# Peanut allergy and loss-of-function mutations in the gene encoding filaggrin, a skin barrier protein: effect of restrictive case definition and atopic asthma

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## TABLE OF CONTENTS

## ABSTRACTS


### Abstract: (English)

Peanut allergy is a condition with high morbidity and mortality. Prevalence of peanut allergy is increasing, and the cause of this is yet unknown. We previously examined for the presence of loss-of-function mutations in *filaggrin* (*FLG*), a gene that encodes a skin barrier protein, in individuals with peanut hypersensitivity. In this work we further our investigation by providing a sensitivity analysis of the effect of the diagnostic criteria used to define peanut allergy. We also examined the relationship of peanut allergy and *FLG* mutations, independent of atopic disease, modeled using logistic regression and self-reported history of asthma. Finally, we examined how error in the self-reported asthma variable and the peanut allergy status variable would affect the results.

### Résumé: (Français)

L'allergie aux arachides est un problème de santé sérieux avec un haut taux de morbidité et de mortalité. La prévalence des allergies aux arachides ne cesse d'augmenter. La raison de cette hausse demeure encore inconnue. Nous avons précédemment étudié chez les patients atteints d'hypersensibilité aux arachides la présence de mutations nulles de filaggrin, un gène codant une protéine qui participe à la barrière cutanée. Dans cet ouvrage, nous raffinons notre recherche en effectuant une analyse de sensibilité de l'effet des critères diagnostiques utilisés pour définir l'allergie aux arachides. De plus, nous étudions la relation entre l'allergie aux arachides et la mutation de la filaggrin, indépendamment des autres maladies atopiques.

Pour ce faire, nous avons employé un modèle de régression logistique ainsi que l'histoire d'asthme rapportée par les patients. Nous avons ensuite évalué comment l'erreur sur les variables d'histoire d'asthme et du statut d'allergie aux arachides pourrait affecter nos résultats.

## LIST OF TERMS, NOMENCLATURE AND ABBREVIATIONS

Allergic rhinitis: here used interchangeably with hayfever
AD: Atopic dermatitis, here used interchangeably with eczema and atopic eczema
DBPCFC: double blind, placebo-controlled, food challenge
Filaggrin (non-italicized): protein involved in cutaneous barrier
*Filaggrin* (italicized): gene encoding the protein filaggrin
*FLG*: abbreviation for the gene encoding the protein filaggrin.
Homozygous: having two identical alleles for a single trait
Heterozygous: having two different alleles for a single trait
IgE: immunoglobulin E
Loss-of-function mutation: the resulting protein from the mutation has less or no function
Null mutation: complete loss of function of the resultant protein
NPV: negative predictive value
OFC: oral food challenge
PPV: positive predictive value
SPT: skin prick test
Wildtype: the typical or most common gene or trait for a species

## LIST OF TABLES AND FIGURES IN TEXT

## STATEMENT OF SUPPORT

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## CONTRIBUTIONS OF AUTHORS

The author of the thesis was responsible for the writing the protocol, questionnaire, consent and assent forms, and obtained ethics approval and grants for the Canadian portion of this project. The author performed the statistical analysis described in this thesis. Drs. Ann Clarke and Celia Greenwood supervised the author, providing logistic, clinical and statistical guidance. Dr. Peter Hull conceived the study idea. Drs Greenwood, Clarke and Hull provided editorial assistance. Greg Shand, Popi Panaritis and Christina Neville were responsible for the data entry and extraction. Peanut allergic case DNA was isolated from the salivary samples in the laboratory of Dr. Peter Hull, with technical assistance of Deborah L. Michel. DNA isolation of the Quebec City newborn blood samples was performed by François Rousseau with the technical assistance of Johanne Bussières. *Filaggrin* genotyping was provided by the laboratory of Professor WH Irwin McLean by Linda Campbell. Alan Irvine, Reza Alizadehfar and Moshe Ben-Shoshan provided clinical input into study design. Kenneth Morgan and Mary Fujiwara provided sample size estimations at the outset of the project.

## STATEMENT OF ORIGINALITY

This is the first study to show that the relationship between mutations in the *FLG* gene and peanut hypersensitivity appear unaffected by peanut allergy case criteria definition and atopic asthma.

## I: INTRODUCTION

Peanut allergy is a source of significant morbidity and mortality. The prevalence of peanut allergy and other atopic diseases has increased over the past few decades, although it appears to have recently stabilized in some countries. The origin of this increase in peanut hypersensitivity is unknown, but is likely due to a combination of environmental and genetic factors. Peanut allergy is strongly inherited, but despite this widely established knowledge, its genetic cause is unknown. This project was instigated to look at the relationship between mutations in the gene encoding a skin barrier protein, filaggrin (*FLG*), a protein integral to the formation of an intact epidermal barrier and peanut allergy. Loss-of-function mutations in this gene have been found in patients with atopic dermatitis[1]. Interestingly, *FLG* mutations are specifically associated with 'extrinsic' atopic dermatitis, which is accompanied by high total serum IgE and sensitization to a variety of allergens[2, 3]. This has led to the suggestion that a defective epidermal barrier may be the site of exposure in this allergic disease[4, 5], a theory that is supported by mouse models that have found a link between a disrupted epidermal barrier and peanut hypersensitivity[6]. Peanut proteins are the main allergens that cause peanut hypersensitivity. As large protein molecules generally do not penetrate the epidermal barrier, our hypothesis is that loss-of-function *FLG* mutations are associated with type I hypersensitivity reactions to peanut protein, and we have previously found evidence of this association[7].

Using a well-established registry of Canadian peanut allergic individuals, we examined the presence of common loss-of-function *FLG* mutations in subjects with peanut allergy compared to two control groups of ethnically similar individuals from Ontario and Quebec City. Knowing that criteria to determine peanut allergy may differ dependent on physician, we constructed a series of case definitions and used logistic regression modeling to examine if the relationship between *FLG* mutations and peanut allergy changed as diagnostic criteria for definition of peanut allergy became more restrictive. We then examined the effect of history of atopic disease on *FLG* mutations and peanut allergy. While neither control group had any information on eczema status, the control group from Ontario had data on asthma and smoking history. Using these data, an atopic asthma variable was created to examine the relationship of peanut hypersensitivity and *FLG* mutations independent of history of other atopic disease. Finally, a sensitivity analysis to investigate the effect of error in asthma diagnosis, as well as the effect of error in peanut allergy status was performed.

The results of this work and the implications of this research on the pathogenesis of peanut hypersensitivity, its prevention and management, and possible interventions for this disease are then discussed.

## II: LITERATURE REVIEW

Peanut hypersensitivity is the subject of intense research. Multiple fields exist within peanut allergy investigation, including studies of prevalence, diagnosis and laboratory testing, accidental ingestions, food labeling, genetics, antibodies, cytokine signaling, treatment and immunotherapy, and public policy. These topics have been covered in several recent reviews [8-10] and a brief summary of areas relevant to this thesis is provided below.

### A: Prevalence of peanut allergy

There is currently increased public awareness of hypersensitivity reactions to peanut (*Arachis hypogaea*). Anecdotally, physicians and the lay public report an increase in prevalence of peanut allergy over the past few decades[11, 12], but few reliable statistics on prevalence of peanut allergy were available until approximately the past decade[13-16].

In both the lay[17, 18] and medical press[19-21] there has been suggestion of a potential peanut allergy hysteria in the general public and medical community. Estimates of prevalence of peanut hypersensitivity are complicated by several issues: first, prevalence data is largely obtained by self-report, which may result in over-estimation of allergy prevalence as compared to prevalence by confirmed by positive food challenge[22-25]. Hypersensitivity is defined as "a condition in which there is an exaggerated response by the body to the stimulus of a foreign agent", while allergy is defined as "hypersensitivity caused by

exposure to a particular antigen (allergen) resulting in a marked increase in reactivity to that antigen upon subsequent exposure, sometimes resulting in harmful immunologic consequences."[26] The general consensus regarding sensitization is that it is the process through which an allergy develops, immunologic recognition of an antigen, which may not necessarily provoke a clinical reaction. Peanut sensitization may be mistaken for peanut allergy, and the immunologic criteria used to make a diagnosis are controversial, which is further discussed below. Increased public awareness of peanut allergy may lead to more individuals being tested or tested at an earlier age – for example, one study found that more children appear to be having reactions at a younger age[27].

In both the United States and United Kingdom, over a five-year period reported allergy in children doubled and sensitization to peanut tripled[16, 28]. In the Isle of Wight, a study looking at two sequential cohorts of children separated by six years, who were skin prick tested and had food challenges, found that there was a strong trend for increase in prevalence of peanut allergy, although it was statistically non-significant[29]. The most recent self-reported US prevalence data finds that peanut and tree nut allergy has increased over 11 years in children, with a current prevalence of 2.1%, but is stable in adults at 1.3%[15].

In Canada, the prevalence of peanut hypersensitivity is similar at approximately 1.7%, and there has been no recent significant increase in prevalence[14, 30, 31]. This is comparable to other Westernized nations, including Denmark, where the prevalence of peanut allergy in young adults was 0.6%[23]. Similar findings

are seen on the Isle of Wight, where the prevalence in young children was 1.2%[32, 33].

A meta-analysis of studies of food allergy found a prevalence of peanut allergy ranging between 0% and 2%, reflecting a marked heterogeneity of prevalence, likely due to differences in population, study design or methodology[34].

Prevalence of peanut allergy may be easiest to understand in the context of anaphylaxis. In a study of severe reactions to food in the UK and Ireland, peanut caused the largest number of both severe and non-severe reactions[35]. Indeed, of the estimated 2,500 food-induced anaphylactic reactions in the United States each year, 125 of which are expected to be fatal[36], peanut and tree nut would be responsible for the majority of these fatalities [37, 38]. Those affected most by anaphylaxis are young; in a study looking at children and young adults in the state of Texas, those under the age of five years were the most likely to have a hospitalization for anaphylaxis[39]. Peanut was the top cause of severe non-fatal food allergic reactions in a study looking at food allergy reactions across the UK and Ireland[40], and caused 2 of the 8 deaths reported in the study. These results are in stark contrast to studies of anaphylaxis in adults in Italy, where peanut was an uncommon cause of anaphylaxis[41]. The discrepancy between countries highlights the importance of environmental and genetic factors in the development of food allergy.

## B: Diagnosis of peanut allergy

The diagnosis of peanut allergy is particularly fraught with controversy. Although some guidelines have been set, there is still lack of consensus over the criteria used to diagnose peanut allergy. Indeed, there may be a disease spectrum that ranges from sensitization to true hypersensitivity. While those at opposite ends of the spectrum may be more easily identified, such as those who present with severe reactions like anaphylaxis, it may be difficult to distinguish between sensitivity and true allergy in individuals who have symptoms that are less severe. The three main diagnostic tests used by clinicians include oral food challenge (OFC), considered the gold standard, as well as serum peanut-specific immunoglobulin E (IgE), and skin prick testing (SPT).

These methods of testing for peanut hypersensitivity have the same issues as all diagnostic tests: primarily that different cut-offs may be appropriate depending upon the purpose of the test. The majority of studies on the methods for diagnosing peanut allergy have focused on the positive predictive value (PPV) in ensuring that an individual has the disease. Fewer studies have been couched in the framework of ruling out peanut hypersensitivity.

Multiple algorithms for diagnosis of peanut allergy exist[42]. The most commonly accepted cut-offs for testing are: 1) a SPT greater than or equal to 8mm 2) a serum peanut-specific IgE of 15kU/L or 3) a positive OFC[43]. Some may also include a SPT

greater than or equal to 4mm if the infant is less than 2 years of age[44].

Skin prick testing for peanut:

The most accepted size of prick test wheal to peanut for diagnosis of peanut allergy is 8mm[43]. Using the gold standard of OFC, this cut-off has been replicated in several studies, with a reported PPV of 100%[45-48]. However, both lower and higher cut-offs for the diagnosis of peanut allergy have been suggested. Some have investigated a SPT cut-off of 7mm, which was 97% specific, 83% sensitivity and had a PPV of 93%[49]. A SPT less than or equal to 7mm was 84% specific and 83% sensitive for tolerance on oral food challenge, and its specificity and sensitivity increased when used in combination with results of peanut-specific IgE[50]. Others have argued for a higher cut-off than 8mm. In a study with 52 OFC positive subjects, skin prick testing was only 67% specific for positive OFC if greater than or equal to 8 mm; the specificity increased to 100% if the cut-off was increased to greater than or equal to 15mm[51]. Similarly, a French study found a PPV of only 90% with a SPT of 8mm, which increased to 100% if an SPT cut-off of at least 16mm was used[52].

Size of skin prick wheal is correlated to both severity of reported symptoms, and anaphylaxis on food challenge. A study of Dutch adults with peanut hypersensitivity found that those with severe allergy symptoms had higher SPT reactions to lower concentrations of antigen, and had a reaction to a greater number of *Arachis* antigens than those with milder

symptoms[53]. A mean SPT size of 10.1 mm was found in those with anaphylaxis when challenged by OFC, versus an SPT of 6.7mm in those who did not have anaphylaxis[54].

Some have suggested that SPT is more reliable than peanut-specific IgE in the diagnosis of peanut allergy[48, 49, 55]. However, SPT results may be influenced by a variety of factors, including area of the body tested, time of day and season[56]. Skin prick test results may also be influenced by age: in children less than 2 years of age who present with a history of food allergy, the PPV is 100% if a 4mm cut-off for peanut is used to diagnose peanut allergy[47, 57].

Peanut-specific Immunoglobulin E:

A person with a peanut-specific IgE less than 0.35ku/L is very unlikely to be peanut allergic, with a negative predictive value (NPV) of 85%[56], but a cut-off at this level gives a poor PPV of only 15%[58]. Indeed, one study found that the peanut-specific IgE level of 0.37kU/L was 98% sensitive but only 33% specific[51, 59]. Another study found that peanut-specific IgE levels of less than 2kU/L were best at identifying children most likely to tolerate peanut[50]. However, it is still possible to have reactions on OFC with IgE levels lower than 0.35kU/L[60].

The most commonly accepted cut-off for peanut-specific IgE level is 15kU/L[43, 45, 46]. However, both lower and higher cut-offs have been recommended, and may vary widely. One study found that a level of 10kU/L was 100% specific for peanut allergy as diagnosed by OFC[59], while another found 14kU/L to

have a PPV of 100%[46]. A Dutch study of 100 children using double blind, placebo controlled food challenge (DBPCFC) as the gold standard found a 95% PPV when the cut-off was 24.1kU/L, which increased to a PPV of 100% if an IgE cut off of 26.5kU/L is used[61]. A French study found an even higher IgE level of 57kU/L had 100% PPV for peanut hypersensitivity[52].
Level of peanut-specific IgE is correlated with anaphylaxis on food challenge. In a study of 52 children in Australia, those who had anaphylaxis on food challenge had a median peanut-specific IgE of 20.5kU/L, compared to a median of 0.68kU/L in those who did not have anaphylaxis on food challenge[59].

Levels of peanut-specific serum IgE tend to decrease with age[62]. Periodic re-testing of peanut-specific IgE has also been recommended as a method of monitoring for development of tolerance, and predicting outcomes of repeat food challenges[63].

Oral food challenge:

Even the gold standard, food challenge, the test to which SPT and peanut-specific IgE are compared, can pose difficulties. These include the need for experienced personnel who are comfortable conducting the test, identifying symptoms appropriately, and managing reactions, as well as the resources required to administer the test in a timely fashion[64], especially considering the possibility of resolution of allergy[60, 65, 66]

The false positive rate of DBPCFC may be as high as 12.9%, and can be due to both objective and subjective symptoms.

Objective symptoms include the observation by the health care provider of signs ranging from angioedema, or urticaria, to coughing, wheezing, tachycardia and hypotension. Subjective symptoms include report of abdominal pain, itchy throat, nausea, and worsening of itch[67]

Logistically, children may refuse to eat the peanut or parental anxiety may lead them to refuse the test[54]. In young children, a refusal to eat may be because they already feel symptoms but are unable to describe them – in this case, a refusal to eat might have clinical significance. Unfortunately, it may be impossible to differentiate between the two situations.

The dose required for food challenge reaction is also an issue. Some have found that the amounts needed to provoke a true reaction may be quite high[54], although minimum eliciting doses do not appear to differ between those with a history of severe reaction to peanut versus those with a history of mild reaction to peanut[68]. However, one study found that the majority of individuals with peanut allergy who had anaphylaxis on food challenge would not have had such a reaction if the food challenge had been stopped at a milder reaction[54].

Although DBPCFCs can be performed safely in children who have peanut-specific IgE and SPT levels lower than the above-discussed cut-offs[69], deliberate incitement of anaphylaxis, or even the risk of potentially causing anaphylaxis, especially in children, may be an ethical issue for practitioners[70, 71]. Since the advent of better laboratory testing methods, some have even suggested that "DBPCFC should no longer be considered a

mandatory diagnostic procedure[;] it has to be used in those rare events where the patient has a clinical history of peanut allergy, but no measurable (peanut-specific) IgE."[72]

Combined methods:

Both the size of the wheal on skin prick test and the level of peanut-specific IgE have been correlated with risk of anaphylaxis on oral challenge[54], although not necessarily with clinical severity[48]. Some researchers have suggested combining the results of multiple testing methods in order to improve specificity of diagnosis of peanut hypersensitivity in the absence of food challenge[50, 59, 73]. Non-laboratory findings such as age, gender, and characteristics of symptoms of the reaction to peanut may also improve the specificity of diagnosis[74]. Characteristics of the reaction are essential to the interpretation of the SPT and peanut-specific IgE[75] and in the context of a convincing history, a lower SPT and IgE may be considered sufficient to diagnose peanut allergy.

## C: Impact and treatment of peanut allergy

The mainstay of treatment of peanut allergy is avoidance. Prophylactic avoidance has been advised by most authors[76, 77]; however, some have argued that decreased exposure may paradoxically increase the risk of peanut allergy[78]. Indeed, for individuals in whom peanut hypersensitivity resolves, at least monthly regular peanut consumption to maintain immunological tolerance and decrease the risk of allergy recurrence has been advised[79]. However, cases have been reported in which

documented frequent consumption of peanut did not prevent peanut hypersensitivity recurrence[80].

Although peanut allergy is most commonly understood to cause morbidity and mortality through anaphylaxis, it is important to realize that peanut hypersensitivity impacts affected individuals and their families both financially and emotionally, due to extra vigilance required for peanut avoidance, preparation for accidental exposure, and reaction of the social environment to the allergic person and their family. Although cost may vary dependent upon location, a prophylactic rescue epinephrine autoinjector is approximately sixty to one hundred dollars, and like all medication must be replaced regularly due to expiration dates. Children generally have more than one autoinjector, for example, one at school and one at home. In children diagnosed with a peanut allergy, the annual incidence rate of accidental ingestions is 14%[81]. Many of these reactions occur at school: in a study of 132 children in Baltimore, 18% of food-allergic children had experienced a reaction at school or pre-school in the past two years[82]. Due to these events, institutions have or are in the process of instituting peanut-free guidelines in an effort to decrease this rate of accidental ingestion, with subsequent success in decreasing peanut-containing food items; however, the success of these guidelines in reducing accidental exposure to peanut is yet to be elucidated[83]. Allergic children can be the targets of school taunting, teasing and bullying[84]. Peanut allergy causes significant disruption in the lives of children with peanut allergy[85], and parental stress and anxiety of the affected child have been well documented[86]. Epinephrine auto-injector prescription without proper training

may induce anxiety, rather than relieve it[87]. Parents of peanut allergic children often encounter individuals who do not believe the allergy is real and give the child food containing peanuts, and describe being made to feel "neurotic, over-anxious, fussy, and 'faddy' when they raised concerns, asked for special arrangements, or requested information"[84]. Indeed, members of the lay press have questioned the increase in prevalence of peanut allergy, likening it to mass hysteria and "Yuppiedom".[17]

The issue of peanut avoidance has societal ramifications as well. Whose responsibility is it to ensure that a peanut-allergic individual avoids exposure? Should schools and other industries be required to change their policies or their practices?[88] The majority of major airlines have switched to peanut-free on-board snacks due to possible in-flight reactions[89-94]. Although guidelines are given to patients with peanut allergy for avoidance[95], current food labeling efforts are found confusing by peanut allergic individuals[96, 97] and children may not even be able to identify peanuts in their intact form[98]. Some authors have advocated the establishment of a system similar to public defibrillators for allergy rescue medication[99]. Both members of the lay press and the medical establishment have questioned the utility of the peanut ban, and many feel that the risk has been blown out of proportion[18, 19].

Apart from peanut avoidance, other treatment options that are being investigated include oral peanut and soy immunotherapy, anti-IgE therapy, alternative medicine, probiotics, and cellular

mediators, including platelet-activating factor and platelet-activating factor acetylhydrolase[10].

## D: Clinical course of peanut allergy

The natural history of peanut allergy has been discussed in some length in reviews [60, 100-102]; a summary is provided below.

It is estimated that up to 20% percent of peanut allergic individuals have resolution of their allergy[103, 104]. Some have questioned whether resolution of peanut allergy indicates a lack of true peanut allergy in the first place, considering the previously discussed controversies in making the diagnosis[105]. Indeed, those who have a peanut allergy that does not persist tend to have mild disease, with individuals with peanut-specific IgE levels of 5kU/L or less having a 50% chance of resolution[60], and several studies did not establish the primary allergy diagnosis using OFC[60, 65, 79]. However, the 20% prevalence rate was replicated in a study that used DBPCFC for diagnosis of allergy[104], which does not support the theory that those individuals with resolving peanut allergy were never truly peanut allergic.

Although some authors feel that initial reaction to peanut does not predict subsequent reactions[64], resolution of peanut allergy appears to be best predicted by the initial reaction type to peanut. Symptoms at unintentional exposure are similar to symptoms of future reactions[106]. Those with mild disease are more likely to resolve[60], and patients who present with anaphylaxis to peanut are highly unlikely to have their peanut

allergy resolve[66], although this is not impossible[107]. In a small case-control study, children whose peanut allergy resolved less commonly had allergies to food other than peanuts, although the prevalence of asthma, eczema, hayfever and rhinitis were similar to those whose disease had persisted[65].

## E: The genetic mechanisms of peanut allergy

Similar to other atopic conditions, there is a strong genetic component to peanut hypersensitivity. Among 14 monozygous twin pairs, 9 (64%) were concordant for peanut allergy compared to 3 of 44 (7%) dizygous twin pairs[108]. In addition, the relative risk of peanut allergy to siblings of an individual with peanut allergy was reported to be significantly higher than that in the general population, with reported odds ratios ranging between 6.7 and 13.5[109, 110]. A survey of 2,000 UK households showed that given one person with peanut allergy, the probability of another was 3.2%, which is six times the UK population prevalence, and 9 of the 10 second cases were in first-degree relatives[111]. The risk of peanut allergy in the siblings of peanut-allergic children is so high that some have recommended that siblings of peanut hypersensitive individuals be assessed by an allergist prior to being exposed to peanut[109].

Despite convincing evidence of strong heritability, there is surprisingly little data regarding the genetic basis of this disorder. Family studies have indicated HLA class II DRB1, DQB1, and DPB1 polymorphisms are associated with peanut hypersensitivity[112, 113], but these associations were not

replicated[114] and very little has been done since. Similarly, a study finding association of a CD14 polymorphism with food allergy was not replicated[115]. Other possible genes of interest include those that have been implicated with related atopic conditions, such as STAT6 polymorphisms with nut allergy[116].

The pathogenesis of peanut allergy is a complex interaction between environment and immune response, which widens the field for potential genetic candidates. Genes of interest include those regulating immune function, such as genes for toll-like receptors, interferon gamma, interleukins, CD14, platelet-activating factor, histamine, immunoglobulins, and B and T-cell regulation, all of which have been implicated in the pathogenesis of peanut hypersensitivity[117-121]. Microarray data of T cells from studies on oral peanut immunotherapy suggest a role for apoptotic genes in induction of tolerance, such as caspases and genes involved in the p53 and tumor-necrosis pathways[122] The strong inheritance of not only peanut allergy but of all atopic diseases, allows inclusion of those genes that have been implicated in related allergic conditions.

## F: Peanut allergy, atopic dermatitis and asthma

Clinicians have long speculated about the inheritance of allergic susceptibility, as clinical observation and research have established that atopic diseases (asthma, eczema or atopic dermatitis, allergic rhinitis and food allergies) tend to cluster together in individuals and families. The 'atopic triad' of asthma, eczema and allergic rhinitis are often found together in individuals[123, 124], as well as families[125, 126].

Of particular interest is the relationship between peanut allergy and atopic dermatitis. Atopic dermatitis (AD) is a risk factor for peanut allergy[12, 127]. In Denmark, it is estimated that approximately 15% of children with AD have food allergy of some sort[128]. Similar to peanut allergy, twin studies for AD have shown much higher concordance rates for AD in monozygotic versus dizygotic twins [129-131].

Asthma is also well known to be related to peanut allergy[11, 132-134]. Coexisting asthma is strongly associated with severe reaction to peanut[135, 136], and is a risk factor for increased mortality with anaphylaxis[39]. A questionnaire study of survivors of anaphylaxis found that those who required epinephrine were more likely to report both peanut allergy and asthma[137]. The opposite relationship is also true: food allergy is also a risk factor for fatal childhood asthma[138-140].

Several loss-of-function mutations in the gene encoding filaggrin (*FLG)* have recently been found in patients with ichthyosis vulgaris, a hereditary condition of dry skin[4] and AD [1], two conditions which often co-exist[141]. Interestingly, *filaggrin* mutations have been specifically associated with AD accompanied by high total serum IgE levels and concomitant allergic sensitizations, often identified as the "extrinsic type" of atopic eczema[2, 142, 143]. *Filaggrin* mutations have also been associated with asthma, although whether this association is only mediated by the presence of eczema is debatable[3, 144-147]. Interestingly, an interaction between *FLG* mutation and food allergy predicts childhood asthma[147]. These findings

have led to the suggestion that a defective epidermal barrier may be the site of exposure in allergic disease[4, 5].

## G: Discovery of null mutations in *filaggrin* in atopic dermatitis

Filaggrin, a protein found in the stratum corneum of the epidermis, was initially linked to ichthyosis vulgaris (IV), a hereditary condition characterized by dry, scaly skin and hyperlinear palms, due to abnormal histological findings, such as absent keratohyalin granules, and lack of profilaggrin and filaggrin expression[148-151]. Eczema and IV frequently co-exist, and a similar finding of decreased filaggrin expression in atopic skin was subsequently discovered in AD[152]. Despite this compelling evidence, the gene proved to be difficult to target for genetic studies due to its structure. *FLG* is a large, complex gene with multiple repeats[153], all of which have slight variation, and both characteristics result in difficulties in finding reliable primers for its sequencing[154].

Beginning in 2006, loss-of-function mutations in *FLG* were linked to IV and AD in a variety of populations[1, 4, 155-160], and were specifically linked to AD associated with sensitization to aero- and food allergens with elevated serum IgE[2, 3, 161]. Since this time, replication studies in a variety of populations have been completed, which have found that although null mutations in *filaggrin* are consistently found in AD, each ethnic population appears to have its own signature set of mutations[144, 155, 160, 162-164]. This is a semi-dominant trait (incomplete dominance) [156, 165, 166], meaning that

having two mutated copies (homozygous phenotype) is more severe than having one mutated copy (heterozygous phenotype), in comparison with complete dominance, where the phenotype of a homozygote is the same as a heterozygote. Since these are all loss-of-function mutations, this means that a compound heterozygous mutation is the same in function as a homozygous null mutation, as there is no protein expression from either copy of the gene. Penetrance was originally estimated to be 80-90% in ichthyosis vulgaris[4, 166], although in eczema this may be as high as 100% if two null mutations are present[167]. The most prevalent European mutations include R501X and 2282del4, which have been found in the original Irish-Scottish population, as well as the Swedish[160] and German populations[166]. The frequency of these mutations in the normal Irish, Scottish, German and European American populations appears to be approximately 1 to 2 percent[1, 4, 166, 168], although more recent studies found a combined prevalence of 8%[169, 170]. The relationship between *FLG* null mutations and eczema show the strongest evidence of any candidate gene in atopic dermatitis[171].

## H: Function of filaggrin

The gene encoding filaggrin is found in a small cluster of genes on chromosome 1q21 called the epidermal differentiation complex, which encodes a number of genes important in the function and terminal differentiation of the epidermis[172]. Filaggrin begins its life as profilaggrin, a large peptide with multiple repeated sections that appears in keratohyalin granules, which give the stratum granulosum its granular quality.

Terminal differentiation of the epidermis, the process by which the skin evolves from a multipotent basal cell to an enucleate, flattened squamous cell that is eventually shed, is a complicated, multi-step process that involves many genes[173]. During terminal differentiation, at the border between the uppermost granular layer and the squamous layer, profilaggrin is released. Here, it is cleaved into filaggrin peptides by enzymes by dephosphorylation[174].

Filaggrin, named thus due to its intermediate filament aggregating properties (**fil**ament **agg**regating prote**in**), gathers the keratin intermediate filaments together at the junction between the stratum granulosum and the stratum spinosum, flattening the cells into a compact layer. This function of filaggrin is confirmed by the appearance of a poorly formed stratum corneum in those with *filaggrin* null mutations[4]. However, its role does not end with the mechanical compaction of the epidermal cells.

Filaggrin is then modified by peptidylarginine deiminase (PAD), which results in a conformational change and dissociation[175, 176]. Deimination by PAD is also thought to affect gene expression and apoptosis[177]. Filaggrin in its intact form does not appear to have any DNA binding activity[178], but it may also have a role in transcriptional regulation of proteins. The C-terminal domain of the profilaggrin molecule is critical for proper processing of profilaggrin to filaggrin[179], while the N-terminus of profilaggrin appears to localize to the nucleus after cleavage, indicating a possible regulatory function of this segment[180].

Filaggrin is broken down into amino acids, and protein dissociation via deimination allows for its appropriate degradation. Calpain I, caspase 14 and bleomycin hydrolase have all been found to cleave filaggrin into peptides of a variety of sizes, and bleomycin hydrolase has been found to co-localize with filaggrin in the skin[181]. Filaggrin's split products become free amino acids, as well as urocanic acid and pyrrolidone carboxylic acid[182], and comprise a major component of natural moisturizing factor (NMF)[183]. The production of these molecules is thought to allow hydration of the stratum corneum despite a dry external environment, as well act as a possible sunscreen[184]. *Filaggrin* genotype has been found to correlate very strongly with reduced NMF in the stratum corneum[183, 185, 186] and increased trans-epidermal water loss (TEWL)[187]. As expected in a semidominant trait[156, 165, 166] milder cases of xerosis are associated with heterozygosity of null mutations in *FLG*[188]. Some have suggested modulation of filaggrin's effects could be due to number of *filaggrin* repeats (intragenic copy number)[189, 190].

## I: Null mutations in *filaggrin* and epidermal barrier defect

Atopic dermatitis and IV skin has higher transepidermal water loss (TEWL)[191, 192] and impaired barrier function, both in affected and unaffected (ie: normal-appearing) skin[191, 193-195]. In atopic dermatitis patients with *filaggrin* mutations, clinical severity of eczema correlates with barrier impairment as determined by TEWL and stratum corneum hydration[187]. The mouse model of AD described above also has an impaired barrier function with greater penetration of allergens[196].

Clinically, those with AD are at higher risk for both irritant and allergic contact dermatitis. A deficiency in filaggrin, through its role in the production of the physical barrier, as well as its role in moisture-retention of the epidermis, could explain this higher risk rate. Although an initial study did not find an association between loss-of-function mutations in *filaggrin* and contact allergy[197], further work has found that allergic contact dermatitis and sensitization to nickel are associated with *filaggrin* mutations[198]. This is thought to be due to the loss of nickel-chelation properties, leading to specific penetration of nickel through the epidermis. Filaggrin is a histidine-rich protein, and histidine and proteins that contain it are known to be strong nickel chelators[199].

In summary, filaggrin's role in the epidermis is at least two-fold: formation of the epidermal barrier, as well as moisturization of the skin[200]. It may have yet undetermined roles in transcriptional regulation or signaling, and UV protection. It has been suggested that the possible evolutionary advantage to this type of mutation, and why separate mutations should arise independently in a variety of populations, is that a barrier defect such as this may provide a low level of antigen exposure, essentially providing a "natural vaccination" to pathogens[201].

## J: Peanut allergy and the epidermal barrier

With the explosion of knowledge about the barrier defect in eczema, the possibility of this defect being the potential starting gate of the atopic march has been proposed[4, 5], possibly due to allergen penetration and provocation of Th2 type responses and IgE production[202].

The barrier defect, concomitant IgE sensitization and loss-of-function mutations in *FLG* have been demonstrated in AD patients in several studies[2, 161, 195]. *Filaggrin* mutations have been found to predispose to asthma, allergic rhinitis and allergic sensitization both with and without the presence of eczema[3], which lends support to the hypothesis that allergen penetration through the skin leads to susceptibility to other allergic disease. This is further supported by the fact that filaggrin is not expressed in bronchial mucosa[203].

Although the "flaky tail" mouse model for AD has a barrier deficit, allergen-specific IgG and IgE induction from topical application of allergens to intact skin, increased TEWL, and other characteristics seen in atopic skin, this mouse model does not appear to develop airway disease[196]. This is interesting, as a recent study looked at the clinical characteristics of IV in patients in an atopic dermatitis clinic and found that those with clinical characteristics of severe IV were more likely to have asthma and allergic rhinitis[141].

An association between peanut allergy and atopic eczema has been previously established[204], and some have even

suggested that cutaneous exposure to peanut derivatives may be the source of sensitization in peanut hypersensitivity[205]. An impaired epidermal barrier has also been suggested as the cause of sensitization for some type IV hypersensitivity reactions, such as those to nickel[198]. This theory of cutaneous sensitization is further supported by mouse models that found that epicutaneous exposure to peanut protein through a disrupted stratum corneum not only enhanced allergic sensitization, but also prevented oral tolerance[6, 206].

Cutaneous peanut butter exposure has been associated with contact reactions[59], and in one study, all children whose initial reaction was at the site of skin contact and who experienced a reaction on subsequent exposure, developed respiratory and/or gastrointestinal symptoms[207]. However, this clinical finding is not consistent[59, 106].

Despite evidence establishing the relationship between peanut allergy and barrier defect, how cutaneous sensitization may occur has been hotly debated. Peanut oil was previously implicated as a possible source[205], as it is ubiquitously present in food, medicinal[208, 209] and cosmetic products[210]. However, although crude peanut oil can contain peanut protein allergens, refined oil contains no peanut allergens by immunoassay, and has not been found to cause reactions in peanut-sensitized individuals. In the United Kingdom, refined peanut oil in food and medicinal products have been deemed to be without risk in allergic individuals[211]. Supplementation of vitamins A and D in peanut oil in infancy shows no increased risk of peanut allergy[212]. However, similar evidence is not

available for other peanut-related additives, such as peanut flour. Household peanut consumption is a risk factor for the development of peanut allergy, and oral exposure to peanut at a young age appears to be protective[213]. However, there is currently no strong evidence regarding trans-epidermal sensitization to peanut allergens, and "a possible induction of sensitization against peanut proteins through contact with the skin via skin care products and the respective protein concentrations is a matter of speculation"[214].

Peanuts contain multiple protein allergens, and peanut-allergic individuals are most frequently sensitized to Ara h2, followed by Ara h1, h6, h3 and h7[72, 215, 216]. Positive peanut-specific IgE in atopic patients is primarily caused by the Ara h8 protein, which is homologous to the pollen-associated allergen Bet v1[72]. In a Swedish study, the levels of IgE to recombinant Ara h8, recombinant Bet v 1 and birch pollen were highly correlated[217]. This has led to the suggestion that sensitization to peanut may occur through cross-reactivity with pollen.

Previously, our research team demonstrated an association between *FLG* null mutations and peanut allergy[7]. In a case-control study of 71 English, Dutch, and Irish patients with a positive OFC to peanut, and 1000 non-peanut-sensitized English population controls, we found an odds ratio (OR) 5.3 (95% CI, 2.8-10.2), which decreased to 3.8 (95% CI, 1.7-8.3) when controlling for history of AD. This association was replicated in 390 Canadian individuals with peanut allergy, diagnosed by peanut-specific IgE≥15kU/L, or SPT≥8mm, or positive OFC, and 891 white Canadian population controls; the OR in this

population was 1.9 (95% CI, 1.4-2.6). The difference in ORs between the populations may be explained by the use of non-sensitized individuals in the English control group, while the Canadian control group likely included both sensitized and possibly even allergic individuals, leading to an inflated association in the European results. An alternative explanation is that since the Canadian peanut allergic cases were not OFC tested, the presence of sensitized individuals in the cases weakened the observed association. We set out to address these questions, and the possible role of atopic disease, by further analysis of the Canadian peanut allergic cases.

## K: Other possible mechanisms of peanut allergy pathogenesis

Some have suggested the hygiene hypothesis to explain the increased incidence and prevalence of atopy and autoimmune disorders[218-220]. Serologic evidence of acquisition of infections is associated with a lower odds of having hay fever and asthma[221], but bacterial flora appears to be the same in both food sensitized and non-food sensitized individuals[222].

The role of maternal peanut consumption in the development of peanut allergy remains a controversial domain. This theory suggests that in-utero or perinatal sensitization to peanut allergens, due to exposure to peanut antigens via maternal consumption during pregnancy or lactation, may be the cause of peanut allergy[110, 223-226]. Food proteins may enter breastmilk in an immunologically intact state[227] and may provoke reactions when given to a sensitized infant[228, 229].

Although some researchers expressed their reservations about instituting such advice with such little data available[230], pregnant women in the United Kingdom were previously warned to avoid peanuts during pregnancy and lactation[231]. This advice was withdrawn nearly a decade later, due to its lack of foundation in evidence-based medicine[232]. There was no significant change in the prevalence of peanut allergy in the United Kingdom during this time, and there was no clear conclusion regarding the effect of peanut avoidance[233]. A recent case-control study concluded that peanut consumption during pregnancy and lactation did in fact increase the risk of peanut allergy in offspring, however this study may have been susceptible to differential recall bias[225]. Recent evidence from a cohort study finds that peanut consumption during pregnancy or lactation may not be significant to peanut allergy development in the child[213], and although evidence is weak, breastfeeding may have a protective effect on development of atopic conditions[234]. Early introduction of peanut into the infants' diet in Israel is associated with a much lower prevalence of peanut allergy[235] than in Great Britain, where peanut introduction is postponed[236].

## III: STUDY OBJECTIVE

In normal conditions, large protein molecules should not penetrate the epidermal barrier. Peanut allergy is caused by hypersensitivity to *Arachis* protein allergens. Therefore, a defect in the epidermal barrier, such as that which is caused by a loss-of-function mutation in *filaggrin*, would allow penetration of protein allergens and predispose to hypersensitivity. Our hypothesis is that loss-of-function mutations in *filaggrin* are associated with type I hypersensitivity reactions to peanut protein allergens. In our previous work, a link between *FLG* null mutations and peanut allergy was made[7], but questions remained regarding the relationship of the genetic mutations and peanut allergy independent of atopic disease, and the effect of diagnostic criteria for diagnosing peanut allergy, considering the difference in OR between the populations studied. The purpose of this study was to see how peanut allergy case definition affects the relationship between peanut allergy and defects in *FLG*, and to make an assessment of how asthma may affect relationship between peanut allergy status and *filaggrin* null mutations.

## IV: METHODS

### A: Recruitment of Cases and Controls

Cases:

Subjects were enrolled from a previously established and well-described peanut case group, which has been recruited through the Montreal Children's Hospital, Anaphylaxis Canada, the Association Québécoise des Allergies Alimentaires and the Allergy and Asthma Information Association [81, 237]. These individuals previously consented to being approached for further studies in peanut allergies. Individuals were invited to participate by a letter sent in the mail or by email. Those who were willing to participate were sent a questionnaire in their language of preference (English or French) and a mail-in DNA salivary sampling kit (Oragene), with an addressed envelope with return postage.

Recruitment occurred from July 2008 to April 2009. Subjects indicated their self-identified ethnicity in accordance with the categories taken from Census Canada in 2001 (White, Chinese, Japanese, Korean, Black, Filipino, Arab, Latin American, South Asian, Southeast Asian, West Asian, Aboriginal, or Other). Questionnaire respondents were able to write free-text information regarding their ethnicity when the "Other" category was indicated. Identification of ethnicity was particularly necessary in this genetic study, as loss-of-function mutations in *filaggrin* are well known to vary across ethnic backgrounds[144, 155, 160, 162-164].

Personal data collected on the patients included: age, sex, personal history of eczema, and family history of atopic conditions (asthma, eczema, rhinitis or hayfever, and food allergies). Data in the registry from previous questionnaires was included, including personal and family history of atopic conditions, as well as results of SPT and serum peanut-specific IgE. All characteristics, other than SPT and serum peanut-specific IgE results, are by self-report. Since atopic diseases such as eczema, asthma and food allergy may spontaneously resolve, with resultant problem of recall, a variable was constructed for each atopic disorder indicating if they had ever had the condition. These variables were constructed from the database information as well as the current questionnaire: if the subject ever answered "yes" on their original questionnaire, follow-up questionnaires, or the current questionnaire, they were considered to have had the disease. Similar variables were constructed for family history of atopic disease.

*Case definition*

A diagnosis of peanut allergy is generally made in the clinical setting based on the symptoms and signs after exposure to peanut, and with confirmatory immunologic testing, either serum-specific peanut IgE, a positive SPT to peanut, or an OFC. Once a diagnosis of peanut allergy has been given to an individual with a strong history, especially if the patient continues to react upon accidental exposure, it is unlikely that he/she will have any subsequent testing done.

There is no firm consensus on the definition of peanut allergy. Different physicians may have different standards of history, SPT results, and IgE levels required to make a diagnosis. Furthermore, the gold standard for peanut allergy testing, an OFC, may not necessarily be performed by physicians who feel that subjecting a patient with a strong history of anaphylaxis and immunologic results indicating a high probability of peanut allergy to an oral challenge is both dangerous and unethical[70, 238]. This dilemma is even more pronounced in children, and many pediatric allergists feel comfortable with a diagnosis made by history, SPT and peanut-specific IgE results alone.

*Original registry case definition:*

Individuals were eligible for registry enrolment if either of the following criteria were fulfilled[239]: (1) the child had a convincing history of an allergic reaction to peanut and a positive SPT to peanut or a peanut-specific IgE level ≥0.35 kU/L, or (2) the child had no history of peanut ingestion or an uncertain clinical history of peanut allergy and either a positive SPT and a peanut-specific IgE level ≥15 kU/L or a positive SPT and positive food challenge with peanut or a positive food challenge only.

Clinical history:

A convincing history of a peanut allergy was defined as a minimum of 2 mild symptoms or signs or either 1 moderate or 1 severe symptom or sign occurring within 120 minutes after peanut contact or ingestion. The severity of the reaction was defined as follows[240]:

(1) Mild: pruritus, urticaria, flushing, and/ or rhinoconjunctivitis
(2) Moderate: angioedema, throat tightness, change in voice, coughing, difficulty breathing (other than wheeze), nausea and/or vomiting, and/or abdominal pain
(3) Severe: wheezing, stridor, cyanosis, and/or circulatory collapse

Diagnostic tests: skin prick test to peanut protein, peanut-specific IgE level and oral food challenges

Skin prick tests were done with commercial extracts. A SPT was defined as positive if the greatest diameter of the wheal was at least 3 mm greater than the negative control (saline) at the time of the child's initial evaluation for peanut allergy[241]. For skin prick testing, some case subjects had been graded by their allergist on a system rated 0 to 4, rather than in millimetres. To allow for the inclusion of these individuals, the system was converted as follows[242] (Table 1):

*TABLE 1: SPT CONVERSION FROM OLD GRADING SYSTEM TO MILLIMETRES*

| Old system | Equivalent in millimeters |
|---|---|
| 0 | <4mm |
| 2+ | 5-10mm |
| 3+ | 10-15mm |
| 4+ | >15mm |

Individuals who had been graded 1+ were considered negative. The individuals classified by this system will be further discussed in the case definition sensitivity analysis below.

The serum level of peanut-specific IgE was measured by the CAP-system Fluoroenzyme Immunoassay (Pharmacia Diagnostics, Uppsala, Sweden). Given that individuals with a peanut-specific IgE level ≥15 kU/L have at least a 95% likelihood of experiencing an allergic reaction to peanut on exposure[45, 46, 52, 243], we defined patients who had never been exposed to peanut or had an uncertain clinical history and a peanut-specific IgE level ≥15 kU/L as allergic to peanut without requiring a food challenge. However, a patient with a convincing clinical history of an allergic reaction to peanut and a peanut-specific IgE ≥ 0.35 kU/L was also considered allergic because, in clinical practice, it is believed that the risk of such a patient reacting on peanut ingestion is so high that a challenge is seldom performed[244].

Oral food challenges to peanut were conducted under medical supervision according to previously published protocols[244, 245]. Either open or single-blind food challenges were performed at the discretion of the treating physician on the basis of the available clinical history.

This original case definition was constructed to be the most inclusive. Those individuals who have a peanut-specific IgE of <0.35kU/L or an SPT of <3mm are unlikely to have peanut allergy[51, 58]. However, it is likely that some individuals who are classified as peanut allergic based on a peanut-specific IgE of

0.35kU/L or greater, or an SPT of 3mm or greater may be sensitized rather than allergic, and this concern will be addressed in the sensitivity analysis described below.

Controls:

Two control populations were used for this study. The first was a control group from the Ontario Population Genomics Platform (OPGP) provided by The Centre for Applied Genomics (TCAG), in Toronto, Ontario. This control group was initially obtained from healthy individuals, collected as controls for the Ontario Familial Breast Cancer and Ontario Familial Colon Cancer Registries studies and who were re-consented to be part of the OPGP, as well as additional participants from across Ontario who were randomly contacted and asked if they would be a part of this project. Participants provided a blood sample and completed a questionnaire in English over the phone with a trained interviewer. Data on ethnicity, age, gender, asthma history and smoking history were available from this control group.

A second, smaller control group of 270 samples was obtained from a collection of cord blood from newborn infants in Quebec City, recruited for a previous study[246]. This second control group was chosen to rule out any possible effect of French-Canadian ethnicity on the results of this study. Although no direct information on ethnicity was available for this group, control subjects were randomly sampled from a primarily French-Canadian area of Quebec (Quebec City), based on French-Canadian last name of the infant. There was no personal or family history of atopy existing for this control set, although

age and gender were available. An additional reason for utilizing this second control group was to identify controls of similar ages to the cases.

## B: DNA isolation of cases and controls

DNA extraction of peanut allergic cases was performed using the "Laboratory protocol for manual purification of DNA from 0.5mL of OrageneTM/saliva" provided by Oragene. The DNA was rehydrated in Tris-EDTA buffer at a pH of 8. The DNA concentration was measured using a NanoVue spectrometer (GE). Samples that contained salivary swabs were heat treated at 50°C and centrifuged to collect the saliva as per protocol, then were processed the same as above. Isolated and plated DNA for the Ontario control group was purchased from the OPGP of TCAG in Toronto, Ontario. Quebec City newborn DNA was extracted from 200 µl of frozen whole blood using the QIAamp 96 DNA Blood Kit from Qiagen. DNA was re-suspended in 200µl AE buffer. All DNA samples were then shipped to Dundee, Scotland for genotyping.

## C: Genotyping

Genotyping was conducted similar to previous studies[1, 165]. Genomic DNA obtained from blood in control samples or from salivary collection in peanut allergic cases was amplified by polymerase chain reaction (PCR) using 9µl of mastermix and 1µl of 10µg/mL DNA, with a final concentration of 300nM for primers and 100nM for probes. Mastermix contained: 510µl 2X ABI Mastermix without uracil-N-glycosylase (Applied Biosystems),

61.2µl of each primer (5µM), 20.4µM of each probe (5µM), and 245.2µl water. PCR was run on a Veriti thermal cycler (Applied Biosystems), then scanned with TaqMan using default conditions (1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, then at 60°C for 1 minute). Changes from the original protocol include the use of the Fwd2 and Rev primers for the 2282del4 mutation, and the use of two new primers designed for the R2447X mutation (Table 2).

*TABLE 2: PRIMERS AND PROBES USED FOR MUTATIONS*

| | Primer | Sequence | Probe | Sequence |
|---|---|---|---|---|
| R501X | R501X Fwd | CAC TGG AGG AAG ACA AGG ATC G | R501X mut | 6-FAM-CAT GAG ACA GCT CC-MGB |
| | R501X Rev | CCC TCT TGG GAC GCT GAA | R501X wt | VIC-CAC GAG ACA GCT C-MGB |
| 2282del4 | Del4 Fwd2 | CCA CTG ACA GTG AGG GAC ATT CA | Del4 Probe1 | 6-FAM- CAC AGT CAG TGT CAG GCC ATG GAC A-TAMRA |
| | Del4 Rev | GGT GGC TCT GCT GAT GGT GA | Del4 Probe2 | VIC-AGA CAC ACA GTG TCA GGC CAT GGA CA-TAMRA |
| R2447X | R2447X F2 | CAC GTG GCC GGT CAG CA | R501X mut | 6-FAM-CAT GAG ACA GCT CC-MGB |
| | R2447X R2 | TCC TGA CCC TCT TGG GAC GT | R501X wt | VIC-CAC GAG ACA GCT C-MGB |
| S3247X | S3247X Fwd | CCA GAA ACC ATC GTG GAT CTG | S3247X Probe 1 | 6-FAM-CAG TCA AGG CAC GG-MGB |
| | S3247X Rev | TGC CTG ATT GTC TGG AGC G | S3247X Probe 2 | VIC-AGC AGT AAA GGC ACG-MGB |

## D: Analysis

Descriptive statistics were calculated for the cases and both controls groups. A binary variable for mutation status was created by grouping the heterozygous, homozygous and compound heterozygous mutations into a single "mutation carrier" category. Odds ratios were calculated for the association between *filaggrin* null mutations and peanut allergy, compared to the Toronto control group, the Quebec control group, and the combined control group. Chi-squared and Fisher's exact tests were calculated for each separate control population, as well as for the combined control group. Analysis was completed in both STATA 11/12 and R statistical programs[247] with the same results to 2 decimal places.

## E: Sensitivity analysis of peanut allergy diagnostic criteria

A sensitivity analysis was then undertaken to examine whether the relationship of *filaggrin* null mutations with peanut allergy is affected by how the diagnosis of peanut allergy is determined. As the case definition of peanut allergy is controversial, a continuum of case definitions was subsequently constructed in order to assess the effect, if any, of stringent versus lenient clinical or immunologic requirements for diagnosis of peanut allergy on the relationship with *FLG* null mutations. This sensitivity analysis was particularly of interest since the relative number of subjects who had undergone a food challenge, the gold standard diagnostic test, was low in this study.

Although the cut-offs of 8mm or greater for SPT, 15kU/L or greater for peanut-specific IgE, a positive OFC, or any combination of the above are currently the most widely accepted for diagnosis of peanut allergy[43], both serum peanut-specific IgE level and size of wheal after SPT have been correlated with both severity of reported symptoms and anaphylaxis on food challenge[53, 54]. We examined if peanut allergy as defined by higher cut-offs would still have the same relationship with loss-of-function mutations in *filaggrin*. After review of the literature, several case definitions were evaluated, using the following criteria (Table 3):

*TABLE 3: LABORATORY CUT-OFFS USED FOR CASE DEFINITION CONSTRUCTION*

| Immunologic test | Cut-off | Reference |
|---|---|---|
| Peanut-specific IgE | 15kU/L or greater | [43, 45, 46] |
| | 26.5kU/L or greater | [61] |
| | 57kU/L or greater | [52] |
| Skin-prick testing | 8mm or greater | [43] |
| | 15mm or greater | [51, 52] |
| Oral food challenge | Positive | [43, 45-48] |

Since there is some evidence that use of multiple test types may improve specificity of peanut allergy diagnosis[50, 51, 73], various permutations of these criteria were evaluated to create the case diagnosis, combining each peanut-specific IgE level *AND* each SPT cut-off, or combining each peanut-specific IgE level *OR* each SPT cut-off. These criteria were also combined both with and without a clinical history of anaphylaxis. A

definition that was a hybrid of the original case definition and the most common clinically accepted standards was also examined.

The originally constructed case definitions were 15 categories as defined below. A category labelled "all cases of peanut allergy" was used to verify conversion and compare subject characteristics.

1) all cases of peanut allergy
2) the original case definition:
    a. convincing history of an allergic reaction to peanut **and**
        i. a SPT of ≥3mm to peanut **or**
        ii. a peanut-specific IgE level ≥0.35 kU/L
    b. no history of peanut ingestion or an uncertain clinical history of peanut allergy **and**
        i. a SPT of 3mm and a peanut-specific IgE ≥15 kU/L
    c. Positive OFC
3) the hybrid definition:
    a. History of anaphylaxis **and**
        i. SPT ≥3mm[239] **or**
        ii. Peanut-specific IgE ≥0.35kU/L[58]
    b. Suggestive history (any history suggestive of an IgE-mediated reaction not compatible with anaphylaxis as defined above) **and**
        i. SPT of ≥8mm **or**
        ii. SPT of ≥4mm if <2 years of age[47, 57] **or**
        iii. Peanut-specific IgE ≥15kU/L
    c. No previous exposure **and**
        i. SPT ≥13mm[239] **or**
        ii. Peanut-specific IgE ≥15ku/L **and** SPT ≥3mm

d. Positive OFC regardless of SPT or IgE

4) peanut-specific IgE ≥15kU/L **or** SPT ≥8mm **or** positive OFC
5) peanut-specific IgE ≥26kU/L **or** SPT ≥8mm **or** positive OFC
6) peanut-specific IgE ≥57kU/L **or** SPT ≥8mm **or** positive OFC
7) peanut-specific IgE ≥15kU/L **or** SPT ≥15mm **or** positive OFC
8) peanut-specific IgE ≥26kU/L **or** SPT ≥15mm **or** positive OFC
9) peanut-specific IgE ≥57kU/L **or** SPT ≥15mm **or** positive OFC
10) peanut-specific IgE ≥15kU/L **or** SPT ≥8mm **and** anaphylaxis, **or** positive OFC
11) peanut-specific IgE ≥26kU/L **or** SPT≥8mm **and** anaphylaxis, **or** positive OFC
12) peanut-specific IgE ≥57kU/L **or** SPT≥8mm **and** anaphylaxis, **or** positive OFC
13) peanut-specific IgE ≥15kU/L **or** SPT ≥15mm **and** anaphylaxis, **or** positive food challenge
14) peanut-specific IgE ≥26kU/L **or** SPT ≥8mm **and** anaphylaxis, **or** positive OFC
15) peanut-specific IgE of ≥57kU/L **or** SPT ≥8mm **and** anaphylaxis, **or** positive OFC

Anaphylaxis was defined as involvement of at least two of the following systems: mucocutaneous, respiratory, gastrointestinal, and cardiovascular. Mucocutaneous involvement was defined by symptoms of redness, itchiness, swelling, conjunctivitis, rhinitis, sneezing, or throat itchiness. Respiratory involvement was defined by symptoms of dyspnea, cough, wheeze, throat tightness, choking or asthma. Gastrointestinal involvement was defined by symptoms of abdominal pain, diarrhea, nausea or vomiting. Cardiovascular involvement was defined by cyanosis, pallor, seizures, loss of consciousness, incontinence, or becoming

unresponsive. This definition is consistent with the consensus statement on anaphylaxis[248].

Following the construction of a continuum of case definitions ranging from most permissive to most restrictive, logistic regression modeling was then used to determine if the relationship between *filaggrin* null mutations and peanut allergy changed with stricter case definition criteria. Here, the outcome variable was the binary *filaggrin* mutation indicator, and case definition was used as the predictor variable. Logistic regression modeling was also used to evaluate whether the characteristics of the peanut allergic cases changed as the case definitions for peanut allergy became more stringent. In this case, each characteristic was used as the outcome, with case definition as the predictor variable. Both STATA 11/12 and R programs were used for statistical analysis with similar results.

As we are aware that the conversion of skin prick test results from the old system of evaluation (0 to 4+) to the current recommended method of millimetres could be a contentious issue, the analysis of the effect of case definition was then repeated a second time, dropping the individuals who were given a peanut allergy diagnosis only on the basis of their skin prick test result as classified by the old system of grading. The results of the case definition sensitivity analysis were then used to decide which peanut allergy diagnostic criteria should be used to determine which cases should be included in the logistic regression modeling that follows.

## F: Modeling filaggrin null mutations with peanut allergy and asthma

Considering the recognized relationship between eczema and peanut allergy, a deficiency of the current study is that neither the Ontario nor Quebec control group has information regarding current or past history of eczema. This makes it difficult to determine the contribution of *filaggrin* null mutations to peanut allergy independent of the effect of eczema. However, the TCAG control group had information on self-reported asthma and self-reported bronchial emphysema, as well as smoking history (current, never or ever), which was used to construct a variable representing atopic asthma. Considering that adult self-identified asthmatics may have significant misclassification with those who have chronic obstructive pulmonary disease or chronic bronchitis, the atopic asthma variable was constructed from the asthma and smoking history. This variable, atopic asthma, was constructed based on several assumptions:

1) Those individuals who have atopic asthma in childhood are less likely to smoke as adults.
2) Those adults who have asthma and have never smoked are more likely to have atopic asthma.
3) If a patient reports bronchial emphysema, they do not have atopic asthma.
4) Asthma reported in the case group is atopic.

As this variable was constructed in order to have a measure for atopy when comparing the peanut allergy cases to the control group in logistic modeling, a fifth assumption must be made:

5) Those who have atopic asthma are more likely to have eczema.

This assumption is reasonable as childhood and later onset eczema are negatively correlated with asthma remission[249], and predicts atopic but not non-atopic adult asthma[250]. Furthermore, filaggrin mutations have been linked to asthma in the context of both those asthmatics who also report a history of eczema and those who do not[3, 144-147].

Univariate logistic regression was conducted on the data using the binary mutation variable as outcome, with peanut allergy status, age, gender and atopic asthma as predictor variables, followed by multivariate logistic regression. Three interaction terms were also constructed and evaluated in the multivariate analysis.

1) interaction between peanut allergy status and atopic asthma
2) interaction between peanut allergy status and age
3) interaction between peanut allergy status and gender

Model selection was conducted by examination of change in effect with the addition of each variable or interaction term, and using the Bayesian Model Averaging (BMA) package in the R statistical software program[251]. This was followed by subgroup analysis of the atopic asthmatic and non-atopic asthmatic groups.

Since the pathophysiological mechanism of loss-of-function mutations in *filaggrin* inherently have the genetic mutation upstream from the development of peanut allergy, the modeling was then repeated, this time reversing the predictors and outcome. With peanut allergy status as the outcome, and age, gender, atopic asthma, and *FLG* mutation as the predictive

variables, we used logistic modeling to assess the effect of defects in *FLG* on peanut allergy status. Again, interaction terms were evaluated, and model selection was conducted by examination of change in effect and using the BMA program.

## G: Effect of asthma reporting and eczema status on the relationship between peanut hypersensitivity and *FLG* mutations

Due to the concern of poor capture of asthma and eczema leading to biased estimates, we next evaluated how error in the atopic asthma variable would change the effect of peanut allergy on presence of *FLG* mutations. The prevalence of atopic asthma in the cases is by self-report, and the atopic asthma variable in controls is a constructed variable from other self-reported conditions, and both may be subject to error. Using random sampling and logistic regression modeling, we undertook a series of analyses, including a 12% increase in asthma reporting in cases, a 6% increase in asthma reporting in controls, a 15% increase in asthma reporting in cases, a 10% increase in asthma reporting in controls. This random sampling and subsequent modeling was repeated one hundred times to see the overall effect of error in asthma reporting on the relationship of peanut allergy and filaggrin null mutations.

As we had no information regarding history of AD in the control groups, we calculated the OR for peanut allergy cases who reported a history of eczema with the combined control group as well as each control group alone, and compared the ORs with

those calculated from peanut allergic individuals who did not report a history of eczema.

Further details are given in Appendix E.

## H: Effect of peanut allergy resolution and peanut allergy in the general population

It is estimated that up to 20% percent of peanut allergic individuals have resolution of their allergy[103, 104]. Due to the large age difference between the case and control groups, we strove to investigate the effect of peanut allergy resolution in our case group on the relationship between loss-of-function mutations in *filaggrin* and peanut allergy. We investigated two circumstances: one in which peanut allergy resolution occurs randomly, and one in which resolution is determined in part by asthma status. Mild allergy is less likely to persist[60], and peanut allergy is correlated with asthma allergy[11, 132-134]. We first randomly selected 20% of individuals from the case group, and changed their peanut allergy status to negative, in order to mimic a resolution of peanut hypersensitivity. A univariate and multivariate analysis of peanut and atopic asthma variables was then conducted. The random sampling and analysis was completed a total of 100 times. The same process was then repeated, but selection occurred only from those peanut allergic individuals who did not report asthma, in order to reflect the fact that asthma is associated with severe hypersensitivity, and severe allergies are less likely to resolve.

Approximately 1% of the North American population reports having peanut allergy[15, 30, 132]. Our control group, a general sampling of the population which had no available atopic history other than asthma, is likely to contain people with peanut hypersensitivity. Again, due to the association of severe peanut allergy with asthma and lower likelihood of resolution, 1% of individuals were first randomly selected, then selected from only those who were positive for atopic asthma. Univariate and multivariate analyses were conducted 100 times in both cases. Finally, the effect of peanut allergy prevalence in the general population as well as the possibility of peanut allergy resolution were examined together, contingent upon asthma status. All analyses were run 100 times.

# V: RESULTS

## A: Demographics and atopic history

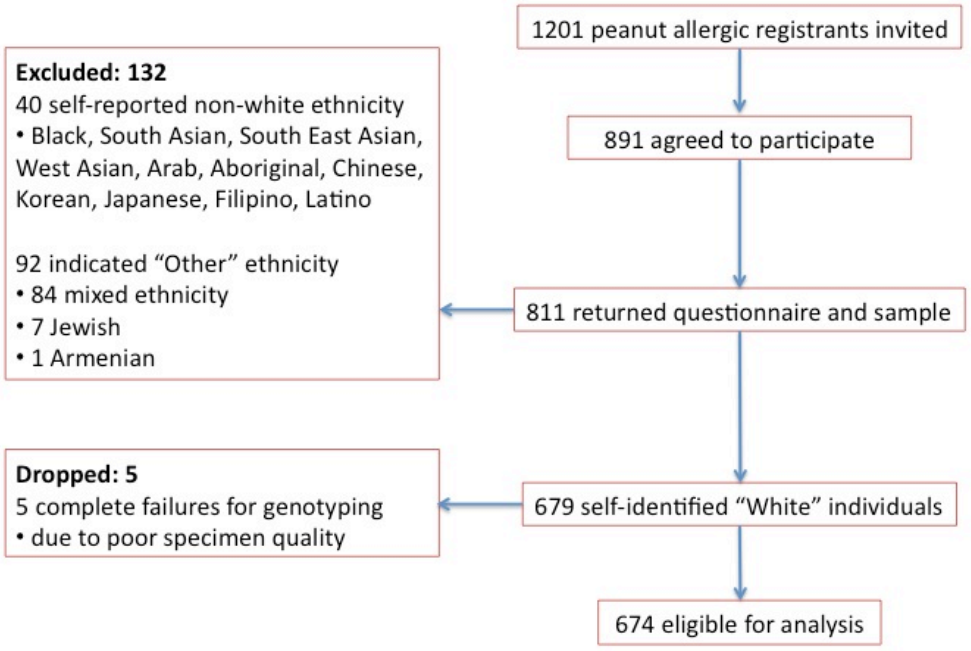
### Cases

From the peanut registry, 1201 individuals were invited to participate (Figure 1a). 891 agreed to participate, and 811 returned their questionnaire and samples. Of these, 40 were of non-white ethnicity, 84 were of mixed ethnicity, 7 were Jewish, and 1 was of Armenian descent. Of the 679 self-identified "white" subjects, 5 were dropped due to complete genotyping failure.

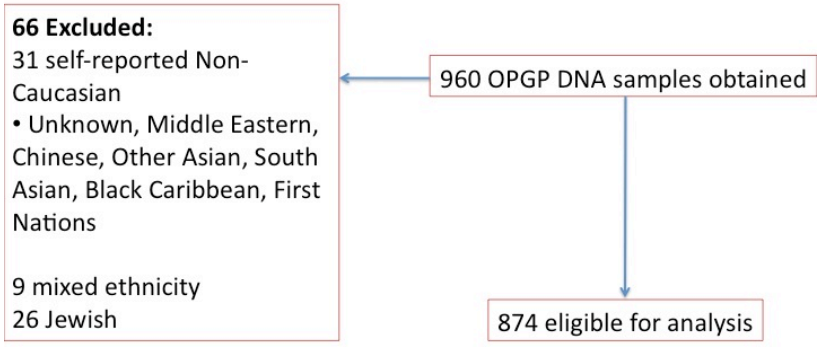
The mean age of the cases is 9.9 years (sd= 4.0), ranging in age from 1.5 to 21.5 years (Table 4). There are more males in the case group, with 61.7% male and 38.3% females.
Approximately half of all of the peanut allergic cases fulfilled the two-system criteria of anaphylaxis (Table A1, Appendix A). A total of 25 were food challenge positive. The mean peanut-specific serum IgE level was 51.1kU/L, with a standard deviation of 40.7kU/L and a range of 0.35, 100kU/L. This large standard deviation is due to the distribution of serum IgE levels, which is skewed to the right and left (Figure A1, Appendix A).

*FIGURE 1: RECRUITMENT OF CASES AND ONTARIO CONTROLS*

*A: RECRUITMENT OF PEANUT ALLERGIC CASES*




*B: RECRUITMENT OF ONTARIO CONTROLS*



Seventy-seven percent of cases reported ever having eczema, based on both the current questionnaire and previous questionnaires. This was much higher than the percentage calculated from responses to previous questionnaires alone (64.5%). From previous questionnaires, 64.5% reported ever having asthma, 58.4% reported ever having hayfever, and 70% reported having another food allergy, other than peanut (Table A1, Appendix A). The cases have a high percentage of ever having a family history of atopic disease, including a family history of hayfever at 88% and 63% with a family history of atopic dermatitis. Fifty four percent reported ever having a family history of asthma. Approximately 43% ever had a family history of food allergy to a food other than peanut. Twelve percent had a family history of peanut allergy; the majority of these were siblings of the affected individual (9%).

*TABLE 4: DEMOGRAPHICS, CASES AND CONTROLS*

| | | Ontario controls | Quebec Controls | Cases (all) |
|---|---|---|---|---|
| Subjects (N) | | 894 | 268 | 674 |
| Age (years) | Mean | 65.5 | 9.7 | 9.9 |
| | S.D. | 10.2 | 0.5 | 4.0 |
| | Range | 33, 84 | 7, 10 | 15, 21.5 |
| | Missing | 2 | 0 | 0 |
| Gender | Male | 281 | 157 | 416 |
| | Prop. | 0.315 (0.284, 0.346) | 0.586 (0.526, 0.645) | 0.617 (0.580, 0.654) |
| | Female | 611 | 111 | 258 |
| | Prop. | 0.685 (0.654, 0.716) | 0.414 (0.355, 0.474) | 0.383 (0.346, 0.420) |

Peanut allergic individuals that had asthma had a higher average peanut-specific IgE level than those who did not (Figure A3, Appendix A). This trend was not seen in those with atopic dermatitis (not shown). Neither eczema nor asthma status appeared to affect skin prick test result.

Controls

Thirty-one individuals in the Ontario control group who self-identified as non-white and 9 of mixed ethnicity were removed from the control dataset, as well as 26 who self-identified as Jewish. This control group was much older than the case group, with an average age of 65.5 years, and was also primarily made up of females, reflecting its initial collection purpose as a breast cancer control group (personal communication, Jo-Anne Herbrick, The Centre for Applied Genomics). There is no overlap between the distribution in age of the cases and the control groups (Figure 2).

Eleven percent of individuals in the control group stated they had asthma. Even without the exclusion of smokers and those with bronchial emphysema, this is much lower than the percentage of individuals with asthma in the cases. The number of controls with a smoking history is approximately half – this also reflects the age of the control group. The constructed atopic asthma variable, based on exclusion of atopic asthma diagnosis on any current or prior smoking history or diagnosis of bronchial emphysema, results in a diagnosis of atopic asthma in 4.5% (Table 5).

*TABLE 5: ASTHMA AND SMOKING HISTORY IN CASES AND ONTARIO CONTROLS*

| | | Ontario controls | | Cases (all) | |
|---|---|---|---|---|---|
| N= | | 894 | Prop. (95% CI) | 674 | Prop. (95% CI) |
| Asthma | Yes | 98 | 0.110 (0.090, 0.131) | N/A | N/A |
| | No | 791 | 0.890 (0.869, 0.910) | N/A | N/A |
| | Miss. | 5 | N/A | N/A | N/A |
| Br. emphy | Yes | 38 | 0.043 (0.029, 0.056) | N/A | N/A |
| | No | 854 | 0.957 (0.944, 0.971) | N/A | N/A |
| | Miss. | 2 | N/A | N/A | N/A |
| Smoking | Yes | 496 | 0.557 (0.524, 0.589) | N/A | N/A |
| | No | 395 | 0.443 (0.411, 0.476) | N/A | N/A |
| | Miss. | 3 | N/A | N/A | N/A |
| A. Asthma* | Yes | 40 | 0.045 (0.031, 0.059) | 426 | 0.645 (0.609, 0.682) |
| | No | 849 | 0.955 (0.941, 0.969) | 234 | 0.355 (0.318, 0.391) |
| | Miss. | 5 | N/A | 14 | N/A |

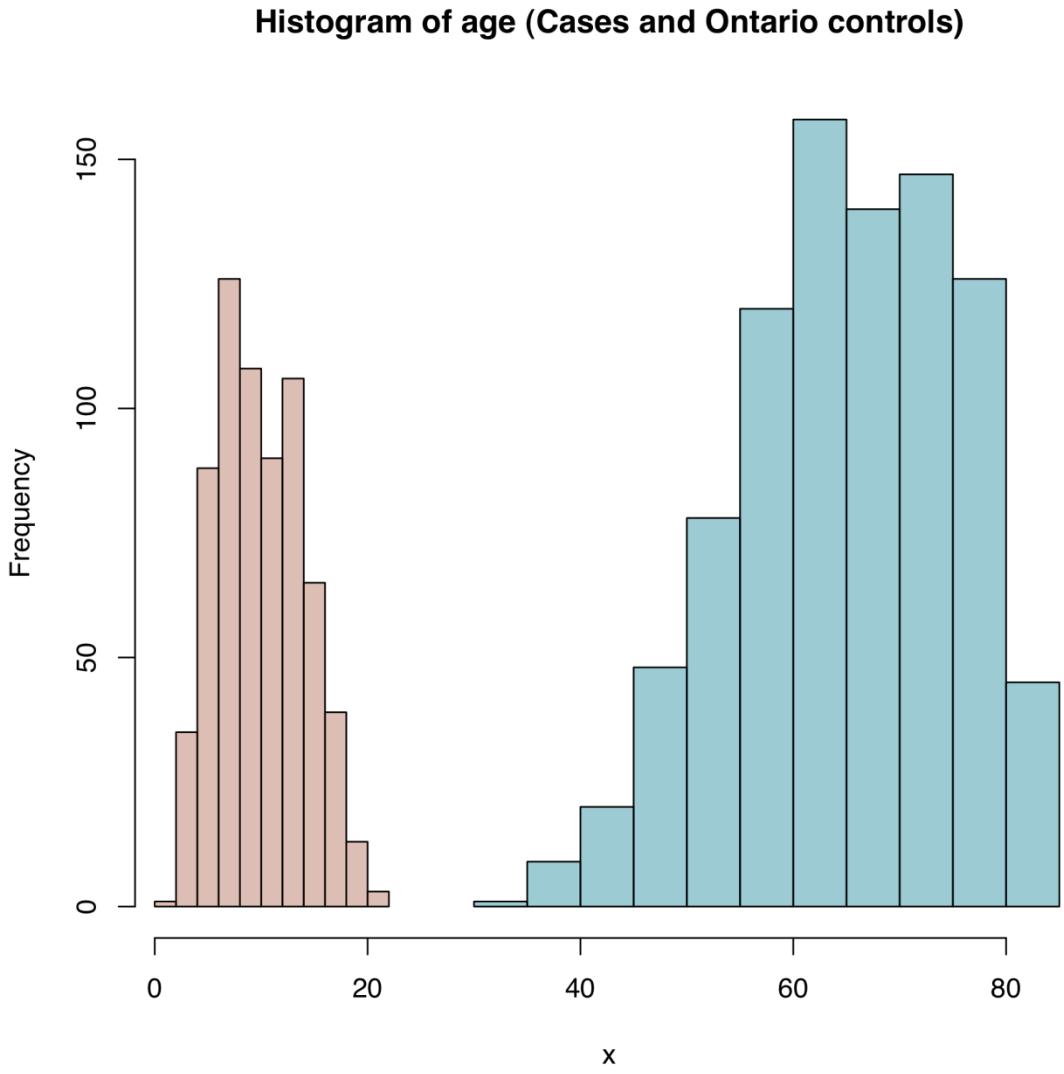
*Abbreviations:* Prop (proportion); Miss (missing); Br emphy. (bronchial emphysema); A. Asthma (atopic asthma)

**We assume case reported asthma is atopic asthma, while atopic asthma in the Ontario Controls is constructed from asthma, excluding anyone with reported history of bronchial emphysema, and cigarette or cigar smoking history.*

The age of the Quebec City control group is comparable to the case group, with a mean age of 9.7 years (sd=0.5). Similarly, the gender ratios are more comparable to the cases, with 41.4% female and 58.6% male. There was no personal or family history available from this control group.

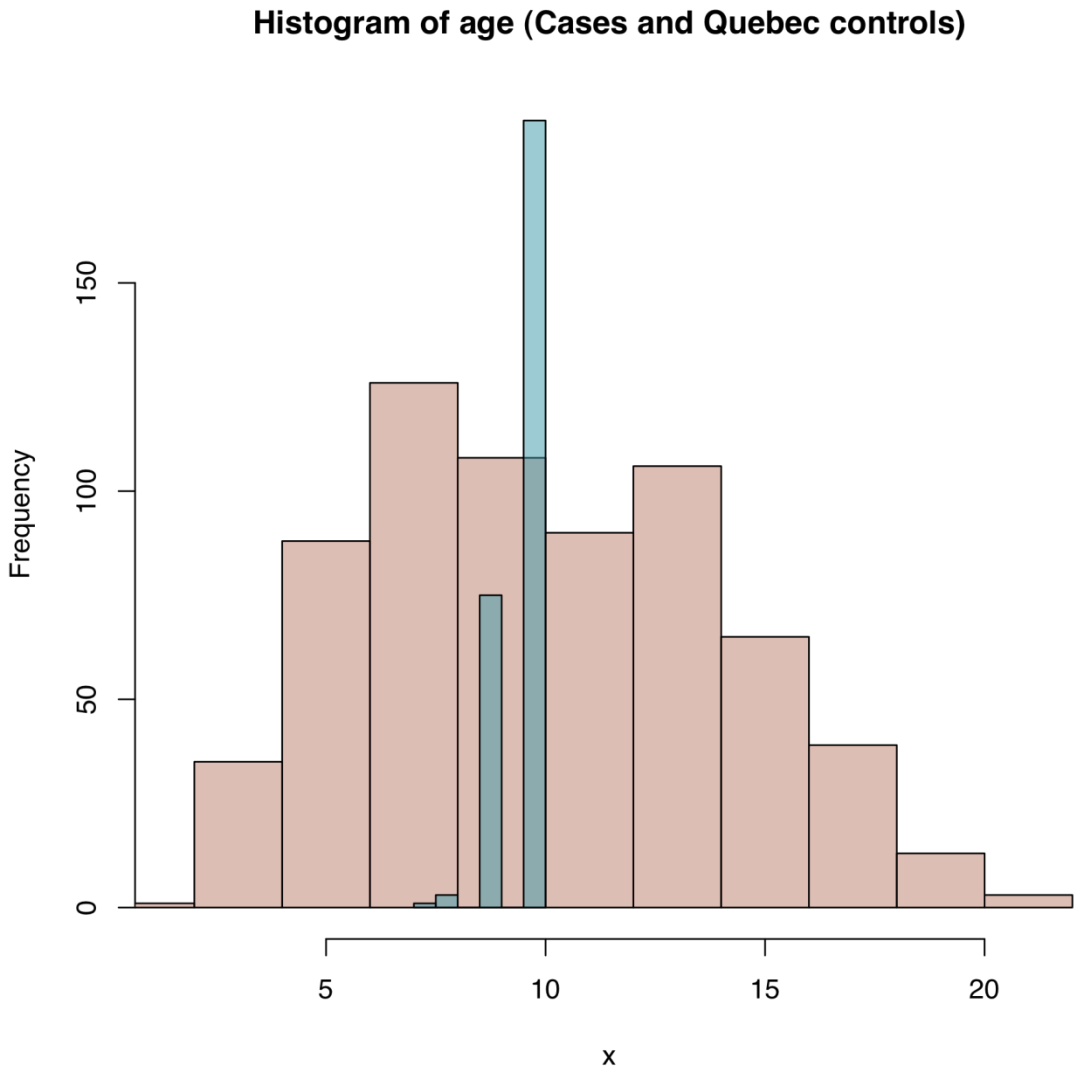
*FIGURE 2: AGE OF CASES AND CONTROL GROUPS*

*A: AGE OF PEANUT ALLERGIC CASES AND ONTARIO CONTROLS*




* pink= cases, blue= controls

*B. PEANUT ALLERGIC CASES AND QUEBEC CITY CONTROLS*




* pink= cases, blue= controls

**B: Genotype frequencies: Cases and controls**

In the 674 peanut allergic cases, 80.4% had the wild-type genotype for the filaggrin gene, while 16.9% were heterozygous and 2.7% were homozygous or compound heterozygous (Table 6). This is much higher than the 10.6% heterozygotes in the

Ontario control group and the 10.9% heterozygotes in the Quebec City control group. Less than 1% of both control populations were composed of homozygotes or compound heterozygotes for mutations in *filaggrin*. When a binary variable is constructed, which groups heterozygous, compound heterozygous and homozygous genotypes together, 19.6% of the peanut allergic cases have the presence of any mutation, which is almost double that of either control group, who have a mutation frequency of approximately 11%.

A higher failure rate in genotyping in the peanut allergic cases compared to the control groups is noted (Table 6), likely due to the DNA collection method; there were more complete failures in the cases than in the control groups (7 complete failures in the cases compared versus none in either control group).

The odds ratios of all of the cases were compared to the combined control group, and each control group individually (Table 7). The results are similar, with an OR of approximately 1.9 in all conditions. The confidence intervals are larger in the Quebec City control group, due to the smaller number of subjects. The odds ratio (OR) for all of the cases compared to the combined control group was 1.96 (95% CI: 1.46, 2.58).

*TABLE 6: FILAGGRIN GENOTYPE, PEANUT-ALLERGIC CASES AND CONTROLS*

| | | Ontario controls | Quebec controls | All cases |
|---|---|---|---|---|
| | | N=894 | N=268 | N=674 |
| Fail | | 5 | 1 | 11 |
| wt | N= | 791 | 237 | 533 |
| | Prop. | 0.890 (0.869, 0.910) | 0.888 (0.850, 0.926) | 0.804 (0.774, 0.834) |
| Hetero | N= | 94 | 29 | 112 |
| | Prop. | 0.106 (0.085, 0.126) | 0.109 (0.071, 0.146) | 0.169 (0.140, 0.198) |
| Homo | N= | 4 | 1 | 18 |
| | Prop. | 0.004 (0.0001, 0.0089) | 0.0037 (-0.0036, 0.0111) | 0.027 (0.015, 0.040) |
| Any | N= | 98 | 30 | 130 |
| | Prop. | 0.110 (0.090, 0.131) | 0.112 (0.074, 0.150) | 0.196 (0.166, 0.226) |

*Abbreviations:* Hetero(heterozygous); Homo(homozygous or compound heterozygous); Prop.(proportion and 95% CI); Fail(failed)

*TABLE 7: ODDS RATIOS OF FLG MUTATIONS IN PEANUT ALLERGIC CASES VERSUS CONTROLS*

| Controls | Ontario | Quebec | Combined |
|---|---|---|---|
| OR | 1.97 | 1.93 | 1.96 |
| 95% CI | (1.47, 2.65) | (1.24, 3.06) | (1.49, 2.58) |
| $X^2$ | 22.33 | 9.37 | 25.22 |
| p-value | $2.30 \times 10^{-6}$ | $2.21 \times 10^{-3}$ | $5.12 \times 10^{-7}$ |
| Fisher's exact | $3.12 \times 10^{-6}$ | $2.05 \times 10^{-3}$ | $8.86 \times 10^{-7}$ |

*Abbreviations:* OR(odds ratio); CI (confidence interval)

## C: Sensitivity analysis of peanut allergy diagnostic criteria

Construction of case definitions:

Due to the low number of subjects that underwent food challenge in this case group, a continuum of case definitions for sensitivity analysis was constructed for logistic regression modeling to examine if the relationship between peanut allergy and filaggrin mutations changed as the peanut allergy case definition criteria became more restrictive. Using a combination of criteria of peanut-specific IgE of 15kU/L or greater, 26kU/L or greater, 57kU/L or greater, a skin prick test result of 8mm or greater, 15mm or greater, positive oral food challenge and clinical history of anaphylaxis, subjects were assigned a case definition group that was the most stringent possible. Initial examination of the case definition groups found that the definition criteria combined with the qualifier "*AND*" (i.e.: SPT ≥ 8mm AND peanut-specific IgE ≥ 15kU/L) were too restrictive and resulted in groupings that were too small in number. This led to the decision to form case definition groups using the criteria above with the qualifier "*OR*" (i.e.: SPT ≥ 8mm OR peanut-specific IgE ≥ 15kU/L).

Originally, fifteen categories of case definitions were made. Descriptive statistics found no observable differences in the personal characteristics (Table B1, Appendix B), or personal atopic history (Table B2, Appendix B) or family history (not shown) across the case definitions, including history of atopic asthma.

Similar to the characteristics of the subjects in each case definition, the odds ratios for the filaggrin mutations and the case definitions did not show any noticeable change as the case definitions became more restrictive (Table 8). Although there appears to be a possible trend in the ORs as case definition becomes more restrictive, the confidence intervals widen considerably due to the smaller samples sizes. We therefore transformed the case definitions into a continuous variable, as there is more power in a continuous variable versus a discrete variable, increasing the ability of detecting more extreme phenotypes, which are more likely to be genetic[252]. When formatting the case definitions for use as a continuous variable for logistic regression analysis, several issues became apparent. Modeling attempts with 15 categories was unwieldy. More importantly, some categories had fewer than 20 subjects, while other categories had no subjects at all, leading to inappropriate gaps in the variable (Figure B1, Appendix B). Subsequently, the selection of the criteria determining the continuum was decided by several factors:

1) The number of categories in the case definition should be no greater than ten, for ease of modeling
2) The categories are continuous; there are no "empty" categories
3) If possible, each case definition group should have 50-100 subjects

*TABLE 8: ODDS RATIOS; THE FIFTEEN ORIGINAL CASE DEFINITIONS VS COMBINED CONTROLS*

| *Case def.* | *1* N=674 | *2* N=660 | *3* N=641 | *4* N=526 | *5* N=510 | *6* N=486 | *7* N=337 | *8* N=309 |
|---|---|---|---|---|---|---|---|---|
| *OR* | 1.96 | 1.95 | 1.96 | 1.97 | 2.02 | 2.07 | 2.03 | 1.96 |
| *95% CI* | (1.49, 2.58) | (1.48, 2.57) | (1.49, 2.59) | (1.47, 2.65) | (1.50, 2.71) | (1.53, 2.78) | (1.44, 2.84) | (1.37, 2.77) |
| *X2* | 25.22 | 24.73 | 24.8 | 22.58 | 23.9 | 25.05 | 18.79 | 15.73 |
| *p=* | $5.12x10^{-7}$ | $6.59x10^{-7}$ | $6.36x10^{-7}$ | $2.02x10^{-6}$ | $1.02x10^{-6}$ | $5.59x10^{-7}$ | $1.46 \times 10^{-5}$ | $7.31x\ 10^{-5}$ |
| *Fisher* | $8.86x10^{-7}$ | $1.05x10^{-6}$ | $1.15x10^{-6}$ | $3.63x10^{-6}$ | $2.03x10^{-6}$ | $1.31x10^{-6}$ | $4.22 \times 10^{-5}$ | $1.61x\ 10^{-6}$ |
| *Case def.* | *9* N=267 | *10* N=266 | *11* N=263 | *12* N=253 | *13* N=159 | *14* N= 146 | *15* N=122 | |
| *OR* | 2.07 | 2.09 | 2.11 | 2.21 | 2.29 | 2.08 | 2.28 | |
| *95% CI* | (1.42, 2.96) | (1.44, 3.00) | (1.45, 3.03) | (1.52, 3.18) | (1.46, 3.52) | (1.29, 3.27) | (1.38, 3.69) | |
| *X2* | 16.93 | 17.37 | 17.82 | 20.21 | 15.7 | 10.91 | 12.66 | |
| *p=* | $3.88x10^{-5}$ | $3.08x10^{-5}$ | $2.43x10^{-5}$ | $6.92x10^{-6}$ | $7.41x10^{-5}$ | $9.54x10^{-5}$ | $3.73x10^{-5}$ | |
| *Fisher* | $9.28x10^{-5}$ | $8.62x10^{-5}$ | $5.73x10^{-5}$ | $2.58x10^{-5}$ | $2.53 \times 10^{-5}$ | $1.83 \times 10^{-3}$ | $1.10 \times 10^{-3}$ | |

*Abbreviations*: case def.(case definition); *see page 39-40 for more detail on construction

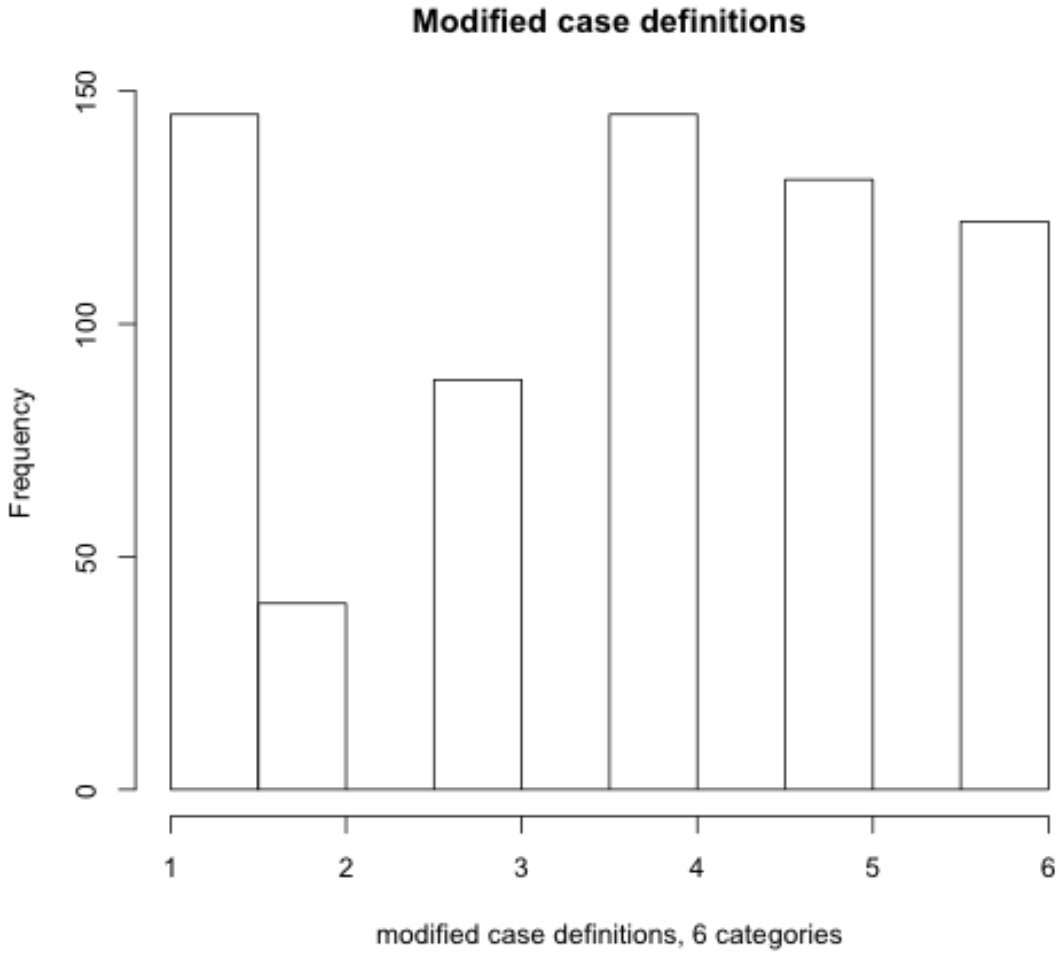
After evaluation, the following six-category case definition continuum was constructed with the following criteria (listed from least to most restrictive):

1) the original case definition
2) peanut-specific IgE of 15ku/L or greater ***OR*** skin prick test of 8mm or greater, ***OR*** positive food challenge
3) peanut-specific IgE of 57kU/L or greater ***OR*** skin prick test of 8mm or greater, ***OR*** positive food challenge
4) peanut-specific IgE of 57kU/L or greater ***OR*** skin prick test of 15mm or greater, ***OR*** positive food challenge
5) peanut-specific IgE of 57kU/L or greater ***OR*** skin prick test of 8mm AND anaphylaxis, ***OR*** positive food challenge
6) peanut-specific IgE of 57kU/L or greater ***OR*** skin prick test of 15mm AND anaphylaxis, ***OR*** positive food challenge

This led to a case definition variable with six categories, the majority of which had greater than 50 subjects (Figure 3).

Logistic regression using the case definition with 6 groupings (as defined above) as the predictor variable with the binary mutation variable as outcome found that there was no change in the relationship of *FLG* null mutations with a change in case definition (OR 1.05, 95% CI: 0.94, 1.17). Similarly, the associations with clinical characteristics of the subjects also showed no noticeable change with a change in method of case definition (Table B3, Appendix B). The sole characteristics which changed with the increase in restrictiveness of case definition were those used to create the definitions, and were thus expected to have an OR greater than the null. These variables included anaphylaxis, and meeting a SPT of 8mm.

*FIGURE 3: DISTRIBUTION OF MODIFIED CASE DEFINITIONS*




Although certain variables achieved statistical significance for an association with case definition severity, such as family history, maternal history or fraternal history of atopic dermatitis, and personal history of atopic asthma, the magnitude of association in seen in these variables is not clinically significant. A trend test of proportions was in agreement with these findings, with a p-value of 0.3966 (df=1, $X^2$= 0.7185).

ANOVA was conducted to examine the relationships between case definition and the continuous variables of peanut-specific IgE and age. As expected, there was a significant difference in

IgE over the case definitions, as this laboratory test was used as a criterion to create the categories. Age was also found to differ between the categories (Table B3, Appendix B). This result is reflected by the mean age of the most restrictive category, 11.1 years (sd=4.2), compared to the mean age of all the cases of 9.9 years (sd=4.0).

Fifty-seven case subjects were diagnosed with immunologic criteria including an SPT graded by the old system. All but six would have met other criteria to keep them in the analysis, albeit with some reclassification. To investigate the impact of the old grading system, these six subjects were dropped and the remaining 51 were reclassified and the analysis of peanut allergy case definition on loss-of-function mutations in *filaggrin* was then repeated. No changes in the conclusions were noted when the input of the old SPT grading system was omitted (Table B4, Appendix B), and all cases were subsequently included for the rest of the analysis.

## D: Effect of atopic asthma on *FLG* mutation

### Logistic regression modeling of peanut allergy and asthma on *filaggrin* null mutations

A logistic regression model using all peanut allergic cases and the Ontario control group was constructed, with peanut allergy status, age, gender and atopic asthma as predictors, and the binary mutation variable as the outcome. Univariate analysis found peanut allergy status to be the strongest predictor (OR 1.97, 95% CI: 1.48, 2.62), followed by atopic asthma (OR 1.67,

95% CI: 1.25, 2.24). Neither age nor gender had an appreciable relationship with presence of *filaggrin* mutations on univariate analysis (Table 9).

*TABLE 9: UNIVARIATE ANALYSES OF PEANUT ALLERGY STATUS, AGE, GENDER AND HISTORY OF ATOPIC ASTHMA ON LOSS-OF-FUNCTION MUTATIONS IN FLG*

| | OR | 95% CI | |
|---|---|---|---|
| Peanut allergy status | 1.97 | 1.48 | 2.62 |
| Age at 1 January, 2009 | 0.99 | 0.98 | 0.99 |
| Male gender | 1.06 | 0.80 | 1.41 |
| History of atopic asthma | 1.67 | 1.25 | 2.24 |

In multivariate analysis, there was no evidence for an effect of age or gender on the relationship between peanut allergy and mutations in *FLG*, even with the addition of interaction terms for the effect of age and peanut allergy status, and gender and peanut allergy status (Table C1, Appendix C). It was previously noted that the age range of the peanut allergic cases and the Ontario control group did not intersect, and in fact the inclusion of the age variable in the multivariate analysis leads to large confidence intervals (Table C1, Appendix C). Based on the results of the univariate and multivariate analyses, age and gender were therefore omitted from the model.

Multivariate logistic regression also found no evidence for an effect of atopic asthma on the relationship between peanut allergy and loss-of-function mutations in *FLG*, even with the addition of an interaction term for the known correlations

between peanut allergy status and presence of atopic asthma (Table 10). This conclusion is supported by the results of the BMA package used in the R program – the first model selected with the highest posterior probability was consistently peanut allergy status alone, through all permutations of all three variables and their corresponding interaction terms.

*TABLE 10: MULTIVARIATE LOGISTIC REGRESSION ANALYSIS OF PEANUT ALLERGY STATUS AND ATOPIC ASTHMA ON NULL MUTATIONS IN FLG*

| Peanut allergy status and atopic asthma on *FLG* mutations | | | |
|---|---|---|---|
| | OR | 95% CI | |
| Peanut allergy status | 1.80 | 1.24 | 2.60 |
| History of atopic asthma | 1.12 | 0.77 | 1.54 |
| Peanut allergy status, atopic asthma and their interaction term on *FLG* mutations | | | |
| | OR | 95% CI | |
| Peanut allergy status | 1.88 | 1.27 | 2.80 |
| History of atopic asthma | 1.44 | 0.59 | 3.53 |
| Interaction: peanut/asthma | 0.74 | 0.28 | 1.98 |

Subgroup analysis of atopic asthma

To further explore the results of the multivariate analysis of peanut allergy and atopic asthma on *FLG* mutation status, atopic asthma was investigated on an *a priori* basis due to its correlation with both peanut allergy and loss of function

mutations in *filaggrin*. The data was then split into subgroups of asthmatic and non-asthmatic individuals (Table C2, Appendix C). The OR for null mutations as determined by asthma status was non-significant, regardless of peanut allergy status. The OR for peanut allergy on mutation prevalence in non-asthmatics was 1.88 (95% CI: 1.27, 2.80), and although it was non-significant due to lack of power, the OR for the association of peanut allergy and *FLG* mutations was 1.40 (95% CI: 0.57, 3.43).

## E: Logistic regression modeling of asthma and *filaggrin* null mutations on peanut allergy

We then repeated the logistic regression modeling, with the peanut allergy status as an outcome, to see which variables best predict presence of peanut hypersensitivity. As expected, the relationship between presence of any mutation and peanut allergy was the same in univariate analysis. However, both male gender and presence of asthma were significant predictors of peanut allergy status in univariate analysis (Table 11), and atopic asthma was a very strong predictor. Age was non-convergent and dropped from the analysis. The interaction terms were evaluated, and only interaction between gender and atopic asthma was significant (Table D1, Appendix D) The best model for prediction of peanut allergy status in multivariate analysis was consistently *FLG* mutation, gender, and atopic asthma (Table 12).

*TABLE 11: UNIVARIATE ANALYSES OF LOSS-OF-FUNCTION MUTATIONS IN FLG, GENDER AND HISTORY OF ATOPIC ASTHMA ON PEANUT ALLERGY STATUS*

| | OR | 95% CI | |
|---|---|---|---|
| Any *FLG* mutation | 1.97 | 1.48 | 2.62 |
| Male gender | 3.51 | 2.84 | 4.33 |
| Atopic asthma | 38.64 | 27.09 | 55.11 |

*TABLE 12: MULTIVARIATE LOGISTIC REGRESSION ANALYSIS OF NULL MUTATIONS IN FLG, GENDER AND ATOPIC ASTHMA ON PEANUT ALLERGY STATUS, WITH INTERACTION OF GENDER AND ATOPIC ASTHMA*

| | OR | 95% CI | |
|---|---|---|---|
| Any *FLG* mutation | 1.84 | 1.26 | 2.69 |
| Male gender | 3.65 | 2.69 | 4.97 |
| Atopic asthma | 30.80 | 2.05 | 47.32 |
| Interaction of male gender and atopic asthma | 3.89 | 1.30 | 11.63 |

## F: Effect of asthma reporting and eczema status on the relationship between peanut hypersensitivity and *FLG* mutations

Sensitivity analysis of asthma reporting

A variety of error situations were evaluated for the reporting of asthma status. In the first, an error rate of 12% in cases only would result in an increased association of atopic asthma with presence of *FLG* mutations (Table 13), and a loss of significance of peanut allergy in multivariate analysis, as the 5$^{th}$ percentile of the lower bound of the 95% confidence interval crosses the null.

If the increase in asthma reporting is 6% in controls as well as an increase of 12% in cases, the association of atopic asthma with *FLG* mutations increases, but to a lesser extent than if asthma reporting increased in cases alone. Peanut allergy remains statistically significant in the multivariate analysis, while the atopic asthma variable was non-significant in the multivariate model, similar to previous analyses. Similar conclusions are made for the circumstance where the atopic asthma increases 15% in cases and 10% in controls (Table 13).

Effect of eczema status

Although there was no control group information on eczema status, we attempted to investigate this relationship by grouping by eczema status. In peanut allergic individuals with eczema, the OR is approximately 2 in both control groups. Similarly to the power issue seen in the asthma subgroup analysis, we see an OR of approximately 1.44 in those without eczema (95% CI: 0.89, 2.33) (Table 14).

*TABLE 13: SENSITIVITY ANALYSIS OF ATOPIC ASTHMA REPORTING. PERCENTILES ARE GIVEN ACROSS 100 RANDOM SAMPLINGS OF ADDITIONAL ATOPIC ASTHMATIC INDIVIDUALS*

| | | OR | | Lower CI | | Upper CI | | OR | | Lower CI | | Upper CI | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Percentile | | 5 | 95 | 5 | 95 | 5 | 95 | 5 | 95 | 5 | 95 | 5 | 95 |
| Assumption | | 12% increase in atopic asthma in cases | | | | | | 12% increase in cases, 6% in controls | | | | | |
| Uni. | Peanut allergy | 1.97 | 1.97 | 1.48 | 1.48 | 2.62 | 2.62 | 1.97 | 1.97 | 1.48 | 1.48 | 2.62 | 2.62 |
| | Atopic asthma | 1.57 | 1.91 | 1.18 | 1.43 | 2.10 | 2.54 | 1.44 | 1.85 | 1.08 | 1.39 | 1.91 | 2.46 |
| Multi | Peanut allergy | 1.52 | 2.06 | 0.98 | 1.36 | 2.32 | 3.10 | 1.57 | 2.12 | 1.07 | 1.45 | 2.31 | 3.10 |
| | Atopic asthma | 0.91 | 1.39 | 0.61 | 0.91 | 1.39 | 2.15 | 0.87 | 1.37 | 0.59 | 0.93 | 1.27 | 2.01 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Assumption | | 10% increase in cases, 15% in controls | | | | | |
| | | OR | | Lower CI | | Upper CI | |
| Percentile | | 5 | 95 | 5 | 95 | 5 | 95 |
| Univ. | Peanut allergy | 1.97 | 1.97 | 1.48 | 1.48 | 2.62 | 2.62 |
| | Atopic asthma | 1.39 | 1.87 | 1.05 | 1.41 | 1.85 | 2.49 |
| Multi. | Peanut allergy | 1.55 | 2.16 | 1.06 | 1.48 | 2.25 | 3.15 |
| | Atopic asthma | 0.84 | 1.41 | 0.58 | 0.97 | 1.23 | 2.05 |

**Abbreviations:* Uni. (univariate); Multi. (multivariate); all reported CIs are 95%

*TABLE 14: ODDS RATIOS OF PEANUT ALLERGIC CASES WITH AND WITHOUT HISTORY OF ECZEMA ON NULL MUTATIONS IN FLG*

| | Peanut allergic cases with history of eczema | | Peanut allergic cases without eczema | |
|---|---|---|---|---|
| | OR | 95% CI | OR | 95% CI |
| Combined controls | 2.20 | 1.55, 2.91 | 1.44 | 0.89, 2.33 |
| Ontario controls | 2.21 | 1.64, 2.98 | 1.45 | 0.89, 2.37 |
| Quebec City Controls | 2.16 | 1.40, 3.34 | 1.42 | 0.79, 2.54 |

## G: Sensitivity analysis of peanut allergy status

The effect of error in peanut allergy status was then examined. Both when a 1% prevalence of peanut hypersensitivity occurs randomly in the control population, or is conditional upon atopic asthma status, the effect of peanut allergy remains significant through the 100 iterations of the model (Table 15). Atopic asthma, while having a significant association with *FLG* mutations in univariate analysis, is not significant in the multivariate model.

Due to the link between peanut allergy severity and asthma, and the fact that severe peanut allergy is less likely to resolve, we modeled both a 20% resolution of peanut allergy cases at random, and a model conditional upon their asthma status (ie: those with asthma do not resolve). Both models led to the loss of significance in the effect of peanut allergy status on loss-of function mutations in *FLG* (Table F1, Appendix F). Atopic asthma was also non-significant. However, if the joint effect of both a

20% resolution in peanut allergy in cases and a 1% prevalence of peanut hypersensitivity in controls are modeled, peanut allergy remains significant in the multivariate model (Table F2, Appendix F).

*TABLE 15:* EFFECT OF 1% PREVALENCE OF PEANUT ALLERGY IN CONTROL POPULATION. PERCENTILES ARE ACROSS 100 RANDOM SAMPLES OF ADDITIONAL INDIVIDUALS WITH PEANUT ALLERGY AMONG THE CONTROLS

| 1% of controls have peanut hypersensitivity | | | | | | |
|---|---|---|---|---|---|---|
| | OR | | Lower 95% CI | | Upper 95% CI | |
| Percentile | 5th | 95th | 5th | 95th | 5th | 95th |
| *UNIVARIATE* | | | | | | |
| Peanut allergy status | 1.91 | 2.04 | 1.44 | 1.54 | 2.55 | 2.72 |
| Atopic asthma | 1.67 | 1.67 | 1.24 | 1.24 | 2.24 | 2.24 |
| *MULTIVARIATE* | | | | | | |
| Peanut allergy status | 1.71 | 1.90 | 1.18 | 1.31 | 2.46 | 2.73 |
| Atopic asthma | 1.09 | 1.17 | 0.75 | 0.80 | 1.59 | 1.71 |
| 1% of controls have peanut allergy, conditional upon atopic asthma | | | | | | |
| | OR | | Lower 95% CI | | Upper 95% CI | |
| Percentile | 5th | 95th | 5th | 95th | 5th | 95th |
| *UNIVARIATE* | | | | | | |
| Peanut allergy status | 1.91 | 2.04 | 1.44 | 1.54 | 2.55 | 2.72 |
| Atopic asthma | 1.67 | 1.67 | 1.24 | 1.24 | 2.24 | 2.24 |
| *MULTIVARIATE* | | | | | | |
| Peanut allergy status | 1.73 | 1.92 | 1.18 | 1.32 | 2.50 | 2.79 |
| Atopic asthma | 1.06 | 1.14 | 0.73 | 0.78 | 1.56 | 1.69 |

## VI: DISCUSSION

Our research substantiates the relationship between peanut allergy and loss-of-function mutations in *filaggrin*, and establishes that this result is independent of diagnostic criteria of peanut allergy. This relationship also appears to be independent of underlying atopic disease in logistic regression analysis, although we did not have sufficient power to detect this in subgroup analysis.

### A: What is the relationship between *FLG* null mutations and peanut allergy?

The combined frequency of loss-of-function mutations previously reported in the English, Dutch, and Scottish general populations ranges between 7-10% [1, 157, 169, 170] with a similar prevalence in the Singaporean Chinese [253], although a lower population percentage of 5% has been described in a German pediatric nested case-control study[254]. Our control groups had a 10.6% and 10.9% combined frequency of mutations (Table 6), which is comparable to these estimates. We are therefore comfortable concluding that we did not have a biased sampling of controls that under-selected for individuals with *FLG* mutations.

In our previous work, we found an association between peanut allergy and *FLG* mutations with an OR of 1.9 using the Ontario control group[7]; unsurprisingly, we found an OR of 1.97 in our current work, which had more cases. A similar association of 1.93 was found of when our cases were compared to the Quebec

City control group (Table 7), which were more similar in age and gender distribution compared to the peanut allergic cases (Table 4), although there were approximately 10% more males in the cases. This slight male preponderance in peanut hypersensitivity is expected[225], and males tend to be at higher risk for atopic disease[255].

There were notable differences between the Ontario control group and the peanut allergic cases. There are more males in the case group, with 61.7% male and 38.3% female, with an almost reversed proportion in the control group, at 31.5% and 58.6%, respectively. The age ranges of the cases and Ontario controls did not intersect, with a mean age of 65.5 years in the Ontario controls compared to the case mean of 9.9 years.

Despite these differences in cases and controls, it is interesting to note the stability of the association when each control group was analyzed separately with the cases. Null mutations in *filaggrin* are the strongest single risk factor for peanut allergy that has been yet discovered, and this result has been replicated in other populations outside of Canada[7].

The peanut allergic cases had a high personal and family history of atopic conditions. Approximately 65% reported a personal history of ever having asthma, and 77% reported a personal history of ever having atopic dermatitis. This was 12% higher than the percentage calculated cumulatively from the previous questionnaires, and may indicate a recall bias, since the information package and consent form discussed the relationship of eczema to *FLG* mutations. Although the reported family

history of peanut allergy was quite low compared to the history of other atopic diseases at 12%, the relatively high proportion of affected siblings (10%) prompted us to examine how many, if any, of our cases were related. By address matching, we discovered 8 sets of individuals (16 subjects) who had the same address – all of these individuals had the wildtype genotype, except one set. These two individuals were twins, as determined by the same birthdate, and were heterozygous for the 2282del4 mutation. Since the number of related individuals was small, and the majority were wildtype, we left all of the individuals in the final analysis, since any effect on estimates of association would likely be minimal and towards the null hypothesis.

**B: What is the impact of restrictive peanut allergy case definition criteria on the relationship between loss-of-function *FLG* mutations and peanut allergy?**

Since a small number of peanut allergic cases in this study had been tested by OFC, we then strove to ensure that our association was indeed robust. There is good evidence to suggest that larger SPT size and higher level of peanut-specific IgE can predict OFC result[53, 54]. The analysis undertaken to examine the effect of more restrictive case definitions, in the absence of OFC to peanut, found that the relationship between peanut hypersensitivity and *FLG* null mutations did not change as case definitions became more stringent. Moreover, there was no evidence to suggest that the characteristics of the individuals who fulfilled more stringent diagnostic criteria differed in any manner from the others included in the study. The exceptions to this included anaphylaxis, peanut-specific serum IgE and age

(Table B1, Appendix B). While anaphylaxis and peanut-specific IgE were criteria used to construct the case definition criteria, the positive finding in the age variable was somewhat surprising. This may be explained by the trend seen in SPT and peanut-specific sIgE results – we see that although SPT size does not change with age (not shown), the peanut-specific IgE seems to increase as age increases (Figure A2, Appendix A). Although this finding contradicts literature that IgE is thought to decrease with age[62], a relationship of age with case definition would not necessarily be surprising, as those who are older may be more likely to have received confirmatory testing during the course of their disease, and those whose peanut allergy resolves tend to be milder cases[60]. This finding may have some portent on the results of the error modeling that was done at the end of the study.

The interpretation of the result that the relationship between peanut hypersensitivity and *FLG* null mutations did not change with more restrictive diagnostic criteria could be interpreted in two ways: 1) that all of the peanut allergic cases are truly allergic, and *FLG* mutations are related to peanut allergy, or 2) that *FLG* mutations are related to peanut sensitization. It could be argued that the discussion of whether null mutations in *FLG* are related to mere sensitization or true peanut allergy is a moot point, since sensitization is bound to occur prior to development of true peanut allergy, and the mutation is likely upstream from both sensitization and hypersensitivity in the pathogenesis of food allergy. In addition, the relationship between the atopic diseases of eczema, asthma, allergic rhinitis and food allergy is complex and not fully understood (Figure 4A-C).

## C: Peanut allergy and *FLG* null mutations: effect of asthma

Family history of atopy, allergy to egg, and eczema are important predictors for peanut allergy[12] and the peanut allergic cases in our study reflected all of these qualities. No AD information was available for either control group. The strong relationship between eczema and *filaggrin* mutations – and the known relationship between eczema and peanut allergy – means eczema could be a possible confounder in this relationship (Figure 4A). Despite the lack of AD data, we were able to model the effect of peanut allergy on *FLG* mutation presence, accounting for other atopic disease using the asthma and smoking data available for the Ontario control group. This is a reasonable substitution, considering the known relationship between eczema and asthma, and especially since FLG association with asthma has been noted in those with atopic dermatitis, specifically those with "extrinsic" asthma – with sensitization to aeroallergens and foods[2, 161].

Furthermore, asthma itself, rather than a "stand-in" for eczema status, may be a possible confounder. *Filaggrin* null mutations have been linked to asthma[3, 144-147], even in the absence of ever having AD[144, 146, 256], and a systematic review and meta-analysis found null mutations in this gene increase the risk of developing allergic sensitization[143]. *Filaggrin* null mutations are associated with both increased asthma severity and number of exacerbations[146, 257]; peanut allergy has a similar association, with increased hospitalization and steroid use of asthmatics[140]. Coexisting peanut allergy has a negative effect on asthma morbidity, but the converse is also true; coexisting

asthma is known to be strongly associated with severe reaction to peanut[135, 136], even more so than previous reaction severity[40], and of peanut allergic individuals hospitalized for anaphylaxis, asthmatics are more likely than non-asthmatics to receive mechanical ventilation[39]. The known relationships between peanut allergy and asthma, and *FLG* mutations and asthma support the argument for using atopic asthma as a confounder for the relationship between loss-of-function mutations in *FLG* and peanut allergy. Intriguingly, despite all of these associations between asthma and *FLG* mutations, filaggrin is not expressed in bronchial mucosa[203], which lends to support to the idea that allergic sensitization in asthma may occur through the skin.

In our own data, this relationship between peanut allergy and asthma is indicated by the higher peanut-specific serum IgE in those peanut allergic subjects with asthma compared to those without (Figure A3, Appendix A). The self-reported asthma in the peanut allergic cases is very high. This in part explains the OR of 30.8 for asthma, when peanut allergy is modeled as the outcome (Table 12). It is reasonable to assume that the asthma reported in the cases is atopic, considering their age and the strong relationship between peanut allergy and asthma. Although reported asthma prevalence in children ranges widely dependent on population and age, the percentage of asthma seen in the peanut allergic cases (65%) is much higher than the reported prevalence in children, which ranges between 7% to 23%[258-261].

There is likely to be substantial error in the atopic asthma variable in the adult control group, due to the assumptions required during construction of the variable. However, the assumption of using asthma to control for atopic disease is reasonable, as childhood and later onset eczema are negatively correlated with asthma remission[249], and predict atopic but not non-atopic adult asthma[250]. Although atopy is still an important factor in adults with asthma[262], it is difficult to find statistics on the prevalence of atopic asthma to specifically compare with our constructed variable. However, the overall prevalence of asthma in the control group is comparable to the figures given for white, non-Hispanic American adults (8.1%)[263]. Approximately 5-6% of Canadian adults report asthma attacks or use asthma medication[264]. The slightly higher percentage of asthma reporting may be related to the excess of females in the Ontario control group – both Canadian and American studies have found that adult females have a higher prevalence of asthma[263, 264]. We feel that the prevalence numbers obtained and assumptions made in this work are reasonable, although one assumption made in this study was that those with atopic asthma were less likely to smoke; this may not be the case.

By investigating the change in the log odds ratio between mutation status and peanut allergy with each additional variable, and by using the BMA program, multivariate logistic regression models were examined looking for the effect of peanut allergy, atopic asthma, age and gender on the prevalence of *FLG* mutation. Despite age and gender differences in our case and control groups, there was no evidence for any effect of age or

gender in univariate or multivariate analysis, even with the inclusion of interaction terms for peanut allergy status and age, and peanut allergy status and gender. Peanut allergy and atopic asthma were the only variables with any significant impact on the presence of filaggrin mutations in univariate analysis, with ORs of 1.97 (95% CI: 1.48, 2.62) and 1.67 (95% CI: 1.25, 2.24), respectively. In multivariate analysis, the impact of peanut allergy status decreased with the addition of the atopic asthma variable to an OR of 1.8 (95% CI: 1.24, 2.60), while atopic asthma itself was non significant (OR 1.12, 95% CI: 0.77, 1.54). The addition of an interaction term to model the known relationship between peanut allergy and atopic asthma was non significant, and there was no significant change in either the peanut allergy or atopic asthma variables (Table 10). The non-significance of gender on *FLG* mutation is unsurprising, as this is not an X-linked trait. The non-significance of age is also unsurprising, since this would only be expected if the gene mutation affects longevity.

The effect of asthma on the relationship between peanut allergy and null mutations in *FLG* was further explored by subgroup analysis. Again, there was no evidence that the presence of asthma, either in peanut allergic individuals or non-peanut allergic individuals, significantly changed the odds of having a common loss-of function mutation in *filaggrin.* Ideally, we would have liked to have seen significance of the 1.44 OR for the association of peanut allergy with *FLG* mutations in the non-asthmatics in the subgroup analysis (Table C2, Appendix C), and in those without history of AD (Table 14). However, the majority of peanut allergic cases reported a history of asthma and/or

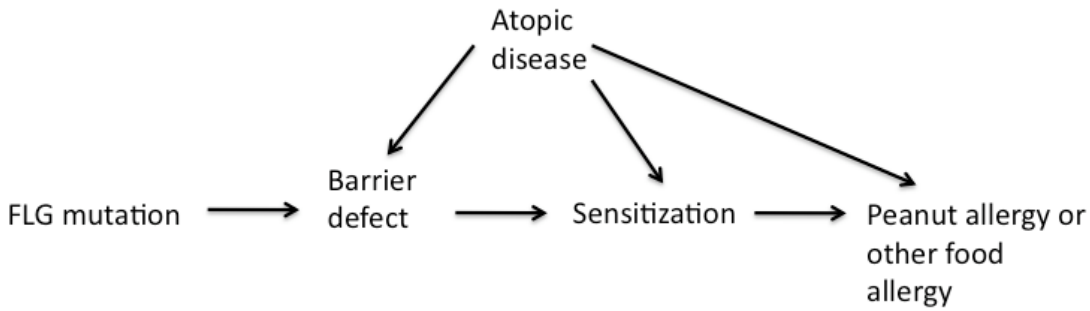
eczema, and the number of individuals without these conditions was insufficient to make any comparisons to the controls, leading to the wide and non-significant confidence intervals. Indeed, the high correlation of atopic asthma and atopic dermatitis with peanut allergy may make this type of comparison impossible – in our study 76.5% of peanut allergic individuals reported ever having a history of eczema, and 64.5% a history of asthma. Despite this lack of power in the subgroup analysis, we are comfortable with the results of the multivariate analysis, which suggest that the relationship of peanut allergy and *FLG* mutations is independent of asthma, especially in light of the stability of this relationship throughout the various models, including reversing the outcome and predictor variables.

When null mutations in *filaggrin* are used to predict peanut allergy status as an outcome, male gender and atopic asthma were significant predictors of peanut allergy status (Table 11, 12), with males having an OR of 3.65 (95% CI: 2.69, 4.97) and atopic asthma having an OR of close to approximately 31 (95% CI: 2.05, 47.32), with significant interaction between the two variables (OR 3.89, 95% CI: 1.30, 11.63). These results are unsurprising, considering the large differences in gender and atopic asthma distribution between the peanut allergic cases and the Ontario control group that were noted on descriptive analysis. Despite this large effect of atopic asthma on peanut allergy status, the effect of *FLG* mutations on peanut allergy is still significant, and similar to the previous odds ratios, with an OR of 1.84 (95% CI: 1.26, 2.69). This lends further support to the theory that the relationship between *FLG* null mutations and peanut allergy is independent of atopic disease.

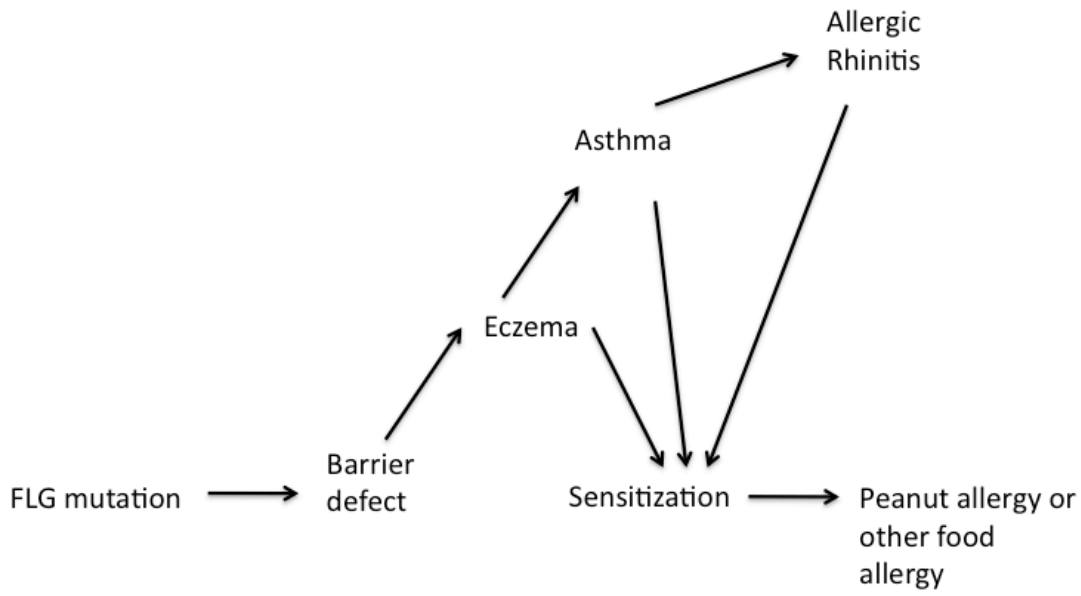
It is easy to argue that it is inappropriate to treat both eczema and asthma as confounders (Figure 4A), since due to the known natural progression of the atopic march, it is possible that either or both conditions could be steps in the causal pathway between loss-of-function mutations in *filaggrin* and peanut hypersensitivity (Figure 4B). Controlling for an intermediary in the causal pathway runs the risk of introducing bias into the results; however, the stability of the relationship between peanut allergy and null mutations in *filaggrin* throughout the various models is reassuring. The continued observance of a significant effect of peanut allergy status on the presence of *FLG* mutations, despite controlling for asthma, is interesting. Although it is possible that the results could be influenced by measurement error such as poor recall of asthma or ineffectual construction of the atopic asthma variable in the control group, our sensitivity analyses looking at error in the atopic asthma variable indicate that the effect of peanut allergy on *FLG* mutations is robust. The retention of the effect of peanut allergy despite controlling for asthma suggests that there may be an association between loss-of-function mutations in *filaggrin* and peanut hypersensitivity that is independent of underlying atopic disease (Figure 4C).

*FIGURE 4: POSSIBLE CAUSAL DIAGRAMS OF THE RELATIONSHIP BETWEEN PEANUT ALLERGY AND LOSS-OF-FUNCTION MUTATIONS IN THE GENE ENCODING FILAGGRIN*

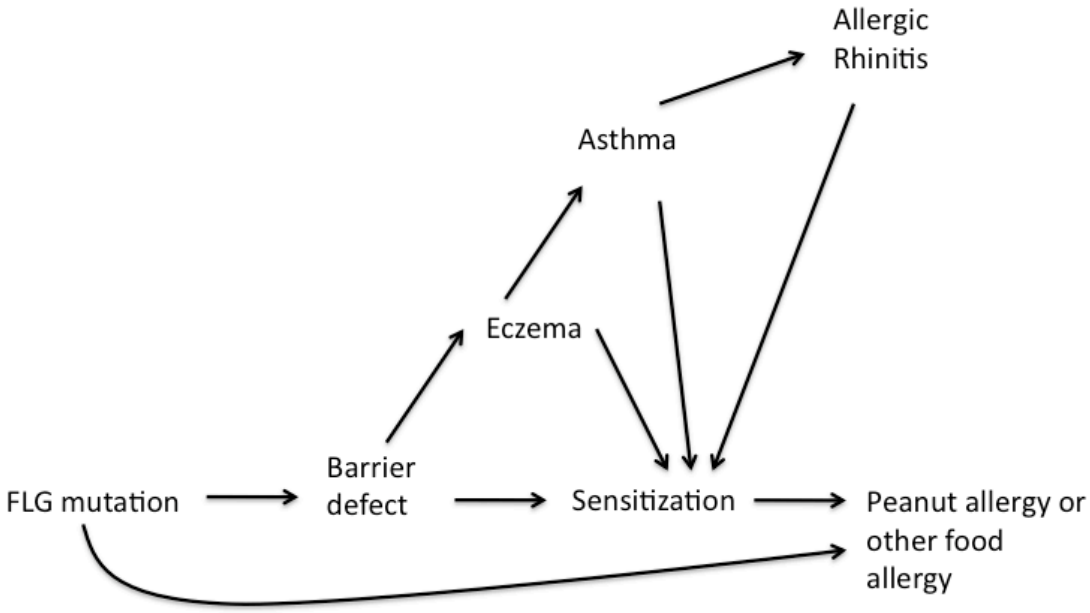
*A. CAUSAL DIAGRAM WITH ATOPIC DISEASE AS A CONFOUNDER*




*B. CAUSAL DIAGRAM WITH ECZEMA AND ASTHMA AS INTERMEDIATES IN THE CAUSAL PATHWAY*



*C: CAUSAL DIAGRAM WITH ECZEMA AND ASTHMA AS INTERMEDIATES, AND ALTERNATIVE PATHWAYS*




## D: Error estimation: peanut allergy resolution and prevalence in the general population

When a 1% prevalence of peanut allergy in the control population was assumed to exist in the multivariate analysis, the estimate of the effect of peanut allergy remained significant, and the estimate of the effect of atopic asthma remained non-significant. This result did not change whether the revised peanut allergy status was assumed to be conditional upon atopic asthma. However, when a 20% resolution of allergy in peanut allergic cases was modeled, the effect of peanut hypersensitivity became non-significant. Although this is the most often cited estimate in the literature[103, 104], this estimate for resolution may be too high in this particular case group. Some of the

individuals included in this study have participated in the peanut allergy registry over many years, and those who continue to participate in the registry are likely continuing to have reactions. Patients who present with anaphylaxis to peanut are highly unlikely to have their peanut allergy resolve[66], and approximately half of our cases reported a history of anaphylaxis. As remarked previously, peanut-specific sIgE is thought to decrease with age[265] and in our case group, it appears to increase (Figure A2, Appendix A). This may indicate that we have a group that is less likely to have their peanut hypersensitivity resolve, and a 20% resolution rate may be too high.

## E: Study limitations

Although our results show that the relationship between peanut allergy and loss-of-function mutations in *filaggrin* is independent of diagnostic criteria of peanut allergy and atopic asthma, there were several limitations to our study, some of which have been already discussed. There were significant demographic differences between our peanut allergic cases and our main control group. Although we have attempted to factor these in through the various interaction terms, there may be other undocumented differences between the groups that could affect the results. Secondly, the atopic asthma variable was a construct, which although based on reasonable assumptions, is likely imperfect. We evaluated this through multiple error models, but it is still possible that these models are insufficient. Similarly, resolution of peanut allergic cases and prevalence of peanut allergy in the general population were unknown, and

were also modeled. Finally, it is possible that this type of analysis may be inadequate for the accurately capturing the complexity of the relationships between peanut allergy, sensitization atopic disease and *FLG* mutations.

## F: Impact of results within the framework of peanut allergy pathogenesis, and possible targets for prevention and treatment of peanut allergy

We have shown that the relationship between peanut allergy and loss-of-function mutations in *filaggrin* is independent of diagnostic criteria of peanut allergy, and also appears to be independent of underlying atopic disease in logistic regression analysis.

If the relationship of defects in the gene encoding filaggrin is mediated through atopic disease as an intermediate step (Figure 4B), we would expect to see the effect abolished, or diminished, when atopic diseases, such as atopic asthma, are controlled for in the multivariate analysis. Introduction of bias through controlling for an intermediate step in the causal pathway is a concern, but the stability of the relationship between *FLG* mutations and peanut allergy is reassuring.

So how could mutations in a skin barrier protein lead to peanut allergy, without requiring other diseases of the atopic march? It is possible that percutaneous exposure to peanut allergens via an impaired epidermal barrier may be the cause, although it is unlikely that this is due to peanut oil in topical preparations, as they contain no peanut protein[266]. Household peanut

consumption is a risk factor for the development of peanut allergy[213], which echoes the evidence in mouse models that epicutaneous exposure to peanut protein increased allergic sensitization[6, 206]. Oral exposure to peanut at a young age appears to be protective[213], and oral tolerance is prevented by epicutaneous sensitization in mouse models[6, 206]. Sensitization to *Arachis* proteins may be related to cross-sensitization to aeroallergens, such as birch pollen proteins that share homology with peanut allergens[72]. A history of eczema may not be necessary to indicate barrier dysfunction, since the condition may resolve, and normal-appearing skin in those with AD has increased susceptibility to irritants and allergens, when compared to normal controls[193, 194, 196, 267]. Although these factors may explain sensitization to peanut via a barrier defect, they do not explain why some sensitized individuals develop peanut allergy and others do not. Cutaneous peanut exposure with subsequent skin reaction does not necessarily result in future systemic symptoms[59]. This suggests that there is a step between sensitization and development of peanut allergy, which may be mediated by some unknown factor.

Although *FLG* is currently the candidate gene with the strongest supporting evidence in both atopic dermatitis[171] and peanut allergy[7], it is evident that this gene is not the sole cause of peanut hypersensitivity, since only 20% of peanut allergic individuals had the presence of any of the screened mutations in our study. It is possible that some individuals may have less common null mutations, which were not screened. Occupational latex allergy in dental workers had the potential of a *filaggrin* defect mediated pathogenesis, due to the regular skin contact

with latex gloves. However, this was not found to be related to genetic mutations in a small study of 41 individuals[268]. Unsurprisingly, hypersensitivity to hymenoptera venom was not found to be related to *FLG* mutations, likely since initial exposure is percutaneous from stinging[269]. Other barrier protein genes that are candidates for investigation include a related protein, filaggrin 2 (*FLG2*), which has a similar structure and regulatory mechanisms as *FLG* and has the same location in the skin[270], and other members of the epidermal differentiation complex[271]. A genome-wide association study (GWAS) conducted in a German population of atopic individuals found significant association with the epidermal differentiation complex on chromosome 1q21[272]; along with a new locus of interest surrounding the hornerin gene (*HRNR*), this study also discovered an association signal for the A allele of rs7927894 on chromosome 11q13.5, recently implicated in Crohn's disease[273]. Investigations in the role of the cutaneous barrier in food allergy have also provoked interest in the gastrointestinal mucosal barrier. Dysfunction in the gastrointestinal barrier has also been proposed in the pathogenesis of peanut allergy[274], and disruption of the gut barrier has been associated with an increased risk of sensitization[275]. However, there is no evidence that *filaggrin* has any effect on susceptibility to inflammatory bowel disease, and is only contributory in cases of coexistent AD and food allergy[276]. Although the general consensus is that oral exposure induces tolerance, mouse models have indicated that oral sensitization to peanut may still occur[277].

There are obviously other factors that determine sensitization and control which individuals will develop hypersensitivity. Several cases of individuals developing peanut allergy after organ and bone transplant have been reported in the literature. Although some of these acquired allergies are transient[278-280] other cases appear to be more permanent[281, 282]. The transient cases may be related to B lymphocyte production of peanut-specific IgE[279]; it is unlikely they are due to passive transfer of IgE, as the majority reported positive SPT results or peanut-specific IgE for several months. In a similar vein, peanut allergy resolution has been reported after bone marrow transplant for primary immunodeficiency[283]. These case reports, particularly those with non-transient acquisition of peanut allergy, indicate that other factors may mediate the final steps to peanut allergy.

One such mediator could be thymic stromal lymphopoietin (TSLP). Thymic stromal lymphopoietin is a potent activator of dendritic cells that is released by epidermal cells[284]. TSLP is crucial for the development of atopic disease in humans and mice, but its expression alone is insufficient for complete disease development, and it requires antigenic co-stimulation[285]. A *filaggrin* mutation, providing constant antigen exposure, could provide this stimulation required for TSLP function. Interestingly, TSLP production in human keratinocytes is decreased by treatment with glucocorticoids, but not calcineurin inhibitors[286], both commonly used topical treatments for eczema. TSLP is also of particular interest because its overexpression can cause worsening of experimental asthma[287]. The TSLP pathway includes genes such as

suppressor of cytokine signaling-7 (SOCS7), whose elimination results in high TSLP production in mouse mast cells and severe cutaneous disease[288], and toll-like receptor 3 (TLR3)[286]. *In vitro* studies of keratinocyte cultures has found that both innate immune signalling by TLR3 stimulation and *FLG* knockdown by short-inhibitor RNA (siRNA) led to increased TSLP expression[289]. Others have suggested that expression of filaggrin could possibly be modulated by inflammatory cytokines expressed in the atopic immune response, such as interleukin-4 (IL4) and interleukin-13 (IL13). IL13 variants have been associated with early sensitization to foods in children with atopic eczema [290] and IL13 promoter variants are linked to development of latex allergy in health care workers, but not to development of allergy in spina bifida or bladder exstrophy patients, who are usually exposed to latex via surgery or percutaneous medical devices[291]. S100A11, a calcium-dependent protein of the epidermis, beta-defensin, an antimicrobial peptide, and filaggrin expression are all decreased by IL4 and IL13[292, 293]. Intradermal injection of IL4 has been found to suppress barrier function recovery after tape stripping[294].

The findings of this study also fit within the framework of the perceived increase in prevalence of atopic disease. While genetic alteration is unlikely to explain of the observed increase in atopic disease seen in recent years, an environmental modification could definitely explain this rapid change in disease prevalence. One method could be through the environmental effects on filaggrin: filaggrin breakdown is reportedly increased after chemical and ultraviolet-induced erythema[295], and appears to

be regulated by the humidity of the external environment[296]. The hygiene hypothesis[218] is another possible environmental component that has gained a wide following in both the lay and medical press to explain the increase in both allergic and autoimmune conditions. However, this may be oversimplifying the concept[220]. Those with atopic dermatitis and a barrier defect are also known to be susceptible to infections, such as recurrent and severe infections with *staphylococcus aureus* and herpes virus. A clinical study of those with a history of eczema herpeticum (disseminated herpes virus infection of the skin) or eczema vaccinatum (disseminated smallpox virus after vaccination) reveals more severe atopic dermatitis, greater body surface area affected, elevated serum IgE and allergic sensitization; these characteristics are consistent with those found in patients with *filaggrin* mutations[297]. However, the subjects in this retrospective study were not genotyped and this theory cannot be confirmed. Interestingly, *s. aureus* epidermolytic toxin has been found to bind *in vivo* to filaggrin[298]. Similarly, in asthma, respiratory viruses have been implicated in the inception of asthma[299]. The interactions between genes, the immune system and the environment is complex, and it may be too simplistic to assume that over-cleanliness alone is the cause of the rise in atopy.

Dietary factors may also play a role in the increase in peanut allergy prevalence. Countries that have early peanut consumption, such as Israel, appear to have lower prevalence of peanut allergy[236], and early consumption of peanut has been found protective for peanut allergy[213, 300, 301]. The method of peanut preparation has also been implicated: roasting may

change allergen conformation and cause greater IgE binding[302-305]. In China, increased prevalence of peanut allergy has been noted at the same time as increased Westernization[306]. Some authors have correlated this with a decrease in use of crude peanut oil in cooking, and thus a decrease in oral tolerance[307]. Early exposure to solid foods was associated with a reduced risk for parent-reported eczema (OR: 0.35; 95%CI: 0.20–0.63), but only among children with allergic parents[308]. In open trials, oral immunotherapy has been found to induce clinical densensitization in peanut allergic individuals, accompanied by changes in gene expression of the apoptotic pathway and decreases in Th2 responses to peanut[122].

The relation between peanut allergy and *filaggrin* mutations has clinical importance apart from the theoretical framework of pathogenesis. Some have suggested that aggressive treatment of eczema may prevent the development of asthma[249, 309]. Indeed, *in vitro* experiments show that inflammatory-type skin keratinocytes have higher engulfment of allergens than their epithelial cell counterparts in the respiratory tract, suggesting that inflamed skin has a higher uptake of allergens and therefore a higher risk of sensitization[310]. A pilot study using emollient for prevention of atopic dermatitis showed some promise[311], although a small case-control study found no evidence for an effect[312]. Topical steroid preparations may improve skin barrier function in inflamed skin, but it is possible this improvement in barrier function may be due to the effects of the vehicle alone[313].

## G: Final conclusions:

Peanut allergy is significantly associated with *filaggrin* null mutations and it is the most significant genetic risk factor for peanut allergy to date, with an OR of approximately 2. The relationship between peanut hypersensitivity and loss-of-function mutations in the gene encoding filaggrin is unaffected by the restrictiveness of diagnostic criteria used to determine peanut allergy diagnosis. The results of this study suggest that this relationship between peanut allergy and *FLG* mutations is independent of atopic asthma, and lend credence to the hypothesis that sensitization in allergic disease may occur through the skin. If true, therapies targeted towards the skin, to either improve barrier function or decrease inflammation, may result in the prevention of allergic disease. Research must be continued in the areas of barrier function, immunologic mediators and environmental interactions to further our knowledge of the pathogenesis of peanut allergy and atopic diseases.

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## APPENDIX A: Descriptive statistics of peanut allergic cases

*Table A1: PERSONAL AND FAMILY HISTORY OF PEANUT ALLERGIC CASES*

| $f$ | Yes | Proportion yes (95% CI) | No | Proportion no (95% CI) | Miss |
|---|---|---|---|---|---|
| Anaphylaxis | 308 | 0.457 (0.419, 0.495) | 366 | 0.543 (0.505, 0.581) | 0 |
| SPT ≥ 8mm | 549 | 0.861 (0.834, 0.887) | 89 | 0.139 (0.113, 0.166) | 36 |
| Asthma | 426 | 0.645 (0.609, 0.682) | 234 | 0.355 (0.318, 0.391) | 14 |
| Eczema | 505 | 0.765 (0.733, 0.798) | 155 | 0.235 (0.202, 0.267) | 14 |
| Hayfever | 385 | 0.584 (0.546, 0.622) | 274 | 0.416 (0.378, 0.454) | 15 |
| Food allergy* | 462 | 0.699 (0.664, 0.734) | 199 | 0.301 (0.266, 0.336) | 13 |
| FHx asthma | 359 | 0.535 (0.497, 0.573) | 312 | 0.465 (0.427, 0.503) | 3 |
| FHx AD | 417 | 0.621 (0.585, 0.658) | 254 | 0.379 (0.342, 0.415) | 3 |
| FHx HF | 592 | 0.882 (0.858, 0.907) | 79 | 0.118 (0.093, 0.142) | 3 |
| FHx of FA* | 286 | 0.429 (0.392, 0.467) | 380 | 0.571 (0.533, 0.608) | 8 |
| FHx of PA | 81 | 0.121 (0.096, 0.146) | 589 | 0.879 (0.854, 0.904) | 4 |

$f$ indicates ever a history; * indicates food allergy other than peanut

*Abbreviations:* FC (food challenge), SPT (skin prick test), AD (atopic dermatitis), HF, FA (food allergy, other than peanut), FHx (family history), Miss (missing)

*FIGURE A1: DISTRIBUTION OF PEANUT-SPECIFIC SERUM IGE IN PEANUT ALLERGIC CASES*

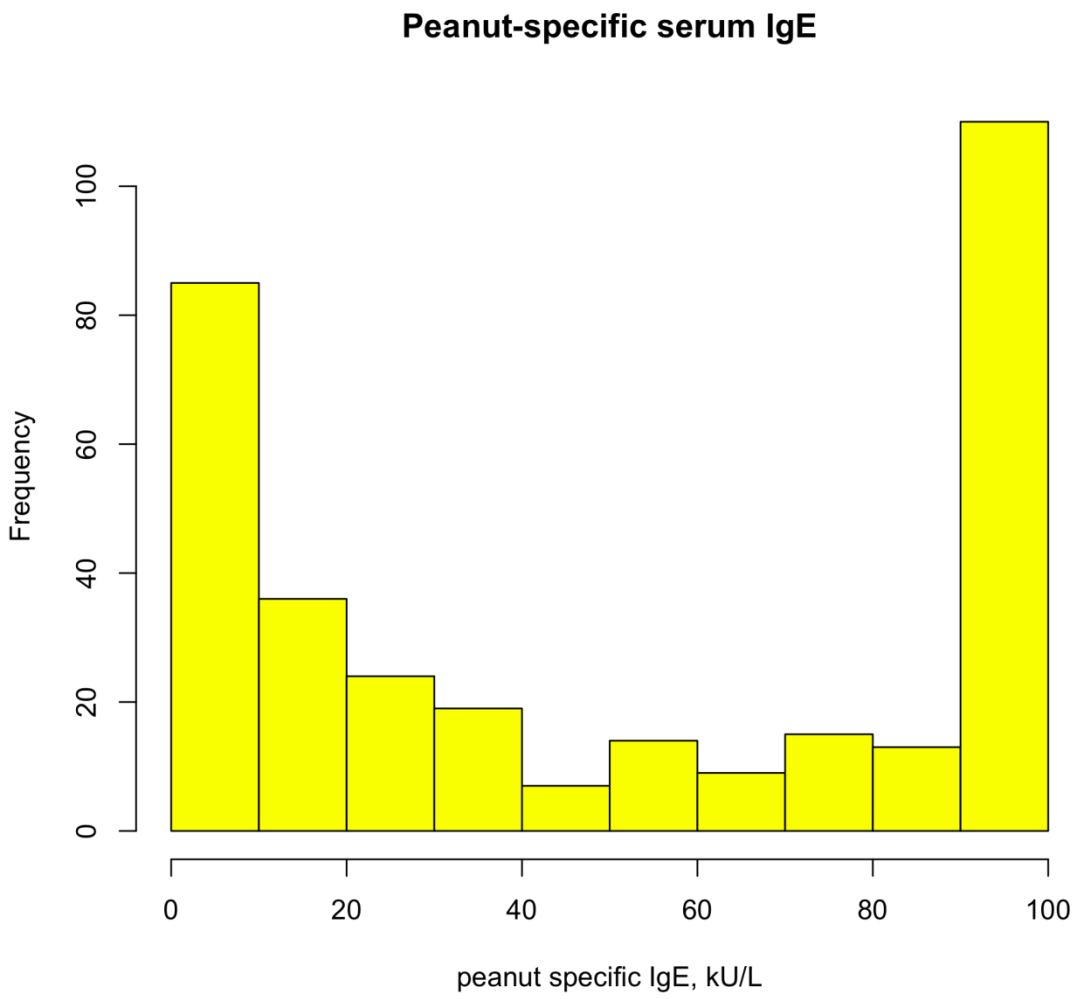
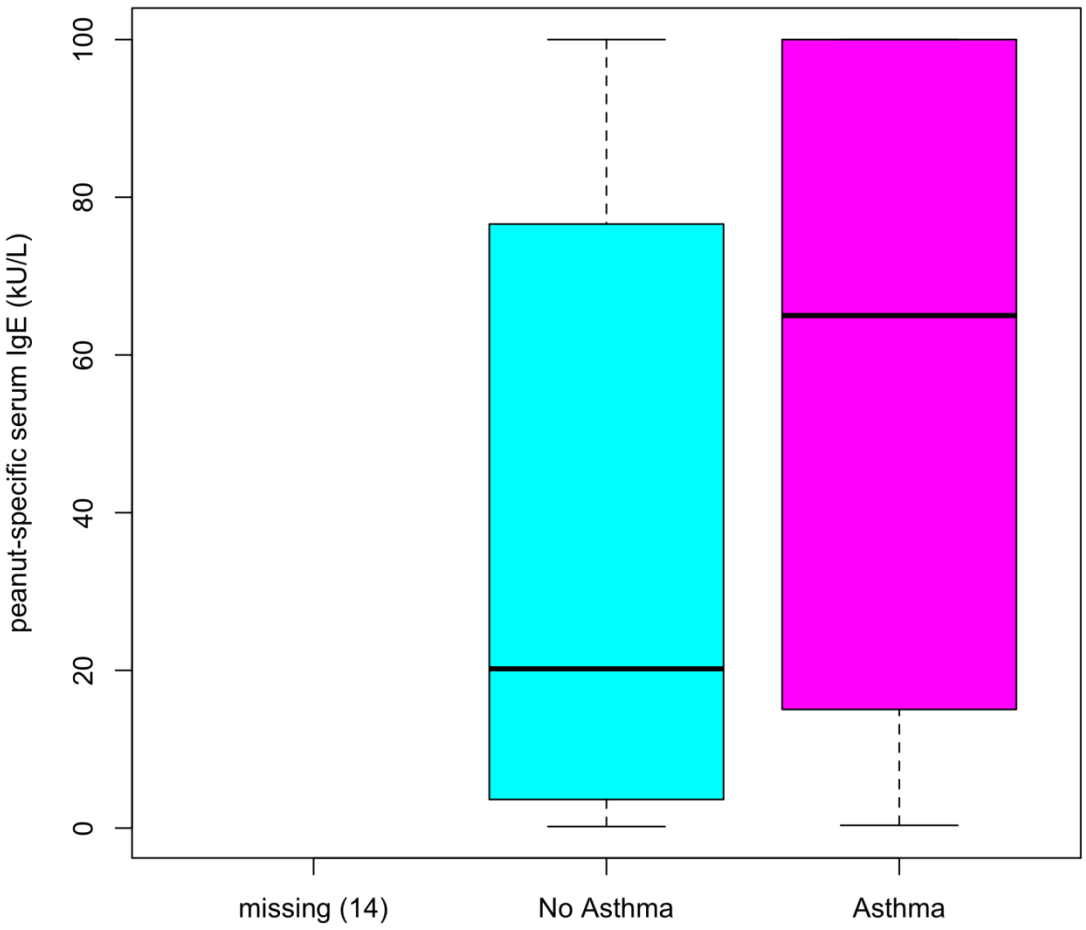


*FIGURE A2: RELATIONSHIP OF PEANUT-SPECIFIC IGE AND AGE AT WHICH THE SAMPLE WAS TAKEN IN PEANUT ALLERGIC CASES*

*FIGURE A3: RELATIONSHIP OF PEANUT-SPECIFIC IGE AND ASTHMA STATUS IN PEANUT ALLERGIC CASES*



## APPENDIX B: Sensitivity analysis of case definition criteria

*FIGURE B1: DISTRIBUTION OF CASES IN ORIGINAL CASE DEFINITION CATEGORIES*

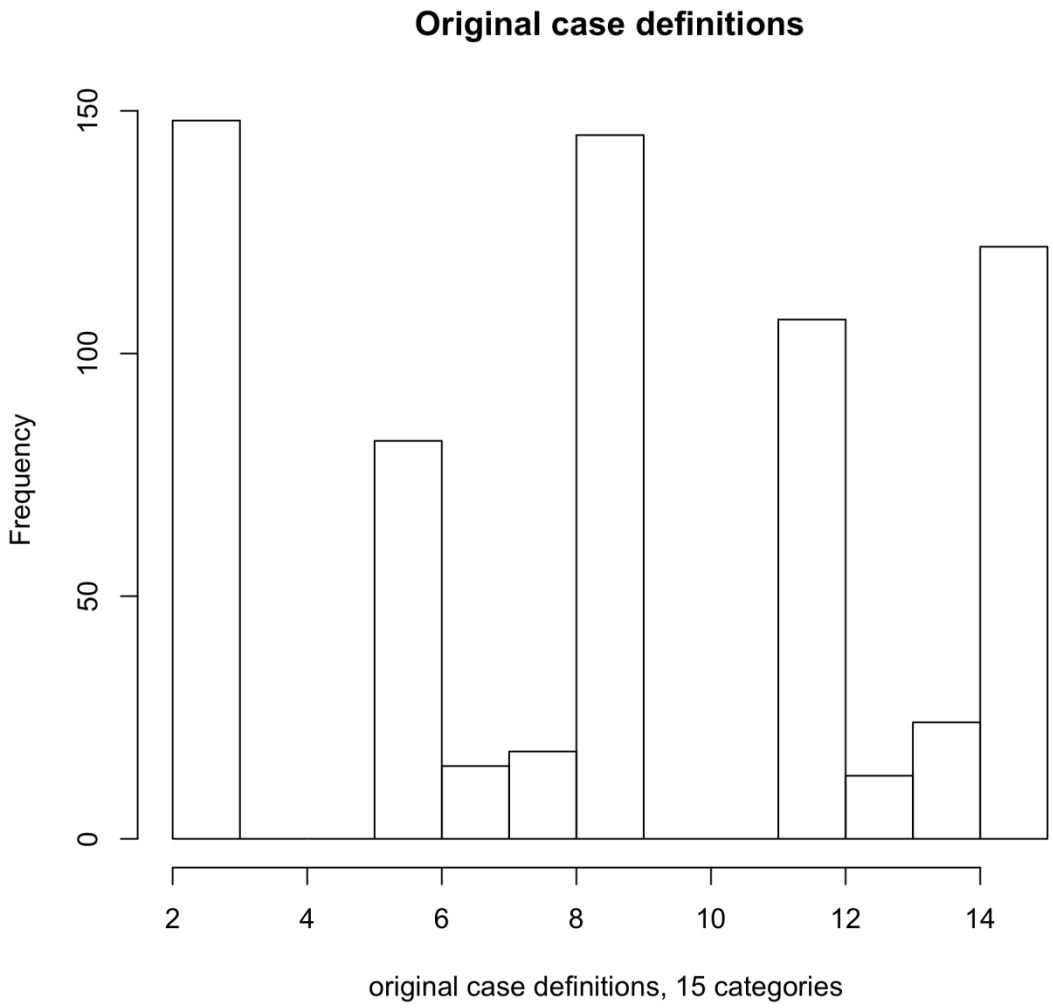


*TABLE B1: DESCRIPTIVE STATISTICS OF CASES OVER ORIGINAL CONTINUUM OF CASE DEFINITIONS (15 GROUPS)*

| N= | | 674 | 660 | 641 | 526 | 510 | 486 | 337 | 309 | 267 | 266 | 263 | 253 | 159 | 146 | 122 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Case def. | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Age | *x* | 9.9 | 9.9 | 9.9 | 10 | 10 | 10 | 11 | 11 | 11 | 10 | 10 | 10 | 11 | 11 | 11 |
| | sd | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.9 | 3.9 | 3.8 | 4.2 | 4.2 | 4.2 | 4.1 | 4.2 | 4.2 |
| Sex | male | 416 | 409 | 392 | 325 | 317 | 299 | 210 | 193 | 163 | 172 | 169 | 162 | 99 | 88 | 72 |
| | miss | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ana | yes | 308 | 353 | 308 | 255 | 252 | 242 | 148 | 135 | 111 | 255 | 252 | 242 | 148 | 135 | 111 |
| | miss | 366 | 307 | 333 | 271 | 258 | 244 | 189 | 174 | 156 | 11 | 11 | 11 | 11 | 11 | 11 |
| OFC | yes | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| | miss | 649 | 635 | 616 | 501 | 485 | 461 | 312 | 284 | 242 | 241 | 238 | 228 | 134 | 121 | 97 |
| psIgE | *x* | 51 | 51 | 52 | 57 | 59 | 61 | 65 | 71 | 78 | 49 | 50 | 50 | 61 | 66 | 73 |
| | sd | 41 | 41 | 41 | 40 | 40 | 41 | 36 | 35 | 34 | 41 | 41 | 42 | 38 | 37 | 39 |
| | miss | 342 | 336 | 315 | 229 | 229 | 229 | 82 | 82 | 82 | 109 | 109 | 109 | 36 | 36 | 36 |
| ≥8mm | yes | 549 | 543 | 521 | 470 | 463 | 451 | 281 | 262 | 232 | 254 | 251 | 241 | 147 | 134 | 110 |
| | miss | 36 | 32 | 36 | 27 | 22 | 17 | 27 | 22 | 17 | 5 | 5 | 5 | 5 | 5 | 5 |

*Abbreviations*: case def(case definition group) *see Table B2 for definitions*; *x*(sample mean); ana(anaphylaxis); OFC(positive oral food challenge); psIgE (peanut-specific IgE); ≥8mm(SPT≥8mm); sd(standard deviation); miss(missing)

*TABLE B2: ATOPIC HISTORY OVER ORIGINAL CONTINUUM OF PEANUT ALLERGIC CASE DEFINITIONS (15 GROUPS)*

| N= | | 674 | 660 | 641 | 526 | 510 | 486 | 337 | 309 | 267 | 266 | 263 | 253 | 159 | 146 | 122 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Case def. | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| A. asthm | yes | 426 | 418 | 404 | 345 | 335 | 321 | 236 | 218 | 191 | 182 | 180 | 172 | 114 | 105 | 86 |
| | miss | 14 | 13 | 13 | 13 | 12 | 12 | 10 | 8 | 8 | 6 | 6 | 6 | 4 | 4 | 4 |
| AD | yes | 505 | 495 | 482 | 394 | 383 | 368 | 250 | 227 | 198 | 194 | 193 | 187 | 112 | 101 | 85 |
| | miss | 14 | 13 | 13 | 10 | 10 | 10 | 7 | 7 | 6 | 7 | 7 | 7 | 4 | 4 | 3 |
| HF | yes | 385 | 374 | 365 | 308 | 300 | 289 | 200 | 185 | 163 | 147 | 146 | 141 | 94 | 87 | 73 |
| | miss | 15 | 14 | 14 | 13 | 12 | 12 | 10 | 8 | 8 | 6 | 6 | 6 | 4 | 4 | 4 |
| FC* | Yes | 462 | 452 | 439 | 374 | 363 | 344 | 248 | 227 | 193 | 179 | 178 | 170 | 112 | 102 | 83 |
| | Miss | 13 | 13 | 12 | 12 | 11 | 11 | 9 | 7 | 7 | 5 | 5 | 5 | 3 | 3 | 3 |

**1:** all cases **2:** original def **3:** hybrid def **4:** psIgE≥15 / SPT≥8 **5:** psIgE ≥26 / SPT≥8 **6:** psIgE≥57 / SPT≥8 **7:** psIgE≥15 / SPT≥15 **8:** psIgE ≥26 / SPT≥15 **9:** psIgE≥57 / SPT≥15 **10:** psIgE≥15 / SPT≥8 & ana **11:** psIgE≥26 / SPT≥8 & ana **12:** psIgE≥57 / SPT≥8 & ana **13:** psIgE≥15 / SPT≥15 & ana **14:** psIgE≥26 / SPT≥8 & ana 15: psIgE≥57 / SPT≥8 & ana. All categories include +FC as a criterion. *Abbreviations:* Case def(case definition); A. asthm (atopic asthma); AD(atopic dermatitis); HF(hayfever); FC*(food allergy other than peanut)

*TABLE B3: ODDS RATIOS FOR CHARACTERISTICS OF PEANUT ALLERGIC CASES VERSUS THE MODIFIED CONTINUUM OF CASE DEFINITIONS (6 GROUPS)*

| All cases, N=677 | OR | 95% CI | |
|---|---|---|---|
| Mutation (binary) | 1.05 | 0.94 | 1.17 |
| Gender | 1.02 | 0.93 | 1.11 |
| Ever anaphylaxis | 1.87 | 1.67 | 2.10 |
| SPT of 8mm | 2.13 | 1.80 | 2.52 |
| History of atopic asthma | 1.17 | 1.06 | 1.28 |
| Ever atopic dermatitis | 0.99 | 0.89 | 1.10 |
| Ever hayfever | 1.04 | 0.96 | 1.14 |
| Ever food allergy other than peanut | 1.06 | 0.96 | 1.16 |
| Ever family history of asthma | 1.01 | 0.92 | 1.10 |
| Ever family history of atopic dermatitis | 1.12 | 1.03 | 1.23 |
| Ever family history of hayfever | 0.99 | 0.86 | 1.13 |
| Ever family history of food allergy | 1.00 | 0.92 | 1.09 |
| Ever family history of peanut allergy | 0.93 | 0.82 | 1.06 |
| **ANOVA** | **df** | **F** | **p** |
| Age at Jan 1st, 2009 | 5 | 7.04 | $2.02 \times 10^{-6}$ |
| Peanut specific serum IgE | 5 | 81.10 | $2.20 \times 10^{-16}$ |

*TABLE B4: ODDS RATIOS OF CHARACTERISTICS OF PEANUT ALLERGIC INDIVIDUALS, WITH AND WITHOUT OLD SYSTEM SPT CLASSIFIED INDIVIDUALS (MODIFIED CASE DEFITIONS, 6 GROUPS)*

| | All cases | | | No old SPT grading criteria | | |
|---|---|---|---|---|---|---|
| | N= 677 cases | | | N=671 | | |
| | OR | 95% CI | | OR | 95% CI | |
| Mutation (binary) | 1.05 | 0.94 | 1.17 | 1.05 | 0.94 | 1.17 |
| Gender | 1.02 | 0.93 | 1.11 | 1.02 | 0.93 | 1.11 |
| Ever anaphylaxis | 1.87 | 1.67 | 2.10 | 1.88 | 1.68 | 2.10 |
| SPT of 8mm | 2.13 | 1.80 | 2.52 | 2.12 | 1.79 | 2.51 |
| History of atopic asthma | 1.17 | 1.06 | 1.28 | 1.16 | 1.06 | 1.28 |
| Ever AD | 0.99 | 0.89 | 1.10 | 0.99 | 0.89 | 1.10 |
| Ever HF | 1.04 | 0.96 | 1.14 | 1.04 | 0.96 | 1.14 |
| Ever food allergy* | 1.06 | 0.96 | 1.16 | 1.05 | 0.96 | 1.16 |
| Ever FHx of asthma | 1.01 | 0.92 | 1.10 | 1.01 | 0.92 | 1.10 |
| Ever FHx of AD | 1.12 | 1.03 | 1.23 | 1.12 | 1.03 | 1.23 |
| Ever FHx of HF | 0.99 | 0.86 | 1.13 | 0.99 | 0.86 | 1.13 |
| Ever FHx of FA | 1.00 | 0.92 | 1.09 | 1.00 | 0.92 | 1.09 |
| Ever FHx of PA | 0.93 | 0.82 | 1.06 | 0.93 | 0.82 | 1.06 |

*Abbreviations:* SPT(skin prick testing); AD (atopic dermatitis); HF(hayfever); FA(food allergy to food other than peanut); FHx (family history); PA (peanut allergy)

## APPENDIX C: Logistic regression modeling of loss-of function mutations in *filaggrin*, predicted by peanut allergy status, age, gender, and atopic asthma

*TABLE C1: UNIVARIATE MULTIVARIATE LOGISTIC REGRESSION MODEL, WITH INTERACTION TERMS AND AGE*

| | | | | |
|---|---|---|---|---|
| *UNIVARIATE* | | OR | 95% CI | |
| | Peanut allergy status | 1.97 | 1.48 | 2.62 |
| | Age at Jan 1st, 2009 | 0.99 | 0.98 | 0.99 |
| | Gender | 1.06 | 0.80 | 1.41 |
| | History of atopic asthma | 1.67 | 1.25 | 2.24 |
| Peanut allergy status, gender, atopic asthma, & interactions | | | | |
| | | OR | 95% CI | |
| *MULTIVARIATE* | Peanut allergy status | 1.89 | 1.16 | 3.08 |
| | Gender | 0.86 | 0.54 | 1.38 |
| | History of atopic asthma | 1.40 | 0.57 | 3.44 |
| | Interaction: peanut/asthma | 0.76 | 0.28 | 2.05 |
| | Interaction: peanut/gender | 1.07 | 0.58 | 1.98 |
| Peanut allergy, age, gender, atopic asthma, & interactions | | | | |
| | | OR | 95% CI | |
| *MULTIVARIATE* | Peanut allergy status | 1.01 | 0.69 | 15.58 |
| | Age at 1 January, 2009 | 3.27 | 0.99 | 1.03 |
| | Gender | 0.81 | 0.49 | 1.33 |
| | History of atopic asthma | 1.42 | 0.58 | 3.49 |
| | Interaction: peanut/asthma | 0.75 | 0.28 | 2.04 |
| | Interaction: peanut/age | 0.99 | 0.94 | 1.05 |
| | Interaction: peanut/gender | 1.14 | 0.61 | 2.16 |

TABLE C2: SUBGROUP ANALYSIS, ATOPIC ASTHMA

| | No peanut allergy | | Yes peanut allergic | |
|---|---|---|---|---|
| Mutation | No | Yes | No | Yes |
| No atopic asthma | 753 | 92 | 187 | 43 |
| Yes atopic asthma | 34 | 6 | 337 | 83 |
| | OR | 95% CI | OR | 95% CI |
| | 1.44 | 0.59, 3.53 | 1.07 | 0.71, 1.61 |
| | No atopic asthma | | Yes atopic asthma | |
| Mutation | No | Yes | No | Yes |
| No peanut allergy | 753 | 92 | 34 | 6 |
| Yes peanut allergy | 187 | 43 | 337 | 83 |
| | OR | 95% CI | OR | 95% CI |
| | 1.88 | 1.27, 2.80 | 1.40 | 0.57, 3.43 |

## APPENDIX D: Modeling of peanut allergy, predicted by null mutations in *filaggrin*, gender, and atopic asthma

*TABLE D1: MULTIVARIATE MODEL OF NULL MUTATIONS IN FLG, GENDER AND ATOPIC ASTHMA(AND INTERACTION TERMS) ON PEANUT ALLERGY STATUS*

| Any *FLG* mutation, gender and atopic asthma on peanut allergy status, plus interaction terms | | | |
|---|---|---|---|
| | OR | 95% CI | |
| Any *FLG* mutation | 1.54 | 0.82 | 2.88 |
| Male gender | 3.45 | 2.47 | 4.80 |
| Atopic asthma | 30.82 | 19.37 | 49.04 |
| Interaction of *FLG* and atopic asthma | 0.99 | 0.33 | 2.97 |
| Interaction of male and atopic asthma | 3.93 | 1.31 | 11.79 |
| Interaction of mutation and gender | 1.46 | 0.64 | 3.33 |

## APPENDIX E: Effect of misclassification error in the atopic asthma variable

The questionnaire associated with this study asked individuals about history of eczema. Twelve percent more reported a history of eczema, compared to previous data in the database. Six percent of individuals had previously indicated they had eczema, but did not indicate so on the current questionnaire. As subjects were not asked about asthma on the current questionnaire, we used the increase in eczema reporting to estimate an increase in asthma reporting. As 426 individuals (63%) with peanut allergy have indicated they have ever had asthma, an increase in about 80 people would signify a 12% increase in asthma. Using the statistical program R, we randomly sampled 80 individuals from those who did not indicate that they had asthma, and changed their asthma status to yes. A univariate and multivariate analysis of peanut and asthma was conducted. The sampling and modeling were repeated for 100 iterations.

We then repeated a similar sampling method, this time investigating a putative 12% asthma increase in cases (80 subjects), and a 6% asthma increase in controls (89 subjects), in order to mimic the effect of those in the control group who may have forgotten they had previously had asthma, in addition to the effect of those in the cases who developed or remembered they had had asthma. Again, the analyses were repeated 100 times. These estimates were then expanded to a 15% increase in asthma in cases, and a 10% increase in asthma in controls, in an effort to reflect probable poor recall of childhood asthma in

adults, and a high risk of development of asthma in those with food allergies.

## APPENDIX F: Sensitivity analysis of peanut allergy status

TABLE F1: ERROR IN PEANUT ALLERGY STATUS ; 20% OF CASES RESOLVE

| 20% of cases of peanut allergy randomly resolve | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | OR | | Lower CI | | Upper CI | |
| Percentile | | 5th | 95th | 5th | 95th | 5th | 95th |
| *UNIV* | Peanut allergy | 1.56 | 3.03 | 1.17 | 1.51 | 2.07 | 2.68 |
| | Atopic asthma | 1.67 | 1.67 | 1.24 | 1.24 | 2.24 | 2.24 |
| *MULT* | Peanut allergy | 1.25 | 1.83 | 0.88 | 1.29 | 1.76 | 2.57 |
| | Atopic asthma | 1.20 | 1.48 | 0.84 | 1.04 | 1.69 | 2.09 |
| 20% of cases resolve, conditional on atopic asthma | | | | | | | |
| | | OR | | Lower CI | | Upper CI | |
| Percentile | | 5th | 95th | 5th | 95th | 5th | 95th |
| *UNIV* | Peanut allergy | 1.62 | 1.99 | 1.22 | 1.50 | 2.16 | 2.65 |
| | Atopic asthma | 1.67 | 1.67 | 1.24 | 1.24 | 2.24 | 2.24 |
| *MUL* | Peanut allergy | 1.18 | 2.08 | 0.71 | 1.30 | 1.91 | 3.29 |
| | Atopic asthma | 0.92 | 1.46 | 0.57 | 0.89 | 1.48 | 2.42 |

*Abbreviations:* UNIV(univariate); MUL(multivariate); CI (95% confidence interval)

*TABLE F2: ERROR IN PEANUT ALLERGY STATUS;* EFFECT OF 1% PREVALENCE OF PEANUT ALLERGY IN CONTROL POPULATION, AND 20% RESOLUTION OF PEANUT ALLERGY CASES

| 1% of controls have peanut hypersensitivity and 20% of cases resolve | | | | | | |
|---|---|---|---|---|---|---|
| | OR | | Lower 95% CI | | Upper 95% CI | |
| Percentile | 5th | 95th | 5th | 95th | 5th | 95th |
| *UNIVARIATE* | | | | | | |
| Peanut allergy status | 1.91 | 1.99 | 1.49 | 1.51 | 2.63 | 2.68 |
| Atopic asthma | 1.67 | 1.67 | 1.24 | 1.24 | 2.24 | 2.24 |
| *MULTIVARIATE* | | | | | | |
| Peanut allergy status | 1.88 | 1.91 | 1.26 | 1.30 | 2.78 | 2.84 |
| Atopic asthma | 1.04 | 1.04 | 0.70 | 0.70 | 1.54 | 1.57 |