs from a PA child. Genes expressed following PBMCs cultured in peanut antigen are displayed in purple (both CD69+ and CD69- Th cells are included) and genes expressed following PBMCs cultured in media alone are displayed in green (both CD69+ and CD69- Th cells are included). Figure 48 displays an RT-qPCR amplification plot for *IL9* in the same PA child whose PBMCs were either cultured in peanut (purple) or media alone (green). The Ct is the intersection between the amplification curve and the threshold line. Thresholds for different genes are displayed as horizontal coloured lines which cross the amplification plots. The amplification plots which start around 8-9 PCR cycles and which cross the threshold between 11-16 PCR cycles are the *18S* gene which is expressed very early on in the PCR cycle. The genes of interest start from 20-33 PCR cycles and cross the threshold between 25-43 PCR cycles. Thus, for example, if the sample contains 32 more copies of the target gene then the threshold of detection is reached after 5 fewer PCR cycles (2^5=32); if the original sample contains 1024 more copies of the target gene then the threshold of detection is reached after 10 fewer PCR cycles (2^{10}=1024). In Figure 48 *18S* is expressed early in the

PBMCs from the PA child both when cultured in peanut protein or media alone; however, *IL9* expression is only found in those PBMCs cultured in peanut (purple amplification plot) and not in those cultured in media alone (green lines are not amplified but show 'noise' at later cycles).

Figure 47: RT-qPCR amplification plot for all genes expressed in PA patient. ΔRn (delta normalised reporter) is fluorescence emission intensity of the reporter dye divided by that of the passive reference dye.

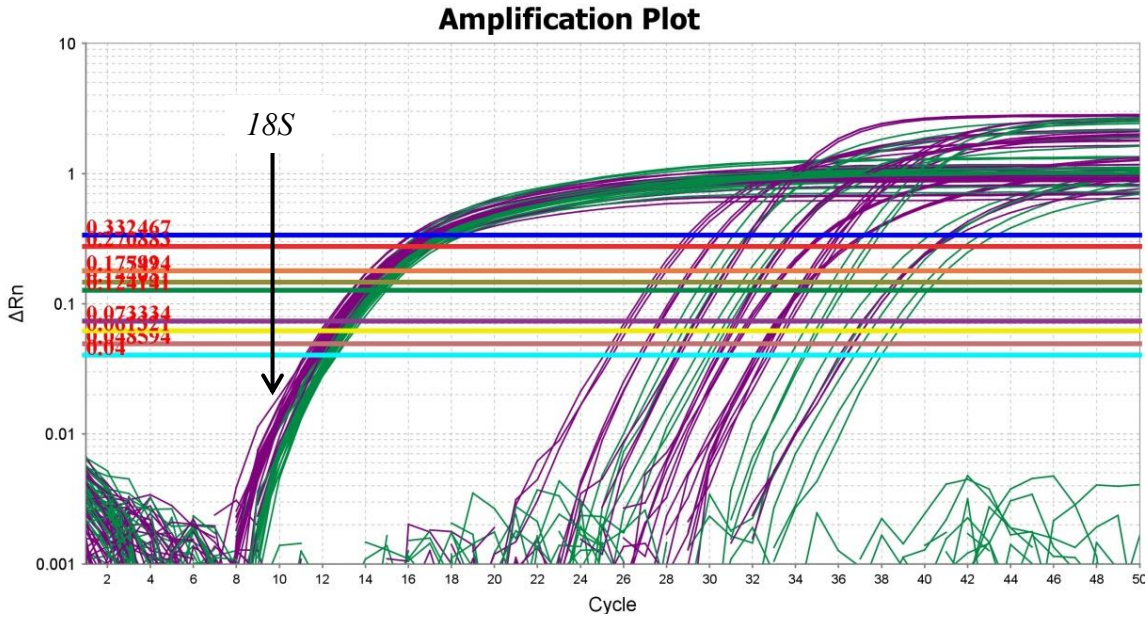


Figure 48: RT-qPCR amplification plot for *IL9* in peanut allergic patient.