NOVEL STRATEGIES TOWARDS REDUCING PEANUT ALLERGENICITY

BY

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ABSTRACT

Food allergy is an adverse reaction mediated by the immune system against normally innocuous food molecules. A small number of foods, such as peanuts, tree nuts, and cow's milk, are responsible for most food allergies globally. The allergenic components found in these foods, which are protein molecules referred to as *allergens*, generally have high stability and consist of complex structures resistant to denaturation. The mechanism is characterized by allergen-specific immunoglobulin E (IgE) antibodies binding and crosslinking to their allergen, triggering a cascade of vasoactive mediators and cytokines leading to the allergic reaction, which in some cases can be lethal.

Peanut, *Arachis hypogaea*, is an important legume crop with high protein and lipid contents. Allergy to peanut is a leading cause of life-threatening anaphylaxis. Evidence published over recent decades demonstrates that the allergenicity of the peanut is greatly influenced by thermal processing, such as dry-roasting or boiling. In this dissertation, we present data on the effect of high-pressure and temperature autoclaving on the peanut, its proteins, and thus on allergenicity. We observed that the high vaporous environment created by the autoclaving process results in a considerable decrease in peanut-specific IgE binding as well as the proportion of intact peanut proteins, including specific allergens. Using ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy, we successfully developed a characteristic signature for the autoclaved peanut that is distinct from that of the raw or roasted peanut. This suggests that autoclaved peanuts consist of an altered allergen profile comprised of peptides at a range of sizes resulting from protein hydrolysis and may thus be exploited in improved diagnostic and treatment methods for peanut allergy.

A second parallel analysis discussed is the evaluation of the proteins and allergenicity of immature peanuts at various reproductive stages of seed development. Total protein content was

lower and water content was higher at earlier stages of the peanut life cycle. Using antibodies specific for allergens Ara h 1, Ara h 2, and Ara h 8, we observed that these peanut allergens increase in proportion and accumulate more in complex protein bodies throughout seed development. This trend was consistent with relative *in vitro* IgE binding using serum samples from patients with high peanut-specific IgE. These data suggest that immature peanuts at the R5 stage, in parallel to the autoclaved peanut, may act as less allergenic forms of peanut, and have the potential to act as safer, more efficacious substrates for peanut allergy treatments such as oral immunotherapy (OIT).

Predictors of response to OIT were addressed in the final chapter. The correlation of cow's milk (CM)-specific IgE levels and other factors at baseline was examined in a cohort of subjects at our centre undergoing CM OIT with treatment outcomes. Specific IgE levels to each of the major CM allergens were quantified in serum samples acquired from subjects in our ongoing CM OIT trial. Uni- and multivariable Cox regression models incorporating these values were used to evaluate sociodemographic factors, co-morbidities, clinical characteristics, and biomarkers at study entry associated with the likelihood of reaching a maintenance dose of 200 mL of CM in 69 children. We found that high CM-specific IgE antibody levels prior to treatment were associated with decreased probability of reaching the maintenance dose, suggesting that assessing these factors prior to therapy can assist in predicting OIT success.

Altogether, the reported data suggest that high-temperature and pressure autoclaved peanuts, as well as immature peanut seeds, contain lower proportions of intact proteins, including allergens. This results in a peanut with reduced allergen potency, and may lead to a safer, more efficacious OIT substrate. Additionally, assessing specific IgE levels prior to OIT may provide information on the success of treatment, as demonstrated with CM OIT.

RÉSUMÉ

L'allergie alimentaire est une réaction indésirable médiée par le système immunitaire contre des molécules alimentaires normalement inoffensives. Un petit nombre d'aliments, tels que les arachides, les noix et le lait de vache, sont responsables de la plupart des allergies alimentaires dans le monde. Les composants allergènes présents dans ces aliments, qui sont des molécules protéiques appelées *allergènes*, présentent généralement une grande stabilité et sont constitués de structures complexes résistantes à la dénaturation. Le mécanisme est caractérisé par la liaison et la réticulation d'anticorps immunoglobulines E (IgE) spécifiques à l'allergène, déclenchant une cascade de médiateurs vasoactifs et de cytokines conduisant à la réaction allergique, qui dans certains cas peut être mortelle.

L'arachide, *Arachis hypogaea*, est une légumineuse importante à haute teneur en protéines et en lipides. L'allergie à l'arachide est l'une des principales causes d'anaphylaxie potentiellement mortelle. Les preuves publiées au cours des dernières décennies démontrent que l'allergénicité de l'arachide est fortement influencée par le traitement thermique, comme la torréfaction à sec ou l'ébullition. Dans cette thèse, nous présentons des données sur l'effet de l'autoclavage à haute pression et température sur l'arachide, ses protéines, et donc sur l'allergénicité. Nous avons observé que l'environnement hautement vaporeux créé par le processus d'autoclavage entraîne une diminution considérable de la liaison des IgE spécifiques à l'arachide ainsi que de la proportion de protéines d'arachide intactes, y compris des allergènes spécifiques. En utilisant la spectroscopie de résonance magnétique nucléaire (RMN) ^{1}H, nous avons réussi à développer une signature caractéristique pour l'arachide autoclavée qui est distincte de celle de l'arachide crue ou grillée. Ceci suggère que les arachides autoclavées sont constituées d'un profil allergénique modifié composé de peptides de différentes tailles résultant de l'hydrolyse des protéines et pourraient donc

être exploitées dans des méthodes améliorées de diagnostic et de traitement de l'allergie aux arachides.

Une deuxième analyse est l'évaluation de l'allergénicité des arachides immatures à différents stades de développement. La teneur en protéines totales était plus faible et la teneur en eau plus élevée aux premiers stades du cycle de vie de l'arachide. En utilisant des anticorps spécifiques pour les allergènes majeurs, nous avons observé que ces allergènes augmentent en proportion et s'accumulent davantage dans les corps protéiques tout au long du développement. Cette tendance est cohérente avec la liaison relative des IgE en utilisant des échantillons de sérum de patients ayant un taux élevé d'IgE spécifiques à l'arachide. Ces données suggèrent que les arachides immatures, comme l'arachide autoclavée, peuvent être des formes moins puissantes de l'arachide en termes de l'allergénicité, et ont le potentiel d'agir comme des substrats plus sûrs et efficaces pour les traitements de l'allergie à l'arachide tels que l'immunothérapie orale (ITO).

Le dernier chapitre de cette thèse décrit la corrélation entre les niveaux d'IgE spécifiques au lait de vache (CM) et d'autres facteurs au départ dans une cohorte de sujets soumis à une ITO au CM, et les résultats du traitement. Les taux d'IgE spécifiques à chacun des principaux allergènes du lait de vache ont été quantifiés dans des échantillons de sérum des sujets participant à notre essai en cours sur l'ITO de CM. Des modèles de régression de Cox uni- et multivariables intégrant ces valeurs ont été utilisés pour évaluer les facteurs sociodémographiques, comorbidités, et biomarqueurs au début de l'étude associés à la probabilité d'atteindre une dose d'entretien de 200 ml de CM chez 69 enfants. Nous avons constaté qu'un taux élevé d'anticorps IgE spécifiques au CM avant le traitement était associé à une probabilité moindre d'atteindre la dose d'entretien, ce qui suggère que l'évaluation de ces facteurs avant le traitement peut aider à prédire le succès de l'ITO.

Dans l'ensemble, les données rapportées suggèrent que les arachides autoclavées à haute température et sous pression, ainsi que les graines d'arachide immatures, contiennent des proportions plus faibles de protéines intactes, y compris d'allergènes. Il en résulte une cacahuète dont le pouvoir allergène est réduit, ce qui peut conduire à un substrat ITO plus sûr et plus efficace. En outre, l'évaluation des taux d'IgE spécifiques avant l'ITO peut fournir des informations sur la réussite potentielle du traitement, comme cela a été démontré avec l'ITO au CM.

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I thank Dr. Stéphane A. Laporte who acted as my academic advisor and a mentor throughout my research project. I also recognize all individuals who have acted as thesis committee members and examiners. I was fortunate to have access to experts in a wide range of fields who offered their support in critically evaluating my research, helping me learn and continuously improve: Dr. Ashraf Ismail (Food Science), Dr. Moshe Ben-Shoshan (Allergy & Immunology), Dr. Albert Berghuis (Biochemistry), Dr. Saji George (Food Science), and Dr. Philippe Bégin (Allergy & Immunology).

Many of the experiments for my project were conducted with the help of other technical experts. I acknowledge Dr. Kurt Dejgaard of the Department of Biochemistry who helped perform mass spectrometry experiments on protein extracts created from differentially processed peanuts and provided his expertise in the analysis of the data. Dr. Anne-Laure Larroque of the Drug Discovery Platform (DDP) at the RI-MUHC was very helpful in setting up HPLC and NMR

experiments and was always there to answer questions with a smile. Dr. Andrée Gravel, a former employee of the DDP, was instrumental in setting up and optimizing the various NMR experiments conducted throughout this dissertation by providing her unique expertise in NMR spectroscopy.

The clinical work described in this manuscript could not have been done without the expert clinical team of allergists, scientists, nurses, coordinators, and other support staff. I am grateful for the growing support from Dr. Moshe Ben-Shoshan who served as a committee member, a significant collaborator, and became a vital mentor. I must also acknowledge Duncan Lejtenyi, the clinical coordinator and our incredible nurses, Liane Beaudette and Manel Ouamrane, for their constant support and positive attitudes, always ready to help. Lastly, I would like to thank my friends and lab mates I was fortunate enough to have met throughout my degree including Diana Toscano Rivero, Eisha A. Ahmed, Wendy (Wei) Zhao, Jana Abi Rafeh, Nicholas Vonniessen, Danbing Ke, Victoria Zismanov, Sara Teimouri-Nezhad, Marieme Dembele, Elliot Goodfellow, Julie Schmitt, Mary Moscone, and many more. Honorable mentions are necessary to Diana and Eisha for their support throughout multiple lab experiments and their assistance with many figures and diagrams throughout Chapter 1.

Finally, I acknowledge my parents, sister, and friends for their support along this journey. Although many of them had little background in related fields, their willingness to listen and effort to understand kept my spirits high. The experience would have been far more difficult without them reminding me to always have Faith and I am truly grateful to them.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The work presented in this dissertation covers a range of topics regarding novel methods towards reducing and treating peanut allergy. This research led to the publication or submission of four manuscripts and the filing of a patent in the United States of America (US20220125916A1), Canada (CA3,097,204), and Australia (AU2020260379) protecting the technology resulting from the work reported in this thesis. Chapter 1 provides a unique overview of the literature covering the topics discussed throughout the following chapters such as food allergy, its epidemiology, development and mechanism, peanut allergy, protein and structural biology, the effect of processing on peanut allergens, analytical methods such as NMR spectroscopy, and more.

One other group has previously assessed the effects of high-pressure and temperature autoclaving on peanut proteins and allergenicity over a decade ago. The current work is the first to evaluate a wide range of autoclaving parameters, such as temperature and time duration of autoclaving. Chapter 2 describes the optimal processing parameters necessary to cross a threshold of protein degradation and fragmentation, providing the first comprehensive evaluation of the effect of autoclaving on peanut proteins, which may potentially be expanded to other food proteins as well. We observed that this processing method leads to a significant decrease in relative allergen content and in IgE binding to sera from highly allergic patients.

Chapter 3 presents novel work using ^{1}H NMR spectroscopy to characterize and obtain molecular signatures of peanuts under various physical states and processing conditions. We used a complementary approach consisting of HR-MAS ^{1}H NMR to evaluate whole intact peanut and defatted peanut flour, as well as traditional solution ^{1}H NMR to analyze solutions derived from soaking peanuts, for each of raw, roasted, and autoclaved peanuts. Spectra of each condition consistently showed lipids in the form of triglycerides predominating the spectrum of intact peanut, as well as the reveal of sugars, mainly sucrose, following peanut defatting. This is the first work

to observe a distinct pattern of peaks in the NMR spectrum resulting from the autoclaved peanut-soaked solution when compared to raw or roasted, characterized by broad peaks, corresponding to greater numbers and diversity of peptide fragments leaching out into solution upon soaking.

Another major approach to mitigating peanut allergy evaluated in this thesis is through harvesting the crop early, at an immature stage. Certain seeds, such as peanut, fully develop their protein matrix only later in development. Chapter 4 describes the composition of peanut seeds at different stages of maturation and peanut allergenicity at these different stages. This work is the first to describe such an approach, analogous to the autoclaved peanut, each of which have high potential for improved treatments and/or diagnostics for peanut allergy.

Finally, an optimized ELISA method for quantifying specific IgE levels to food allergens in serum was used to analyze patient serum samples from a large cohort of cow's milk-allergic patients. Chapter 5 comprises a manuscript published in 2022 in the *Journal of Allergy and Clinical Immunology: In Practice*. This article reported the associations of cow's milk-specific IgE levels and other factors at baseline with outcomes of oral immunotherapy (OIT). This report found that allergic individuals of a multi-center study across Canada with elevated cow's milk component-specific IgE levels at baseline have a decreased the likelihood of reaching the maintenance dose of OIT. These outcomes were novel, and may shed light on other studies, including those evaluating peanut OIT.

CONTRIBUTION OF AUTHORS

Author of current dissertation: **Casey G. Cohen (CGC)**

Supervisors: Bruce D. Mazer (BDM) and Bertrand J. Jean-Claude (BJC)

Chapter 1: Introduction and literature review conducted entirely by **CGC** – literature search, writing, editing, reviewing. BDM and BJC contributed to editing and reviewing process.

Chapter 2: **CGC**: Conducted all experiments, including SDS PAGE, Western blot, and ELISA analyses; Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – reviewing & editing. Eisha A. Ahmed: Conceptualization, Methodology, Investigation, Writing – reviewing & editing. Diana Toscano-Rivero: Methodology, Investigation, Writing – reviewing & editing. Wei Zhao: Methodology, Validation, Writing – reviewing & editing. Kurt Dejgaard: Performed mass spectrometry and proteomics experiments; Investigation, Validation, Writing – reviewing & editing. Albert M. Berghuis: Investigation, Validation, Writing – reviewing & editing. BJC: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. BDM: Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing.

Chapter 3: **CGC** and BJC conceived and designed the experiments; **CGC** performed all experiments; **CGC**, and BJC analyzed the data; **CGC** drafted the manuscript; **CGC**, BDM, and BJC reviewed and edited the manuscript.

Chapter 4: **CGC:** Acquisition of the protein analysis, creation of figures, and writing of manuscript; YL: Design and acquisition of the work; EM: Acquisition the histochemical immune-staining; BDM and BJC: Contributions to the conception and substantial revision; RH: Co-originated the study and managed the plant-based study; RK, NA: Participated in the recruitment and management of patients; MK: Co-originated the study and managed the clinical trials.

We received lyophilized samples from peanuts of each stage of development from Israel. I was invited to perform analyses of protein levels, including major allergens, as well as peanut-specific IgE binding to allergic sera. Experiments evaluating gene expression levels, immunohistochemical staining, and SPT studies were performed by our collaborating partners in Israel.

Chapter 5: **CGC** analyzed all serum samples by ELISA, performed statistical analyses, drafted manuscript, reviewed, and edited the manuscript. Other authors helped in patient recruitment and sample acquisition.

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LIST OF ABBREVIATIONS

ALA:	α-Lactalbumin
BCCH:	British Columbia Children's Hospital
BLG:	β-Lactoglobulin
CI:	Confidence Interval
CMA:	Cow's Milk Allergy
ELISA:	Enzyme-Linked Immunosorbent Assay
FA:	Food Allergy
HR:	Hazard Ratio
HR-MAS:	High-Resolution Magic Angle Spinning
IgE:	Immunoglobulin E
kDa:	Kilodalton
MCH:	Montreal Children's Hospital
MS:	Mass Spectrometry
NMR:	Nuclear Magnetic Resonance
OFC:	Oral Food Challenge
OIT:	Oral Immunotherapy
PA:	Peanut Allergy
sIgE	Specific IgE
SKH:	The Hospital for Sick Children
SPT:	Skin Prick Test

CHAPTER 1: *Introduction & Comprehensive Review of the Relevant Literature*

1.1. Food Allergy (FA)

All living organisms require a form of nutritional support to continue living. As humans, we consume food containing essential nutrients, such as proteins, lipids, carbohydrates, vitamins, and minerals. Geographic and cultural differences have led to the creation of several cuisines and culinary arts, including a wide array of ingredients, herbs, spices, and cooking techniques. While these aspects of food have evolved greatly over the past centuries into a broad diversity of dishes, a small – but increasing – proportion of the global population are at risk of dangerous adverse reactions to certain foods.

Food allergy (FA) occurs when an individual's immune system treats harmless food molecules as harmful, sending out signals and chemicals to defend the body. This leads to symptoms such as hives, swelling, redness, difficulty breathing, and in a very small number of cases, death. The term "allergy" comes from the Ancient Greek words *allos* meaning "other" and *ergon* meaning "work", describing an "altered reaction" in the body. This concept was first introduced in 1906 by Viennese pediatrician Clemens von Pirquet after he noticed that patients who had received injections of smallpox vaccine using horse serum often had accelerated and more severe responses to the second or subsequent injections.[1, 2] This was the first ever recognition of the idea that the immune system, an inherently protective system, could also harm its host.[3]

1.1.1. Epidemiology of FA

Today, FA is often referred to as the second wave of the allergy epidemic, lagging decades behind the first wave consisting of asthma and allergic rhinitis.[4] The prevalence of FA ranges considerably across regions, populations, and studies. Approximately 8% of children in the USA

are estimated to be affected by FA, while over 11% of children self-perceived or are perceived by their parents as having food allergies, suggesting a significant disease burden.[5] These proportions represent the equivalent of 6 million American children, translating to 1 in 13 children, or about 2 students per classroom.[6] FA affects approximately 10.8% of adults in the USA, while self-reported rates in adults reach as high as 19%.[7] In Canada, the prevalence of self-reported FA increased from 7.1% to 9.3% between 2010 and 2016, likely due to increasing awareness, while physician-diagnosed FA increased from 5.9% to 6.1%.[8, 9] In school-age children across Europe, prevalence estimates of FA and food sensitization range from 1.0% to 5.6% and from 11.0% to 28.7%, respectively, demonstrating the substantial geographic variation in prevalence across European regions and cultural backgrounds.[10]

The prevalence of FA has not only increased over recent decades, particularly in urban areas, but hospitalizations due to food-induced anaphylaxis have accelerated as well.[11-13] Although it is possible for any food to cause an allergy, over 90% of food reactions are caused by 8 foods, formerly referred to as the "Big 8" major allergens: milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans, leading to the Food Allergen Labeling and Consumer Protection Act of 2004 (Figure 1.1).[14] Effective January 1, 2023, sesame was added as the 9th major food allergen in the USA.[15] In Canada, the most common allergens in food are referred to as the *priority allergens*; in addition to the Big 9 allergens described above, mustard seed and sulphites are also on the list of priority

Figure 1.1. The 9 major food allergens in the USA. The priority allergens in Canada additionally include mustard seed and sulfites.

allergens. Although sulphites are a food additive that do not cause true allergic reactions, they are grouped with the priority allergens because sulphite-sensitive individuals may react to sulphites with allergy-like symptoms. Milk and egg allergies often resolve later in childhood, while peanut and tree nut allergies tend to persist.[16]

1.1.2. Impact on quality of life

Childhood FA results in significant medical costs for health care systems and even larger costs for families with food-allergic children. It is estimated that the overall economic cost of FA in the USA is $24.8 billion annually, or $4,184 per year per child.[17] As the prevalence of FA has grown, its impacts on the quality of life (QoL) of those affected have also been amplified. Importantly, those affected by FA are not only the allergic individuals, but also anyone they are living with, those caring for them, and extending out to almost everyone they interact with daily. For allergic children, this places a heavy burden on their parents and/or caretakers, which spreads to other non-allergic siblings living at home and then further to schools, summer camps, and more.[18] Although severe food allergies are less common in adults than in children, they still exist and parallels could be drawn in that the burden reaches others living in the same household and in the workplace. While certain social pressures indicate that people should "do their part" to make allergic individuals safe in public spaces (e.g., gatherings with family and friends, school, workplace, etc.), it is not always clear where to draw the line in terms of efforts expected from others.

A range of measures and instruments have been developed and validated for the assessment of health-related QoL in patients with FA in the form of various surveys and questionnaires.[19, 20] Assessing QoL in FA is rather unique when compared to many other chronic illnesses due to the nature of the disease. In the case of FA, patients rarely suffer from physical symptoms in their day-

to-day lives.[21] Specific aspects associated with the burden living with FA include, among others, the fear of and vigilance for accidental reactions, the fear of hidden ingredients or being able to treat a reaction, the burden of food avoidance or label reading, and an overall reduced sense of empowerment.[22] Numerous families across the globe avoid travel, restaurants, and even traditional schooling by choosing to home-school their children in order to limit potential exposures to food allergens. Trying to improve the QoL of allergic individuals and their families demonstrates the importance of further research in this field and has led to our studies on FA.

1.1.3. Development of FA

Currently, there is no confirmed mechanism for the development of FA. However, a wide range of potential hypotheses and contributing factors have been explored such as genetic components, the presence of asthma and other allergies, the maternal state throughout pregnancy, or the gut microbiota.[23]

One major factor is the route of initial exposure to the allergen. The development of the allergy, referred to as *sensitization*, may be occurring through routes other than oral exposure, such as through the skin or inhalation. Evidence shows early-onset atopic dermatitis (AD), particularly in the first 3 months of life, is associated with a substantially increased risk of FA at 12 months old.[24] Moreover, filaggrin is a filament-associated protein that plays an important role in the skin's barrier function by linking together structural proteins in the outermost skin cells to create a strong barrier.[25] Evidence has shown that common loss-of-function variants of the filaggrin protein are a major predisposing factor for AD[26] and more recently, for peanut allergy.[27, 28] These reports support the concept of trans-cutaneous sensitization and the causal link between AD and FA: reducing both the severity and duration of AD could potentially reduce the incidence of FA.[29] Miyaji et al. (2020) found that early, aggressive use of topical corticosteroids followed by

proactive maintenance therapy to shorten the duration of AD in 177 infants efficiently suppresses the development of FA at 2 years of age.[30] Although early environmental peanut exposure was found to increase the risk of developing peanut sensitization and allergy, exposure to peanut via the oral route demonstrated a protective role in developing peanut tolerance, even in the presence of environmental peanut exposure.[31] These findings support the hypothesis that infants and children are sensitized via non-traditional routes of exposure for foods, such as through the skin, and that tolerance is induced in the gut via traditional oral exposure.

In addition to the route of exposure, the timing of exposure has proven to be another crucial factor in food sensitization, leading to the debate on whether early or late introduction to major food allergens is beneficial for FA prevention.[32] In 2008, Du Toit et al. found a considerably lower incidence of peanut allergy in regions where peanut is a primary food early in childhood, such as in Israel, when compared to regions where avoidance of major food allergens in early childhood was recommended.[33] These findings led to the development of a major landmark study in allergy and immunology, referred to as the Learning Early About Peanut, or LEAP, study where 640 infants with severe eczema, egg allergy, or both (i.e., considered "high-risk" to develop FA) were randomly assigned to consume or avoid peanuts until 5 years of age. The prevalence of peanut allergy in the avoidance group was 13.7% compared to 1.9% in the consumption group, demonstrating that the early introduction of peanuts resulted in a significant decrease in the frequency of development of peanut allergy among children at high risk.[34]

Altogether, these findings and other recent studies suggest that early, oral exposure may be required to induce tolerance to foods via the gut.[35-37] Delayed oral exposure, and avoiding major food allergens early in development, increases the likelihood of being exposed via other routes, such as through the skin or via inhalation, and may increase the risk of FA development. Though

disparities exist, early allergen feeding practices, particularly with peanut, are gaining traction among US parents and caregivers.[38]

1.1.4. Diagnosis of FA

The first line of food allergy diagnosis is asking the patient and family about any history of allergic reactions. In the clinic, the first test that is done to evaluate any suspected food allergies is the skin prick test (SPT).[39, 40] This test consists of placing a drop of protein allergen extract followed by a scratch on the forearm of the suspected allergic individual. After 10 to 15 minutes, a wheal (raised, itchy bump) develops, and its size correlates with the presence of specific IgE to the given food. The SPT is a simple, cost-effective, and rapid test that can be performed in the clinic by a nurse, making it an effective first screening for diagnosis. However, the SPT has poor specificity; it has a good rate of negative predictability, i.e., patients who experience a negative SPT result are not likely to be allergic, but it has a poor positive predictability rate, i.e., positive SPT tests are not good predictors of true allergy.

Thus, upon positive SPT results, the patient should be offered an oral food challenge (OFC), if possible. The gold standard of food allergy diagnosis is the double-blind placebo-controlled food challenge (DBPCFC). Here, the individual consumes small increasing amounts of their allergen or of placebo until objective signs of a reaction are observed and the challenge is stopped. Doses of allergen are given 30 minutes apart and placebo or allergen are given at random each on a separate day. While the DBPCFC remains the most reliable diagnostic method for food allergy, it requires a lot of time, energy, and resources for both the patient and their family, as well as for the physician and medical staff.

Other tests exist such as serum IgE testing in the laboratory, though these tests have similar specificities as the SPT, as they are detecting specific IgE for the allergen, and cannot specifically

test for the presence of allergy.[41] More recently, advances in component-specific IgE testing via evaluating IgE levels to specific component allergens of a given food have proven greater predictive values of true allergy.[42, 43] Importantly, a detailed history of allergic reactions or tolerance to foods should be recorded before any testing is done. Clinicians should only use specific IgE tests (SPT or serum tests) as diagnostic tools, and test results alone should not be diagnostic of food allergy.[39]

1.1.5. Immune mechanism of FA

The immune system responds to allergies and atopic diseases primarily through adaptive immune responses. CD4 T lymphocytes can differentiate into many different subtypes of T helper (T_H) cells, including: T_H1, T_H2, T_H17, T_{reg} (regulatory T cell) and T_{FH} (follicular helper T cell).[44, 45] Type 2 responses involving T_H2 cells are traditionally involved in parasite immunity, but in Western countries, this is no longer as relevant. Within a complex network of immunologic mechanisms of atopy and allergic diseases, type 2 immune responses represent the clinically most relevant immune response.

Additionally, when the immune system mistakenly identifies harmless substances as harmful, we refer to this as a *hypersensitivity reaction*. There are four established types of hypersensitivity reactions and FA is an immediate, type 1 reaction highlighted by the formation of specific immunoglobulin E (IgE) antibodies against innocuous food substances, most often protein molecules, referred to as *allergens*. IgE antibodies normally play a role in host defense against parasitic infections. However, in the context of allergy, they aberrantly recognize short, specific sequences of their antigen called *epitopes,* which can be either linear or conformational. This is described in further detail in Section 1.2.4.

Type 2 immune responses involve, among others, T_H2 cells, B cells, group 2 innate lymphoid cells (ILC2's), basophils, eosinophils, and mast cells. These cells play a variety of roles in the immune system, most relevant to allergy is the production of specific cytokines referred to as interleukins (ILs): secreted proteins that bind to their specific receptors, playing a role in intercellular communication among white blood cells.[46] The major cytokines contributing to the development and intensity of T_H2 responses and inflammation are IL-4, IL-5, IL-9, and IL-13 produced by effector T_H2 cells, and IL-25, IL-31, IL-33, and thymic stromal lymphopoietin (TSLP) produced by epithelial cells and other T_H cells.[47-49] It has become clear that both innate and acquired immune responses contribute to type 2 response endotypes, which are subtypes of disease defined by distinct pathophysiological mechanisms.[50]

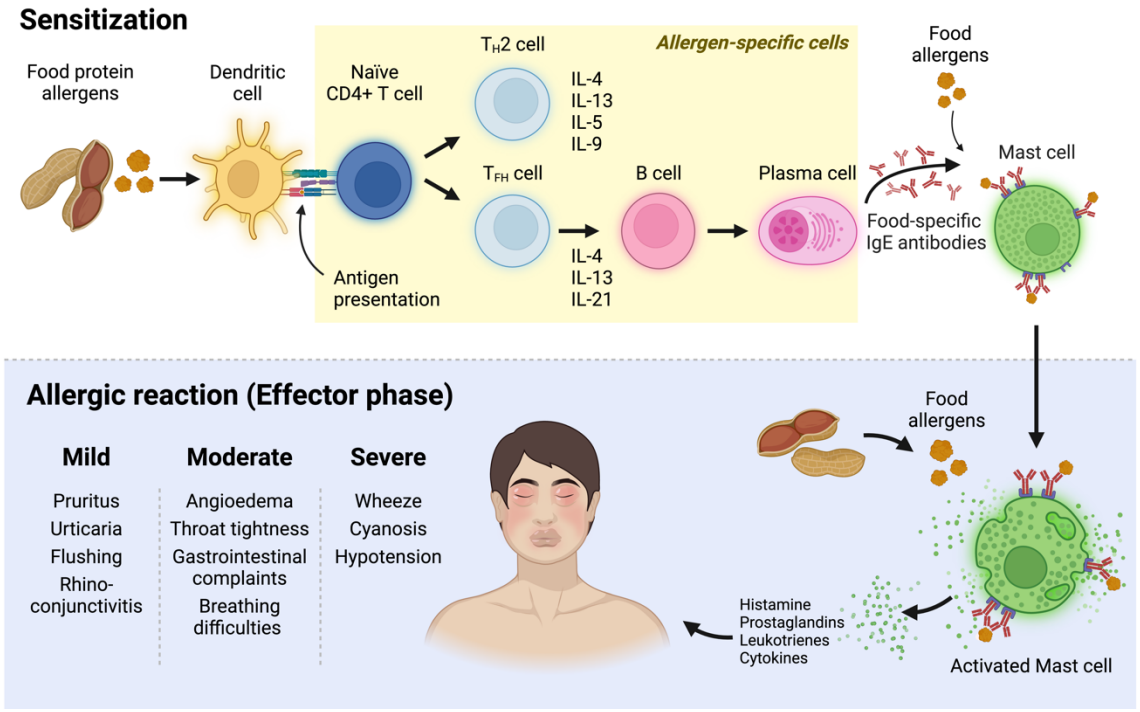

Figure 1.2. Two phases of allergic inflammation: sensitization and effector phase. Upon exposure to the culprit food, likely through non-traditional routes such as through the skin or inhalation, the allergen interacts with antigen presenting cells such as dendritic cells, leading to a cascade of events highlighted by the formation of allergen-specific IgE antibodies which bind to the surface of mast cells. Upon subsequent exposures to the food, the allergen binds and cross-links to IgE, resulting in the activation and degranulation of mast cells, releasing mediators into the bloodstream, triggering the symptoms of allergy, which can be lethal.

As depicted in Figure 1.2, the allergic inflammation process to foods can be divided into two major phases: first, a sensitization phase, with the outcomes of IgE production and antigen-specific memory cell production, and the individual becomes sensitized to a food. Second is an effector phase, with the outcomes of loading mast cells and basophils with IgE, priming the system for the allergic reaction.[51, 52]

1.1.5.1. Sensitization to foods

Sensitization describes when an individual becomes allergic, or sensitive, to a foreign substance. Sensitization to food allergens is most common early in life (<2 years of age), while sensitization to inhalant allergens is more common at older ages (>3 years of age).[53] There is growing evidence that the specific route of the primary exposure to the allergen is critical for sensitization.[54] Particularly, as described in Section 1.1.2., exposure via the gut is believed to have a tolerizing effect (i.e., the traditional way of ingesting foods), while exposure via untraditional routes such as through the skin or through inhalation are more likely to lead to sensitization.

Mucosal-resident dendritic cells capture, internalize, and process the allergen into peptides and transport it to draining lymph nodes. Here, the processed peptides are presented via major histocompatibility complex (MHC) class II molecules to the T cell receptor (TCR) on naïve $CD4^+$ T cells, which can differentiate into T_H2 or T follicular helper (T_{FH}) cells. T_H2 cells in the periphery and in circulation produce type 2 cytokines such as IL-4, IL-13, IL-5, and IL-9, functioning as effector cells that drive many aspects of allergic inflammation, while T_{FH} cells in lymphoid tissues produce IL-21, IL-4, and IL-13, inducing IgE class switch recombination in B cells, memory and plasma cell differentiation, and allergen-specific IgE production.[55] Allergen-specific IgE antibodies that are released into circulation and can bind to FcεRI molecules on the surface of mast cells and basophils.[56] Populations of allergen-specific memory T_H2 and B cells increase via clonal

expansion, which are primed to respond upon further allergen encounters. An individual, now sensitized to their allergen, may or may not have experienced any clinical symptoms of allergy. They have produced IgE which, bound to mast cells, is primed to cause allergic symptoms on future exposures.[57] More recently, a novel subset of IL-13-producing T_{FH} cells ("Tfh13 cells") were identified and reported to be required for production of exclusively high-affinity IgE and subsequent allergen-induced anaphylaxis.[58]

1.1.5.2. Effector phase

The second phase of allergic inflammation is the effector phase, manifesting as the allergic reaction.[59] This phase is characterized by type I hypersensitivity reactions triggered by exposure to the culprit food. Allergens bind and crosslink to IgE antibodies previously bound to FcεRI receptors on mast cells and basophils. These cells are activated upon IgE crosslinking, degranulating and releasing a myriad of chemicals and mediators such as histamines, leukotrienes, prostaglandins, and cytokines into the bloodstream.[51] This release is responsible for the immediate symptoms of acute inflammation such as hives, swelling, redness, and in a significant number of cases, anaphylaxis, the severe and life-threatening allergic reaction.[55, 60]

1.1.5.3. Mechanisms of tolerance and desensitization to foods

Individuals with FA cannot tolerate specific allergens. However, in some instances, tolerance can be induced naturally over time, often termed *outgrowing* an allergy. This is most common in young children with egg and cow's milk allergies, and significantly less common with peanut or tree nut allergies.[16]

Additionally, there are many cases where tolerance does not occur naturally. An exciting change in the approach to treating children with food allergy is that there is increasing evidence that tolerance to foods can be induced. Induction of tolerance has long been applied to allergic

diseases such as allergic rhinitis or venom allergy using allergen-specific immunotherapy.[61, 62] With this treatment, individuals are exposed via injection to or oral administration of small amounts of allergen, with the goal to increase the dose of allergen that an allergic individual can tolerate without adverse reactions, and thus decrease symptoms when exposed.[63, 64]

Two distinct terms when discussing immunotherapy exist in the current literature: desensitization and tolerance.[65] *Desensitization* relies on ongoing exposure to the allergen following therapy. A desensitized individual may be nonreactive while regularly receiving the allergen but could return to the previous state of reactivity when the regular administration ends. The best paradigm for this is the treatment of medication allergy.[66] *Tolerance*, also termed *sustained unresponsiveness*, refers to the retention of the protective benefit achieved through therapy and is not reliant upon ongoing exposure. While the ultimate goal for any immunotherapy treatment is complete tolerance, desensitization is a helpful alternative, though it is still unclear what immune mechanisms are involved in leading to one or the other.[67, 68]

One of the most relevant mechanisms of desensitization and developing tolerance in allergic individuals with FA is through the induction of allergen-specific IgG_4 antibodies.[69] Of the four human IgG subclasses, IgG_4 is the least abundant in serum at approximately 5% of total IgG, although its levels can reach as high as 75% of total IgG after chronic exposure to the antigen, such as through allergen immunotherapy.[70, 71] The structure of IgG_4 may mitigate inflammation; it may act as a blocking antibody to compete with IgE for allergen binding and direct mast cell and basophil suppression by cross-linking of FcεRI and FcγRIIb with IgE and IgG_4.[70, 72] Although evidence suggests that the production of IgG_4 is believed to be confined to human IL-10–producing regulatory B cells that suppress antigen-specific immune responses,[73] it is possible that other cell subsets also play a role in IgG_4 production.

IgG_4 is most widely viewed as a marker of tolerance and desensitization in allergic disease as demonstrated by high levels of allergen-specific IgG_4 in subjects who undergo various forms of immunotherapy,[74] venom-specific IgG_4 in non-allergic bee workers,[75] or cat-specific IgG_4 in non-allergic cat owners.[76] IgG_4 has been associated with the development of immune tolerance to allergens, including foods, and the ratio of specific IgE to IgG_4 might be useful in the context of desensitization.[77] Conversely, IgG_4 is also believed to play an instrumental role in the development and mechanism of eosinophilic esophagitis (EoE), a type of non-IgE mediated food allergy,[78] and in an inflammatory condition known as IgG_4 syndrome.[79, 80]

Additionally, regulatory T cells, or Tregs, play an important role in suppressing the allergic response by secreting suppressive cytokines such as IL-10, TGF-β, and IL-35.[81] Tregs directly or indirectly inhibit allergen-induced activation and degranulation of mast cells and basophils.[82] Although this goes beyond the scope of this thesis, further understanding mechanisms of suppression of the allergic reaction and induction of tolerance are important points for further research.

1.2. Proteins: An Essential and Complex Class of Biomolecules

Food allergens, which trigger the allergic reaction via binding to IgE antibodies, are most commonly **protein** molecules.[83, 84] Understanding their composition and structure is essential for treating food allergy and for related research in the field. Of the four major biomolecules essential for life, proteins are large macromolecules composed of one or more chains of amino acids, referred to as polypeptides. Proteins are essential in performing a wide array of functions in all living organisms such as providing tissue structure, forming antibodies, catalyzing chemical reactions, cell signaling, and many more.[85, 86]

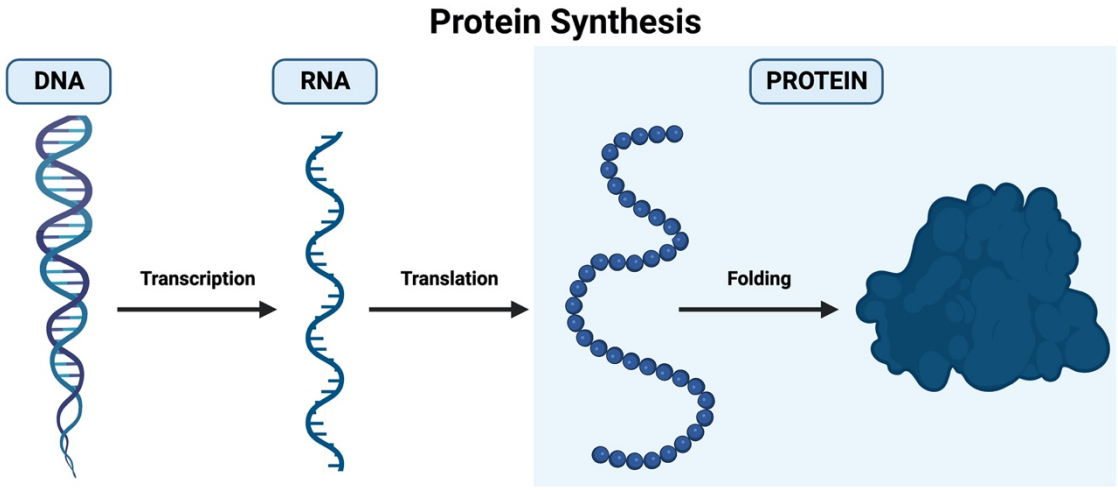

Figure 1.3. Protein synthesis. DNA is transcribed into RNA, which is then translated into a polypeptide chain composed of amino acids. The polypeptide chain is then properly folded to form a functional protein.

Genes can be described as instruction codes written in DNA for thousands of different proteins that interact with each other and the cellular environment. The central dogma of molecular biology states that genetic information flows only in one direction: DNA is transcribed into RNA, which is then translated into polypeptide chains composed of amino acids that fold into proteins (Figure 1.3).[87] A particular sequence of amino acids is what differentiates one protein from another, and the specific folding of the polypeptide chain provides a three-dimensional structure, which is essential for the function of the protein.

Amino acids are connected to each other along a polypeptide chain via peptide bonds, which are amide covalent bonds linking two

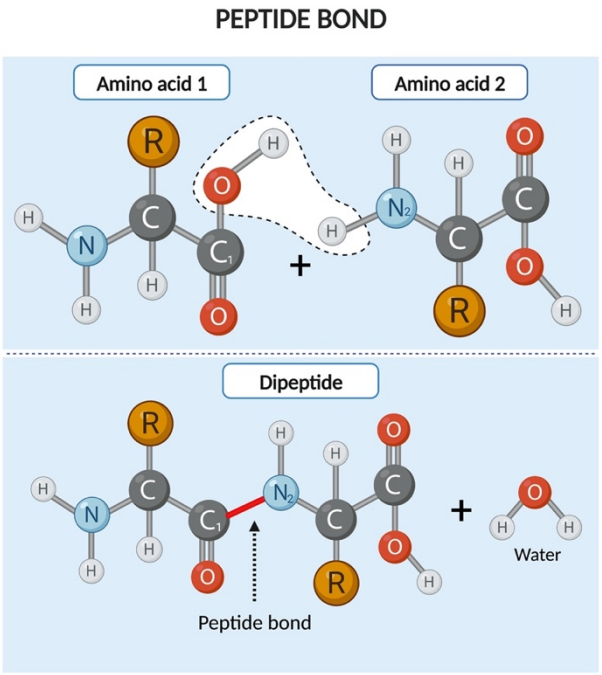

Figure 1.4. Peptide bond between carbon and nitrogen of two adjacent amino acids, producing a dipeptide and releasing one water molecule.

consecutive α-amino acids from the carboxy or C-terminal carbon (C1) of one amino acid to the amino- or N-terminal nitrogen (N2) of another (Figure 1.4).[88] When two or more amino acids combine to form a peptide, a water molecule is released. Amino acids as a quantified unit are often called amino acid *residues*.

1.2.1. Protein structures

A defining characteristic of protein molecules is that their unique folded structure controls their function.[87] In general, proteins naturally fold into their native conformation following their synthesis through chemical interactions of the side chains of the amino acids comprising the protein.[88] There are four defined levels of a protein's structure (Figure 1.5):

1. The primary structure of a protein corresponds to the amino acid sequence of the polymer. Even when the protein no longer has any three-dimensional structure and is in a linear form, the protein's primary structure remains intact, so long as the protein's peptide bonds are conserved.
2. The secondary structure corresponds to regular repeated structures resulting from and stabilized by hydrogen bonds between atoms of the peptide backbone rather than from

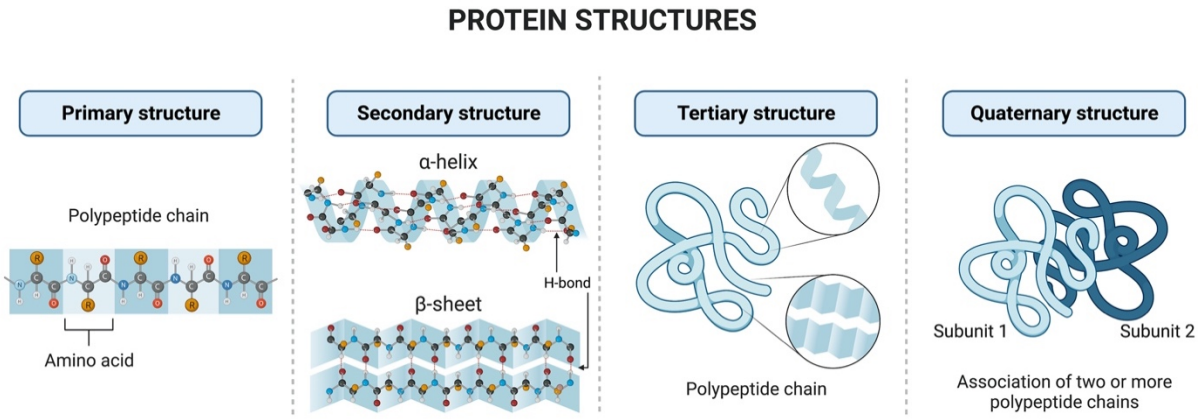

Figure 1.5. Sequential protein structures. The primary structure of a protein consists of the amino acid sequence. The secondary structure consists of regular repeated structures formed and stabilized by hydrogen bonds (H-bonds). The tertiary structure is the overall fold of a protein, resulting from many different interactions. The quaternary structure represents the association of multiple polypeptide chains, or protein subunits, to form a functional oligomer.

amino acid side chains. The most common secondary structures in proteins are α-helices and β-sheets:

a. The **α-helix** is a right-handed helix conformation in which the hydrogen of the backbone amino group (N-H) of a given amino acid bonds, via a hydrogen bond, to the oxygen of the backbone carbonyl group (C=O) of another amino acid located 2 to 5 residues earlier (average = 3.6 residues) earlier in the primary structure.[89]
b. The **β-sheet** consists of β-strands connected laterally by two or more hydrogen bonds between the backbone of consecutive strands, forming a twisted, pleated sheet.

Secondary structures are local, meaning they occupy only certain portions of the overall protein structure, and thus one protein molecule can have multiple regions of different secondary structures.

3. The tertiary structure of a protein is the ultimate three-dimensional structure, often referred to as the "fold", which determines the basic function of the protein. This is the overall shape and structure of a polypeptide chain, composed of multiple secondary structures and their spatial relationship to one another. The tertiary structure can be stabilized by local or non-local interactions, such as hydrogen bonding, disulfide bonds, salt bridges, hydrophobic interactions (often through the formation of a hydrophobic core), and sometimes post-translational modifications.[90]
4. The quaternary structure of a protein describes the interaction of two or more polypeptide chains, often referred to as protein subunits, each with their own tertiary structure, which function together as a single protein complex. An example of a quaternary structure is a protein dimer, a complex formed by two protein monomers, usually non-covalently bound,

which can be a homodimer (two identical proteins) or a heterodimer (two different proteins).[91] Some proteins may only be functional in their multimeric forms.

Proteins can be very fluid molecules, constantly shifting between states, or conformations, depending on the environment and required function. They can undergo conformational changes, i.e., shifts in their three-dimensional structure, to transition between active and inactive states.

1.2.2. Protein denaturation & susceptibility to heat and processing

Under certain conditions, proteins can undergo physical changes that result in the unfolding of their secondary and/or tertiary structures. This process is referred to as *denaturation* or *linearization*; the primary structure of the protein remains intact via its covalent peptide bonds along the backbone, but the overall folding is lost, impairing the function of the protein.[92] Denaturation can occur following a variety of conditions such as high temperature, change in pH, use of detergents, or vigorous shaking. These environments result in the disruption of the weaker bonds within a protein, often hydrogen bonds, which are responsible for the highly ordered structure of the protein in its native state. Denatured proteins have a looser, more random structure and are often insoluble. The term *labile* is used to describe proteins that are susceptible to denaturation or degradation, while *stable* proteins are more resistant to alterations in their structure.

In some cases, the original protein structure can be regenerated by restoring the conditions favoring the native state. In other cases, denaturation is irreversible; for example, when an egg is cooked, the proteins denature, cross-link, and aggregate forming a solid, fixed network of proteins, and nothing can be done to restore the egg to its raw, liquid state.

1.2.3. Proteins as allergens

What distinguishes an allergen from countless other harmless proteins? In susceptible individuals, allergens have the ability to sensitize an individual and elicit an IgE response. Upon

subsequent exposures, this can lead to an allergic reaction to the same protein. Importantly, it is not yet entirely clear what characteristics lead to various proteins having the ability to induce allergenic responses or why some proteins are more allergenic than others. However, today there are hundreds of known allergens among both inhaled substances such as pollens, molds, and animal dander, as well as many foods.

Despite increasing knowledge of the amino acid sequences and structural information of identified allergens, only a limited number of biochemical characteristics are associated with food allergens.[84] Two characteristics of a food protein have been established to greatly influence its ability to become an allergen: the stability of its structure and its relative abundance compared to other proteins in the food.[93, 94] Recent findings suggest, however, that the correlation between abundance, stability, and allergenicity is not a linear relationship as has been previously believed; instead, there are defined ranges for both parameters within which a protein is most likely to become allergenic.[93]

Food allergens have certain biochemical characteristics influencing their stability, which allow them to survive the extreme conditions of food processing, escape various digestive enzymes of the gastrointestinal tract, and then interact with the immune system. For example, proteins that are members of the 2S albumin group, such as the major peanut allergen Ara h 2, contain a conserved cysteine skeleton held together by four disulfide bonds, providing 2S albumins with great stability during proteolytic digestion and heat or chemical treatment.[95] Moreover, many 2S albumin proteins from other species have also been identified as allergens such as those in cashews, walnuts, soybeans, or sesame seeds.[96, 97] Other protein families producing common allergens also exist, such as globulins, profilins, defensins, or non-specific lipid transfer proteins.[98]

1.2.4. Epitopes & epitope mapping

The specific region of an antigen, or allergen, that is recognized by antibodies and certain cells of the adaptive immune system is referred to as the *epitope*. While both B cells and T cells may bind to the same allergens, it is important to note that they each bind to different epitope regions of a given allergen via distinct mechanisms.[99] B cell epitopes, particularly IgE binding epitopes in the context of food allergy, can be conformational or linear. They bind directly to B cells on the B cell receptor (BCR) or to the corresponding antibody produced by the B cell. Epitopes are considered conformational when the secondary and/or tertiary structure of the allergen is required for recognition and binding while in contrast, linear epitopes require only the primary amino acid sequence and can be recognized even if the protein is denatured or fragmented.[100] Linear epitopes can range from as low as 4 amino acids in length up to 12, though they most commonly range from 7 to 9 amino acids long.[101] Because conformational epitopes consist of key residues brought together by folding, their length can range from 5 to 30 amino acids long.[102] The number and structure of epitopes recognized by IgE antibodies in a given allergen is an important factor correlating with its overall allergenicity.

Although beyond the scope of this work, T cell epitopes are distinct from B cell epitopes, even within the same allergen.[103] They are peptides, usually 17 to 21 amino acids in length, which are presented to T cell receptors (TCR) via antigen presenting cells (APC).[104] APCs will internalize and process the intact antigen into peptides, and then present it to the TCR via major histocompatibility complex (MHC) class II molecules on their surface.[105] In this thesis, we will discuss and evaluate only B cell epitopes in the context of IgE binding to food proteins.

Many linear IgE-binding epitopes of food allergens have been identified and mapped using overlapping peptides and IgE in sera from patients with documented hypersensitivity to the food

in question.[106] This process is referred to as *epitope mapping*; experimentally identifying the binding sites, or epitopes, of antibodies on their target antigens or protein allergens.[107, 108] This is often done using *peptide microarrays*, a collection of consecutive, overlapping peptides displayed on a solid surface.[109] By examining how IgE can bind to the series of short peptides corresponding to the primary sequence of a given allergen, antibody epitopes could be mapped and key residues for binding discovered. An important limitation to the peptide microarray, however, is that this technique is most useful for linear epitopes. Because only short peptides are evaluated, conformational epitopes cannot form in the same way as they would in the context of the entire allergen, and thus binding to these epitopes cannot be evaluated reliably.[110] More recently, new methods are being developed to predict and map conformational epitopes effectively using novel techniques such as mass spectrometry or NMR spectroscopy.[111-113]

Some epitopes are considered *immunodominant* because peptides containing these epitopes bind IgE in 90% or greater of the sera tested. For example, Han et al. (2016) performed an epitope mapping experiment via peptide microarray and identified 12 sequential IgE binding epitopes corresponding to the major peanut allergen Ara h 2, three of which were immunodominant epitopes.[114] The use of peptide microarray mapping and analysis of epitope characteristics can provide important information to assess the allergenicity of food proteins such as critical residues for IgE binding. These results are helpful to determine what areas of an allergen could be targeted for novel treatments for food allergy such as peptide immunotherapy, which uses injections of synthesized peptides derived from common foods to desensitize allergic patients.[115, 116]

1.3. Peanut Allergy

Of all food allergies, peanut allergy is one of the most severe. **Novel strategies to reduce, treat, and diagnose peanut allergy is the focus of this dissertation.**

1.3.1. The peanut: *Arachis hypogaea*

1.3.1.1. Composition

Peanut, *Arachis hypogaea,* is an important legume crop worldwide, serving as an excellent and affordable source of protein, fat, vitamins, and minerals.[117] Approximately half of the peanut mass is composed of lipids (49.2%), a quarter is attributed to protein (25.8%), and the final quarter is composed of carbohydrates, water and other nutrients (Table 1.1).

Table 1.1 Composition of raw, unprocessed peanut by mass per 100 g. Adapted from U.S. Department of Agriculture (USDA) FoodData Central Search Results.[118]

Nutrient	**Amount (g)**	**Element**	**Amount (mg)**
Protein	25.8	Calcium, Ca	92
Lipid	49.2	Iron, Fe	4.58
Carbohydrate	16.1	Magnesium, Mg	168
Fiber	8.5	Phosphorus, P	376
Sugar	4.72	Potassium, K	705
Water	6.5	Sodium, Na	18
		Zinc, Zn	3.27
		Copper, Cu	1.14
		Selenium, Se	7.2 (µg)

The earliest report of the study of peanut and its proteins date back to as early as 1880 from Germany, which focused on the protein composition of peanut and other oilseeds, where only a single peanut protein globulin was reported.[119] In the early 20th century, the growing of cotton declined in many regions of the USA due to ravages of the boll weevil beetle. Peanut became a major supplementary crop grown in place, sparking peanut production in North America. Johns &

Jones (1917) published their findings of at least two globulins present in the peanut, which they titled *arachin* and *conarachin*, later renamed as Ara h 3 and Ara h 1, respectively.[120] They found that these globulins contain a high percentage of basic nitrogen (primarily from arginine and lysine amino acids) when compared to other seed proteins, making peanut a more cost-effective crop for feeding the human population than cereals such as wheat and corn.[120]

1.3.1.2. Species & varieties

Four major peanut varieties exist, which include Runner, Virginia, Spanish, and Valencia market types. The peanut species is divided into two subspecies: *hypogaea*, the Virginia peanut variety which accounts for approximately 19.9% of all peanut production in the U.S., and *fastigiata,* which is then further divided into two varieties: *fastigiata vulgaris,* the Spanish variety, and *fastigiata fastigiata,* the Valencia variety, both of which account for a combined 1.4% of U.S. peanut production. Lastly, the Runner variety, primarily used in the production of peanut butter, accounts for the majority of production at 78.7% and is a hybrid of both *hypogaea* and *fastigiata* subspecies.[121] Runner peanuts have become the dominant peanut type grown in the U.S. due to the introduction of the Florunner variety in the early 1970s, which was responsible for a spectacular increase in peanut yields.[122] Peanuts from the main four market types are highly comparable in their total protein content and in their allergen profiles, indicating that allergenicity and thus safety considerations are not dependent on the peanut cultivar in question.[123]

1.3.1.3. Peanut development

Most proteins of the peanut have the role of seed storage. In the mature seed, they accumulate in large and rigid protein bodies of about 5–10 μm in diameter.[124] These organelles are bound by a single membrane and retain a highly stable homogeneous matrix in which crystalloids and/or globoids, inclusions containing nutrients for plant growth, may be embedded. Most peanut

allergens are members of these storage proteins, and this complex structure of the peanut seed likely contributes considerably to the major allergenicity of this legume. This will be described in more detail in the following section.

The peanut seed undergoes several changes during its developmental process. Upon anthesis (flowering), the pod, a simple fruit structure that originates from a carpel, grows quickly and develops a large and juicy shell wall or pericarp.[125] Initially, the pericarp occupies most of the fruit volume and serves as a temporary source of nutrients that are transferred to the nascent seed. As development progresses, the seed accumulates more nutrients and grows rapidly, eventually constituting most of the total pod volume at maturity, a process known as "pod filling".

Peanut growth and development were described by K.J. Boote in 1982 based on visually observable reproductive events related to flowering, pegging, fruit growth, seed growth, and maturity.[126] These events were divided into distinct stages and are described by physical description and time since planting in Table 1.2. Five distinct stages in peanut pod development were identified: R4 – pods with tiny embryos, R5 – seed growth, R6 – fully expanded but immature seeds, R7 – expanded and fully mature wet seeds, and R8 – mature dry seeds suitable for commercial use.[126] Storage proteins start to accumulate as early as the R4 stage, while later in seed development, oil and fats, along with other storage nutrients, accumulate.[127, 128] Consequently, during the early stages of seed maturation, when fats and fat globules are relatively scarce, the weight per volume concentration of peanut proteins, including major allergens, is relatively high. A given reproductive stage is reached when at least 50% of the plants in the sample have one or more flowers, pegs, pods, or seeds exhibiting the specified trait. Protein profiles and relative IgE binding to allergens of each developmental stage from R4 to R8 will be further discussed in Chapter 4.

Table 1.2. Peanut reproductive stages (Florunner variety) and days since planting as described by Boote (1982).[126]

Reproductive (R) stage	Physical description	Days since planting
R1	Beginning bloom	31
R2	Beginning peg	42
R3	Beginning pod	51
R4	Full pod	60
R5	Beginning seed	62
R6	Full seed	74
R7	Beginning maturity	93
R8	Harvest maturity	123
R9	Over-mature pod	129

1.3.2. Peanut allergens

As described in Section 1.1.4, allergic reactions to foods are triggered by IgE antibodies binding and cross-linking to food proteins. In peanut, there are 17 established allergens titled Ara h 1 through Ara h 18 (Ara h 4 renamed to Ara h 3.02, an isoform of Ara h 3), all of which are protein molecules defined by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee (Table 1.3).[129, 130] This committee is responsible for maintaining and developing a unique, unambiguous, and systematic nomenclature for all allergenic proteins, with the goal of defining a common language for scientists. An allergen is considered 'major' if over 50% of allergic individuals have serum IgE specific for that allergen. To date, the major allergens in peanuts are **Ara h 1** and **Ara h 3** of the cupin superfamily of proteins, as well as **Ara h 2** and **Ara h 6** of the prolamin superfamily.[131] It is estimated that over 97% of subjects allergic to peanuts have serum IgE specific for at least one of Ara h 1, Ara h 2, or Ara h 3.[132]

Table 1.3. List of peanut allergens Ara h 1 through 18 defined by the WHO/IUIS Allergen Nomenclature Sub-Committee.[129] Allergens in bold are discussed in further detail in this thesis.

Allergen	Biochemical name	Molecular Weight	Date Created
Ara h 1	Cupin (Vicillin-type, 7S globulin); formerly *conarachin*	64 kDa	24-06-2003
Ara h 2	Conglutin (2S albumin)	17 kDa	27-10-2003
Ara h 3	Cupin (Legumin-type, 11S globulin, Glycinin); formerly *arachin*	60 kDa, 37 kDa (fragment)	24-06-2003
Ara h 4 (Ara h 3.02)	renamed to Ara h 3.02 (Ara h 4 not available for future submissions)		24-06-2003
Ara h 5	Profilin	15 kDa	24-06-2003
Ara h 6	Conglutin (2S albumin)	15 kDa	24-06-2003
Ara h 7	Conglutin (2S albumin)	15 kDa	18-07-2008
Ara h 8	Pathogenesis-related protein, PR-10, Bet v 1 family member	17 kDa	25-04-2004
Ara h 9	Nonspecific lipid-transfer protein type 1	9.8 kDa	30-10-2007
Ara h 10	oleosin	16 kDa	11-07-2008
Ara h 11	oleosin	14 kDa	11-07-2008
Ara h 12	Defensin	8 kDa (reducing), 12 kDa (non-reducing), 5.184 kDa (mass)	03-07-2012
Ara h 13	Defensin	8 kDa (reducing), 11 kDa (non-reducing), 5.472 kDa (mass)	03-07-2012
Ara h 14	Oleosin	17.5 kDa	27-03-2015
Ara h 15	Oleosin	17 kDa	27-03-2015
Ara h 16	Non-specific lipid transfer protein type 2, nsLTP-2	8.5 kDa by SDS PAGE reducing	21-05-2015
Ara h 17	Non-specific lipid transfer protein type 1, nsLTP-1	11 kDa by SDS-PAGE reducing	21-05-2015
Ara h 18	Cyclophilin-peptidyl-prolyl cis-trans isomerase	21 kDa	11-13-2020

1.3.2.1. Ara h 1

Ara h 1 is a 63.5-kilodalton (kDa), vicilin-type 7S globulin and seed storage protein. As the second most abundant protein in peanuts at approximately 12 to 16% of total protein content,[133] Ara h 1 forms highly stable homotrimers mediated through hydrophobic interactions.[134] Natural Ara h 1 forms higher molecular weight aggregates in solution, while in contrast, the full-length recombinant protein is partially unfolded, existing as a monomer, as demonstrated by small angle

x-ray scattering experiments.[135] Upon boiling, Ara h 1 solubility is decreased via further aggregation, forming branched rod-shaped aggregates with loss of secondary structure, causing a decrease in its capacity to bind IgE, though this does not lead to a hypoallergenic peanut.[136, 137]

1.3.2.2. Ara h 3

The most abundant protein in peanuts at 71% of total protein is Ara h 3,[123] a legumin-type 11S globulin and seed storage protein. The crystal structure of Ara h 3 has been reported and has 21% sequence homology with Ara h 1.[135, 138] Ara h 3 can also function as a trypsin inhibitor.[139] The structure of Ara h 3 consists of a hexamer composed of two Ara h 1-like tetramers stacked one on top of the other.[131] Native Ara h 3 exists as a 60 kDa protein that is post-translationally modified by proteolytic cleavage into acidic and basic subunits, resulting in a common 37 kDa fragment upon SDS-PAGE analysis and rendering experiments evaluating the allergenicity of Ara h 3 with a recombinant, whole form of the protein less relevant than to its fragments.[140]

1.3.2.3. Ara h 2

Ara h 2 is a 2S albumin and member of the conglutin family of seed storage proteins containing 12 sequential IgE binding epitopes, three of which are immunodominant and recognized by IgE in over 90% of allergic individuals.[114] Two isoforms exist, Ara h 2.01 and Ara h 2.02, which measure 16.7 kDa and 18.1 kDa, respectively. Ara h 2.02 is characterized by a 12-amino acid insertion that contains a third repeat of the immunodominant IgE binding epitope, DPYSPS, resulting in Ara h 2.02 being a more potent cross-linker of peanut-specific IgE than Ara h 2.01.[141, 142] Sensitization to Ara h 2, along with Ara h 6, another 2S albumin measuring 15 kDa in size, are considered to be the best predictors of severe peanut allergy and anaphylaxis to peanut among all allergens, as demonstrated by correlating elevated specific IgE levels to these proteins with oral food challenge outcomes.[143-145] A recent publication emphasizes that although Ara h 6

shares approximately 60% sequence identity and multiple epitopes with Ara h 2, Ara h 2 remains the dominant peanut allergen.[146]

1.3.2.4. Ara h 8

Ara h 8 is part of the pathogenesis-related (PR)-10 group of proteins, the most prominent of which is the birch pollen protein Bet v 1. Allergic reactions to birch-pollen-related foods are triggered by the immunologic cross-reactivity of IgE antibodies with structurally homologous PR-10 proteins.[147] Cross-reactivity arises from the highly similar amino acid sequences and three-dimensional structures of these proteins, leading to analogous and overlapping epitopes recognized by IgE antibodies. This leads to individuals experiencing reactions to a wide range of plant food sources cross-reactive with Bet v 1 such as apple, hazelnut, peach, kiwi, and peanut. Allergic symptoms to Bet v 1 homologs in these foods primarily consist of oral allergy syndrome, characterized by itching and swelling of the mouth and throat.[148] Importantly, anaphylactic reactions to PR-10 proteins do occur, but are rare in birch-pollen-sensitized individuals.[149, 150]

Ara h 8 is a minor, heat-labile allergen whose crystal structure has been defined.[151] It is a unique peanut allergen in that it is quite low in abundance out of the total protein content in the peanut at less than 0.1 %,[152] but still garners considerable attention. This stems from the finding that subjects with IgE against Ara h 8 alone experience primarily only oral symptoms upon ingestion of peanut. These individuals have an extremely low probability of undergoing anaphylaxis following ingestion of peanut, in contrast to other peanut allergens.[153] This makes sensitization and allergy to exclusively Ara h 8 important to diagnose, which will be evaluated in more detail in Chapter 2.

1.3.2.5. Properties of peanut allergens

As previously noted in section 1.2.3, two major characteristics of a protein influence its ability to become an allergen: abundance and stability.[93, 94] Indeed, the majority of total protein content in peanuts is accounted for by a small number of allergens (Table 1.4). Koppelman et al. (2016) used reverse-phase high-pressure liquid chromatography (RP-HPLC) to quantify the relative amounts of major allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6 present in peanut extracts as a percentage of the total protein content.[123] They found Ara h 3 to be the most abundant protein in peanuts at approximately 70% (± 8.6%), making it a major allergen. However, Ara h 3 is not the allergen most closely linked to anaphylaxis; this title belongs to Ara h 2, a much less abundant peanut protein at approximately 6.2% (± 1.3%) of total peanut protein. The severe symptoms associated with specific IgE to Ara h 2 may be due to its high stability, provided by four disulfide bonds in its structure.[154] An exception to the abundance and stability rule of protein allergens is Ara h 8, known to be both heat labile (i.e., low stability) and much less abundant than other peanut allergens (<0.1%).[151, 152] Despite it not being abundant nor stable, Ara h 8 is still considered an allergen (although minor).

Table 1.4. Percentage of total protein content of individual peanut allergens. Adapted from Smits et al. (2018).[155]

Allergen	Proportion of Total Protein Content (% ± SD)
Ara h 1	17.1 (± 3.4)
Ara h 2	6.2 (± 1.3)
Ara h 3	70.6 (± 8.6)
Ara h 6	5.8 (± 1.8)
Ara h 7	0.5
Ara h 8	<0.1

As described in Section 1.3.1.3, most peanut allergens play a major role in seed storage, tightly bound into specialized circular organelles in the cell called protein bodies.[156, 157] These structures range from 5 to 10 μm in diameter and strengthen throughout seed germination.[158] This

complex peanut protein matrix may be largely responsible for the high allergenicity of the legume, resulting from elaborate structures difficult to naturally degrade, leading to immune systems recognizing them as foreign and harmful more frequently than most other foods.

1.3.3. Epidemiology & development of peanut allergy

Despite peanut's high nutritional value and abundance, for a small but increasing percentage of the population, allergy to peanuts prevents their consumption and requires constant vigilance to avoid any accidental exposure. Of all food allergies, those to peanuts are considered to be the most severe as it is a leading cause of fatal anaphylaxis and utilization of hospital emergency room resources.[159, 160] Peanut allergy (PA) is extremely common, affecting approximately 1.5% of children in North America, Australia, and the UK.[8, 161, 162] The prevalence of self-reported PA increased steadily from the 1990's to the 2010's, with rates of 0.4%, 0.8% and 1.4% reported in 1997, 2002, and 2008, respectively, via telephone surveys in the USA.[163, 164] Similar rates of increase and stability of PA have been reported in the UK as well.[165, 166] Interestingly, while the overall prevalence of food allergy in Asia is comparable to Western countries, reported rates of PA prevalence in Asia is considerably lower.[167] This is likely due to differential processing methods over the course of many generations, which will be discussed in more detail in Section 1.3.5.

The first reports in North America to corroborate patient clinical history with confirmatory tests for PA were published by Ben-Shoshan and Kagan et al. (2003 & 2009) in a 5-year follow-up study in primary school children in Montreal, Canada.[168, 169] Using questionnaires regarding past peanut ingestion, they found an increase in PA prevalence from 1.3% in 2002 to 1.6% in 2007.

Subsequently, publication in 2008 of the finding that early consumption of peanuts in infancy was associated with a low prevalence of PA, as well as the landmark LEAP study

published in 2015 (described in Section 1.1.3),[33, 34] together suggest it is possible that rates of PA incidence have declined in recent years. However, given the consistent increase in PA prevalence over the previous decades, there is still a generation of peanut-allergic individuals living at risk of dangerous allergic reactions with significant impacts on QoL. PA is a current problem that will not fade away soon, requiring substantial continued research.

1.3.4. Current treatments for peanut allergy

Today, most individuals with PA are not treated; rather, they strictly avoid peanut-containing foods and carry precautionary injected epinephrine to treat accidental ingestion. Recently, however, clinical trials in allergen immunotherapy have shown promising results by exposing allergic individuals to small, incremental doses of peanut with the goal of increasing the threshold that triggers a reaction.[170] Over extended periods of time, the immune system appears to be re-educated by allergenic proteins themselves as the active compound, and in most cases, this leads to some degree of tolerance to the culprit food. These therapies include ingesting the allergen through the mouth via oral immunotherapy (OIT),[171] leaving allergen tablets under the tongue via sublingual immunotherapy (SLIT),[172] keeping a patch on the skin slowly releasing allergen via epicutaneous immunotherapy (EPIT),[173] and injecting doses of modified allergen into the skin via subcutaneous immunotherapy (SCIT).[174] In addition to whole allergens being used to modulate the immune system, the use of peptides corresponding to epitope sequences has also been evaluated for inducing tolerance via peptide immunotherapy.[116, 175, 176] This thesis will discuss only OIT in further depth, correlating outcomes of OIT with baseline factors in Chapter 5.

1.3.1.4. Oral Immunotherapy

Oral immunotherapy (OIT) is a treatment for food allergy that consists of the regular consumption of the allergen, starting at very low doses that do not cause overt reactions, and increasing the dose slowly over time until a maintenance dose is achieved (Figure 1.6). OIT is the most common and effective form of immunotherapy for peanut allergies to date.[177] A recent major advance in OIT is Palforzia™, the first ever United States Food and Drug Administration (U.S. FDA)-approved treatment for peanut allergy launched in 2020. This treatment consists of standardized peanut proteins measured and placed into capsules, which are taken daily. Patients have regular follow-up visits with an allergy specialist to increase the dose of the medication up to a maximum of 300 mg per day.[178] However, despite the successes in OIT, caution is needed as subjects experience frequent adverse events, particularly with peanut and milk.[179, 180]

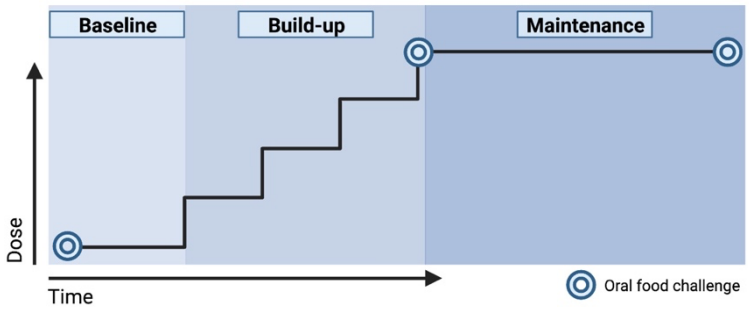

Figure 1.6. Schematic diagram of oral immunotherapy (OIT). Following confirmation of true food allergy via oral food challenge, allergic subjects ingest small, gradually increasing doses of the food they are allergic to over time until a maintenance dose is achieved.

1.3.4.2. Challenges with current PA treatments

Current peanut OIT protocols may lead to complete tolerance or the ability to eat all forms and amounts of peanuts securely in some cases. Although peanut OIT has improved the margin of safety for some individuals, treatments with consistent small doses to maintain this level of protection pose a significant risk of allergic reactions during therapy, including anaphylaxis.[179] Thus, the debate is ongoing over whether providing this therapy outside the context of well-controlled trials is more harmful than beneficial.[178, 180]

After almost two decades of OIT trials for food around the world, there is now a wealth of data in which researchers have analyzed to help predict OIT outcomes. Baseline specific IgE (sIgE) values for peanut or egg have previously shown to be useful in predicting the safety of OIT in allergic children.[181, 182] Correspondingly, high cow's milk-sIgE levels have been previously associated with the persistence of cow's milk allergy.[183, 184] More specifically, elevated levels of sIgE antibodies for casein, the major cow's milk allergen accounting for approximately 80% of total protein in milk, have been reported to be strongly associated with milk allergy in children and are useful in predicting reactivity to both unprocessed and baked milk.[185, 186] This is likely due to casein being a more stable allergen, retaining its conformation following thermal processing more than the whey proteins α-lactalbumin (ALA) and β-lactoglobulin (BLG). Whey proteins are less stable during heating, resulting in more denaturation and less allergic reactions to heated milk in children sensitized to ALA or BLG,[187, 188] as described in the following section. Chapter 5 will discuss an analysis that we performed on serum samples from a cohort of patients from centres across Canada correlating cow's milk-specific IgE levels and other baseline factors with outcomes of OIT.

1.3.4.3. Insights from egg & cow's milk allergies

The rate of spontaneous resolution for peanut is considerably lower than for some of the other food allergies, specifically hen's egg and cow's milk. Indeed, children with egg or milk allergy can frequently introduce small amounts of well-cooked egg or milk into their diets safely as they age.[187, 189] Normal cooking processes denature or linearize egg and milk proteins, which may explain their decreased allergenicity.[190, 191] In association with egg- and milk-allergic patients being able to increase the cooked form of the allergen into their diets, a significant number evolve to complete tolerance.[192-194] Prolonged, frequent exposure to cooked forms of egg or milk allergens

may act as a form of OIT, albeit with more safety than conventional OIT, which often uses uncooked foods as a substrate.

Peanut and its proteins, on the other hand, do not appear to denature under normal cooking conditions, such as roasting. Structural biology analyses have attributed the resistance of peanut denaturation at high temperatures to the three-dimensional structure of the major peanut allergens. Recent reports have thoroughly described the X-ray structures of common allergens[135, 151, 154] and observed that they are rich in disulfide bridges, providing an additional barrier to degradation.[195] Given what we have learned in the case of egg and cow's milk allergies, an alternative, non-traditional processing method may be able to disrupt the peanut protein matrix in a unique way, providing a novel, safer, and potentially more efficacious substrate for peanut OIT.

1.3.5. Thermal processing of peanut

Peanuts are processed in different ways prior to consumption throughout the globe. In North America and Western Europe, peanuts are commonly dry roasted, particularly for the preparation of peanut butter. In Asia, it is more common to boil peanuts in briny, salty water. While the overall prevalence of food allergy in Asia is comparable to Western countries, reported peanut allergy prevalence in Asia is considerably lower.[167] The difference in peanut processing is believed to play a role in explaining such large discrepancies in peanut allergy incidence across different regions around the world. The following sections will discuss a variety of processing methods, some conventional and some not, and the reported findings regarding their effects on peanut allergenicity.

1.3.5.1. Roasting & the Maillard reaction

Roasting is a cooking method that uses dry heat at high temperatures where hot air evenly covers the food. This is achieved using an oven, an open flame, or another heat source. Evidence

suggests changes in the allergenic properties of the peanut following dry roasting; glycation at high temperatures is proposed to be a mechanism of enhancement of allergenic responses to peanut, primarily resulting from the *Maillard reaction*.[196] Named after French chemist Louis-Camille Maillard in 1912, the Maillard reaction is a form of non-enzymatic browning that occurs at temperatures above 140°C. This reaction is responsible for the distinctive flavour and aroma of many foods such as toasted bread, coffee, maple syrup, or beer.

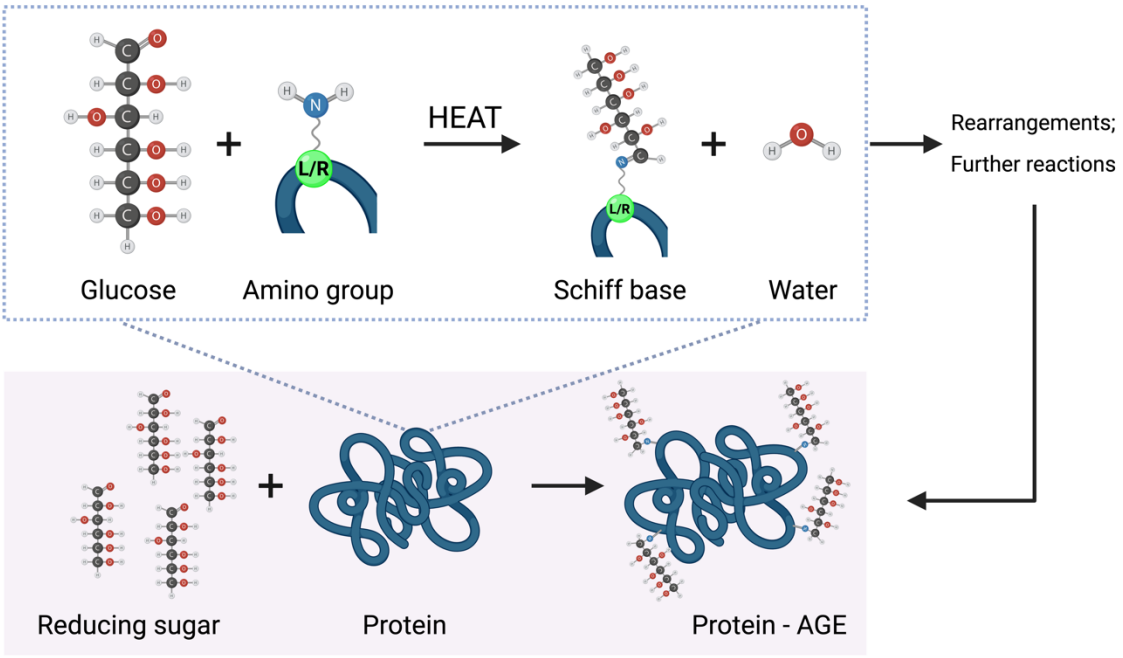

Figure 1.7. Schematic of the Maillard reaction and protein glycation. The carbonyl group of a reducing sugar, such as glucose, reacts with the amino group of a protein, peptide, or free amino acid (lysine (L) or arginine (R) residues) in the presence of heat. This reaction releases water and forms a Schiff base, which then undergoes a series of rearrangements and further reactions, resulting in a protein-Advanced Glycation End product (AGE) complex.

The Maillard reaction is a chemical reaction between the reactive carbonyl group of reducing sugars, such as glucose, and the nucleophilic amino group of amino acids, either free or as part of an intact protein or peptide. This leads to the release of water and the production of Schiff bases that rearrange to form a wide range of products, of which advanced glycation end-products (AGE) are said to play a role in enhancing of allergenicity (Figure 1.7).[197-200] The most relevant

amino acids to the Maillard reaction are lysine and arginine due to the terminal amino groups on their side chains.

Even though the effect of thermal processing, particularly roasting, on peanut allergens and allergenicity has been widely investigated, the specific methods used to process peanuts vary considerably across publications, making comparison of the studies tenuous at best. For example, Maleki et al. (2000) found a 90-fold increase of IgE-binding in roasted peanuts when compared to raw; their "roasted" peanut samples were composed of purified peanut proteins or whole peanut extracts heated in solution.[200] In contrast, Zhang et al. (2016) roasted peanut kernels at 170°C for 15 minutes and found similar levels of aggregation and protein secondary structure of major peanut allergen Ara h 2 when compared to raw.[201] Moreover, Rao et al. (2016) roasted peanuts in an oven at temperatures ranging from 105°C to 150°C and found roasting over 130°C resulted in reduced IgE binding capacity of Ara h 1 and Ara h 3, but increased Ara h 2 and Ara h 6 IgE binding capacity.[202] Given that peanut proteins are embedded in a complex structural matrix, **processing whole kernels, the way peanuts are traditionally consumed, is essential to faithfully represent roasted peanut for analysis rather than using methods such as heating proteins in solution.**

Importantly, evidence of IgE antibodies specific for the glycated forms of proteins or glycated structures is lacking, and the debate is ongoing over whether the Maillard reaction plays a significant role in food allergy.[203-205] Although the molecular composition of the peanut is now well known (i.e. proteins, amino acids, metal ions, and sugar content),[206-208] **the specific contribution of free sugars and amino acids to the allergenicity of peanuts at high temperatures has yet to be defined and studies designed to identify process-dependent molecular signatures are lacking**.[209]

1.3.5.2. Boiling

Previous studies suggest a decrease in IgE-binding in boiled peanuts when compared with raw, as a result of the transfer of low-molecular-weight proteins from the peanuts into the cooking water, particularly the 2S albumins Ara h 2, Ara h 6, and Ara h 7.[202, 210] Moreover, Comstock et al. (2016) found that the combination of boiling peanuts in water for 5 minutes followed by pan frying in oil for 130 seconds led to decreased Ara h 1 and Ara h 2, but did not eliminate IgE binding.[137] Blanc et al. (2011) purified Ara h 1 from raw peanuts then heated it in solution at 100°C and found that Ara h 1 aggregates from this form of boiling had lower allergenic activity than from roasting.[136]

Given these promising results, recent clinical studies have evaluated the tolerance of boiled peanuts in allergic individuals. It was found that, as shown by immunoblot experiments to both boiled peanut and the resulting cooking water, the loss of allergenic 2S albumins throughout boiling results in tolerance to boiled peanut.[211] These studies have resulted in registered clinical trials using boiled peanut as a potentially safer substrate in the treatment of peanut allergy via oral immunotherapy.[212, 213] Grzeskowiak et al. (2023) recently published results from their open-label, single-arm clinical trial designed to test the effectiveness of boiled peanut OIT prior to switching over to roasted peanut in 70 participants and found that desensitization was successfully induced in 80% of participants, with treatment-related adverse events reported in 61% of participants at a rate of 6.58 per 1000 OIT doses.[214] Although these results are promising, the effect of boiling on peanut proteins may not be uniform throughout the whole seed, and it can be challenging to establish consistent boiling protocols. Thus, alternative, reproducible methods of reducing peanut allergenicity would be beneficial, either via processing or some other strategy.

1.3.5.3. Frying

The cooking of food in hot oil, either shallowly in a pan or immersed completely in a deeper vessel is defined as *frying*. Peanuts are fried in a variety of regions around the world. Fried peanuts have not been evaluated as much as roasted or boiled peanuts in the context of allergenicity. It is believed that, as in the case of boiling, low molecular weight proteins leach out into the cooking oil throughout frying, reducing the potency, and thus allergenicity of peanuts.[137] Beyer et al. (2001) evaluated peanuts fried in vegetable oil for 5 or 10 minutes compared to roasted or boiled peanuts and found that both frying and boiling result in a similar reduction of IgE binding intensity, particularly to Ara h 1, Ara h 2, and Ara h 3 when compared to roasted peanuts as observed via immunoblot experiments using serum samples from allergic patients.[215] Furthermore, Zhang et al. (2016) observed that frying and boiling peanuts induced the down-regulation of Ara h 2 allergenicity in Balb/c mice, resulting in the collapse of the protein's tertiary and secondary structures, and a reduction in the core epitope binding capacity when compared with raw or roasted peanuts.[201]

1.3.5.4. Autoclaving

As demonstrated with boiling and frying, moisture during thermal processing plays a significant role in altering the allergen content in peanuts.[202] While moisture is a major component of boiling in water, allowing proteins and allergens to leech into solution, autoclaving is a processing method that uses both elevated temperature and vapour pressure, leaving no route for matter to escape. Cabanillas et al. (2012) reported the first study evaluating the effect of autoclaving on peanut allergens, a method commonly used to sterilize lab equipment. They found that autoclaving roasted peanuts at a pressure of 2.56 atm for 30 minutes produced significant decreases in IgE-binding capacity of peanut allergens as determined via ELISA using individual

patient sera and decreases in skin prick test (SPT) measurements.[216] They also observed the unfolding of peanut proteins and the reduction in overall secondary structure using circular dichroism, likely affecting epitope regions crucial for IgE binding, which may explain the observed reduction in binding to autoclaved peanut extracts. More recently, the same group evaluated the effect of high temperature and pressure on raw, roasted, and fried peanut samples and once again found a decrease in IgE binding properties by means of immunoblot and ELISA inhibition assay, along with an altered capacity to activate basophils sensitized with sera containing IgE from allergic patients.[217] It is important to note the small sample size of 7 peanut-allergic patients used in their SPT study and the description that these subjects had very low specific IgE levels to peanut. Therefore, further work is required to assess IgE binding to autoclaved peanuts in allergic individuals with high peanut-specific IgE levels.

1.3.5.5. Other processing methods

A wide range of unconventional processing methods have been evaluated in the context of peanut allergenicity in recent years.[218] High-moisture extrusion resulted in large reductions in total major allergen content (range: 55%-91%) for allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6, including rearrangements of protein structure, degradation, and aggregation of proteins.[219] Additionally, treatment of raw peanuts with digestive enzymes alcalase, papain, and cellulase reduced Ara h 1, 2, 3, and 6 in raw peanuts significantly, while roasting of peanuts treated with the same enzymes significantly reduced the total protein solubility and enhanced IgE binding slightly, but still much lower than untreated, raw peanuts.[220] Such alternative processing methods may reduce peanut allergenicity by decreasing levels of intact protein, while keeping the flesh of the seed intact.

1.3.5.6. Processing of other food allergens

Thermal processing has also been evaluated on other food allergens and further sheds light on its effect on allergenicity. Heat and pressure treatments via autoclaving of cashews and pistachios, as seen with peanuts, diminished IgE-binding via ELISA and reduced the SPT wheal sizes to their proteins.[221] Moreover, autoclave treatment at 256 kPa at 138°C of walnuts was able to diminish the IgE cross-linking capacity on rat basophil cells more efficiently than high hydrostatic pressure treated walnuts, a method of preserving and sterilizing food using very high pressure, but not high temperature.[222]

The effects of heating (65 to 95°C) and glycation via incubation with each of 6 different sugars in water at 60°C on cow's milk allergen β-lactoglobulin (BLG) were evaluated.[223] Under these conditions, BLG was denatured, as demonstrated by the loss of its secondary and tertiary structures, and this was associated with weaker binding of IgE, measured by competitive ELISA using a pooled serum from 14 cow's milk-allergic patients. High degrees of glycation were found to have a "masking" effect on BLG's recognition by IgE.

1.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

The chemical composition of peanuts consists primarily of lipids, proteins, and carbohydrates as well as small molecules such as amino acids, reducing and non-reducing sugars. At high temperatures, a myriad of chemical reactions occur, leading to a complex, multifarious mixture of protein products. Accordingly, monitoring changes in molecular profiles of intact seeds or soaked extracts from peanuts requires a technique that can capture the signature of both small molecules and proteins under the different processing conditions. Nuclear magnetic resonance (NMR) spectroscopy is a technique that provides detailed, characteristic information about the

structure, dynamics, and chemical environment of molecules. Thus, we chose NMR spectroscopy to obtain molecular distribution profiles reflecting the various peanut components across conditions.

The physical phenomenon of NMR is based on the principle that in the presence of a strong magnetic field, atomic nuclei will resonate at different frequencies when perturbed by a second, weak oscillating magnetic field. NMR has evolved into an extremely powerful technology, providing detailed information about the identity, structure, and connectivity of molecules.

1.4.1. The principle of NMR

The phenomenon of NMR was discovered in 1937 by a scientist named Isidor Rabi who described how nuclei could be induced to flip their magnetic orientation by applying an external, oscillating magnetic field.[224] One year later, Rabi performed an experiment where he passed a molecular beam of lithium chloride molecules in a vacuum through a hairpin coil producing a constant radiofrequency of 3.5 MHz surrounded by an electromagnet of strength 0.2 Tesla (T), producing an oscillating magnetic field.[225] He noticed energy absorption and resonance peaks for both lithium and chloride and then coined the term "nuclear magnetic resonance" for this phenomenon.

The principle of NMR has evolved considerably over the past century and has led to two major uses: magnetic resonance imaging (MRI), using the spin relaxations of hydrogen atoms to obtain images of the human body, and NMR spectroscopy. NMR spectroscopy is a powerful analytical technique that takes advantage of the principles of NMR to obtain a characteristic signature of a given sample. The most common nuclei used are hydrogen (^{1}H) and carbon (^{13}C), but other nuclei such as fluorine (^{19}F), phosphorus (^{31}P), and sulfur (^{33}S) are used as well.[226]

1.4.2. The technique of NMR spectroscopy

All matter is composed of atoms, each of which contains a nucleus. Nuclei with an odd number of protons or neutrons have a non-zero net spin, an intrinsic property of angular momentum, and can exhibit the phenomenon of nuclear magnetic resonance.[227] Under an applied magnetic field, the spin of every such nucleus aligns in one of the two possible orientations: either in a low-energy state parallel to the field or in a high-energy state anti-parallel to the field. Nuclei can transition, or *resonate*, from one state to the other by absorbing (low to high energy) or emitting (high to low energy) a photon equal in energy to the energy difference between the two states.[227] This is where the term *Nuclear Magnetic Resonance*, comes from: nuclei resonate between spin states in the magnetic field. The signal measured results from this energy difference between both states and is proportional to the ratio of the number of nuclei in each spin state. NMR spectroscopy can measure this extremely small population difference and, as such, is a very sensitive technique.

The magnetic resonance frequencies in NMR generally correspond to the radio frequency portion of the electromagnetic spectrum. The energy difference between nuclear spin states and the resulting resonant frequencies are dependent and proportional to the strength of the external magnetic field. High costs of NMR spectrometers (>$10 million) are due to the expensive costs of the powerful magnets required to increase the applied field, however this results in higher resolution spectra and provides greater structural information.

1.4.2.1.Shielding & chemical shift

The frequency at which nuclei resonate is also dependent on the molecular environment.[228] Depending on the proximity of other nuclei, the time it takes for a nucleus to return to its original state will vary, leading to very specific spectral peaks corresponding to each nucleus in a given sample. Nearby electrons carrying a spin create their own small magnetic fields, which shift the

resonant frequency of a given nucleus if in close enough proximity.[229] Electron density around a nucleus will alter its resonance frequency slightly, a phenomenon called *shielding*.

In NMR spectroscopy, we measure how weak this induced field is, or how low in frequency the nucleus resonates at, using a property called the *chemical shift*.[230] Given by δ and measured in parts per million (ppm), the chemical shift enables very accurate measurements of the magnetism of electrons and quantifies the degree to which a given nucleus is shielded. Tetramethylsilane (TMS) is often used as a reference point, assigned a value of δ = 0.0 ppm. TMS is composed of an electron-donating silicon atom conjugated with four methyl groups, greatly shielding the hydrogen and carbon nuclei of this molecule (i.e., high electron density around them), making it a useful reference. For biological samples, which are generally composed of polar molecules, trimethylsilyl-propanoic acid (TSP) is often used instead of TMS due to TSP's solubility in water.

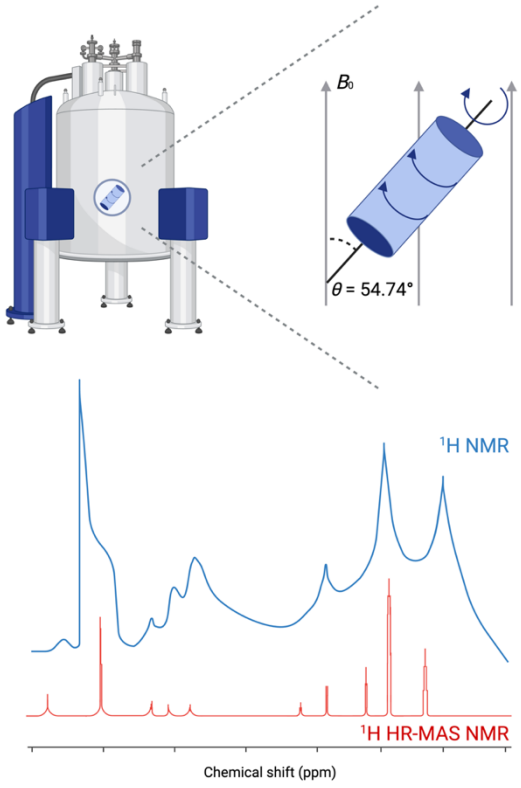

Figure 1.8. Schematic of HR-MAS NMR spectroscopy. By spinning a semi-solid sample at the specific, or magic, angle of 54.74° with respect to the external magnetic field (B0), we minimize line-broadening effects and obtain high-resolution spectra. HR-MAS, High-Resolution Magic Angle Spinning; NMR, Nuclear Magnetic Resonance.

1.4.3. High-resolution magic angle spinning (HR-MAS) NMR

Traditionally, NMR spectroscopy is performed on samples **in solution**. In liquids, the free motion of molecules allows an averaging of line-broadening magnetic interactions such as dipole-dipole interactions, a type of intermolecular attraction between two molecules. **In solid samples**, however, these

interactions cannot be averaged; the spins of the nuclei are no longer able to rotate and tumble freely in the tissue, resulting in inhomogeneity in the applied magnetic field and hence lower-resolution signals with broad peaks. This inhomogeneity arises due to sample heterogeneity, dipolar couplings, and chemical shift anisotropy (CSA) related to varying magnetic shielding and orientation of molecules relative to the external magnetic field.[231] As a result, we obtain very broad signals with little information.

However, recent advances in solid state NMR have led to the development of a particular NMR technique titled High Resolution Magic Angle Spinning (HR-MAS). Magic angle spinning with high power proton decoupling averages out all anisotropic NMR interactions, such as CSA and residual dipolar coupling.[232] At the particular orientation when the CSA is minimized, i.e., when $3\cos^2\theta - 1 = 0$, line-broadening effects are virtually eliminated. This occurs at the specific, or *magic*, angle of $\theta = 54.74°$ between the dipole moment of the molecule and the applied magnetic field (i.e., vertical axis; Figure 1.8). By spinning the sample at a high frequency to homogenize it relative to the detector, we minimize the CSA and maximize resolution, resulting in very clear, sharp peaks in the spectra.[232] **This technique has been proven to be very useful to study biological tissues**, which are often heterogeneous samples composed of both solid and liquid (i.e., *semi-solids*), greatly increasing its popularity in medical and biological research in recent decades.[233, 234]

1.4.4. NMR spectroscopy of foods and plants

Whole intact plants and their seeds have been metabolically profiled *in vivo* using HR-MAS NMR spectroscopy.[235, 236] The non-destructive nature of the technique makes it useful for studying a range of properties throughout the plant life cycle such as early growth, seed germination, or dormancy breakage.[234]

Solution ^{1}H NMR spectroscopy has also been used to compare metabolite levels of peanut allergic and peanut tolerant patients before and after a peanut food challenge by measuring plasma and saliva samples of each group.[237] Clear differences were observed between their NMR spectra, where allergic patients demonstrated different metabolic profiles even before peanut consumption, i.e., before the onset of any allergic reaction. This study suggests that peanut allergic patients have distinct metabolite levels, and that this technique can serve as a means to discover new biomarkers for food allergy.

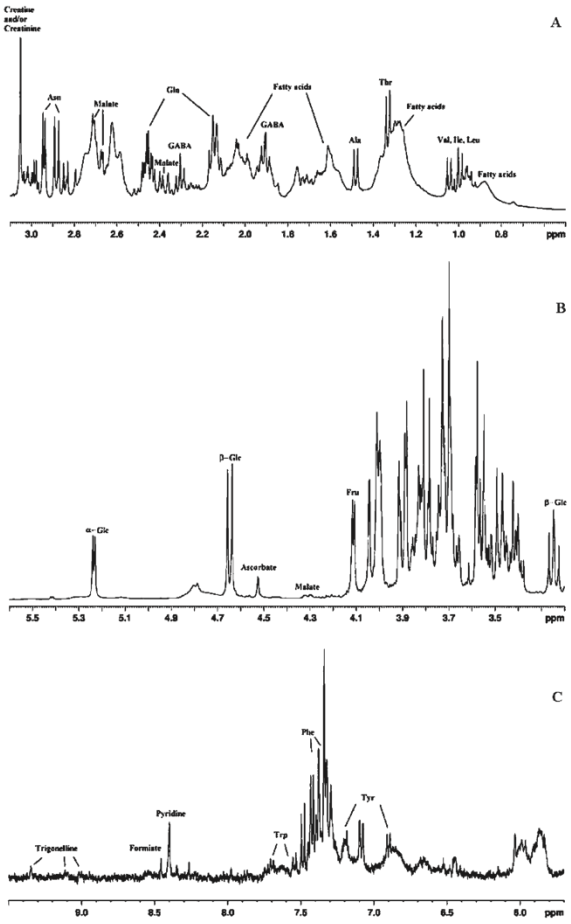

Figure 1.9. ^{1}H HR-MAS NMR spectrum of sweet pepper. (A) high-field region; (B) middle field region; (C) low field region.
Reprinted with permission from Ritota M, Marini F, Sequi P, Valentini M. Metabolomic characterization of Italian sweet pepper (Capsicum annum L.) by means of HRMAS-NMR spectroscopy and multivariate analysis. *J Agric Food Chem* 2010;**58**(17):9675-84. Copyright 2023, American Chemical Society.

Ritota et al. (2010) performed HR-MAS NMR on freeze-dried Italian sweet pepper to obtain a metabolic characterization of the vegetable.[238] This report provided a very thorough analysis of the ^{1}H NMR spectrum of sweet pepper and assigned chemical shifts and peaks to various amino acids, organic acids, fatty acids, carbohydrates and other metabolites (Figure 1.9). In the upfield region (A), from 0.0 to 3.0 ppm, there are signals present pertaining to fatty acids, organic acids and aliphatic side chains of amino acids. These molecules are high in CH_2 and CH_3 groups with few electronegative atoms nearby to draw electron density away (e.g., oxygen or nitrogen), resulting in highly shielded nuclei. In the middle field

region (B), from 3.0 to 5.5 ppm, there are strong, sharp peaks arising from sugars such as glucose and sucrose, suggesting a high concentration of these molecules in the vegetable. In the downfield region (C), from 5.5 to 10.0 ppm, the signals correspond to aromatic and phenolic side chains of amino acids, and to those nuclei in the vicinity of very electronegative atoms such as oxygen, resulting in the most de-shielded nuclei. These signals are weaker, suggesting a lower concentration of these molecules. We can see from this report that unique information on molecular structure and connectivity can be gathered from simple NMR experiments.

Chapter 3 describes the analysis of peanuts under raw, roasted, and autoclaving conditions using a complementary ^{1}H NMR spectroscopy approach, using HR-MAS to analyze the intact seed and defatted flour as well as solution NMR to evaluate peanut-soaked solutions.

1.5. Hypothesis

The overarching hypothesis for this project was that a modified peanut, via thermal processing or by using an immature form of the seed, may result in lower total peanut- and component-specific IgE binding, thereby reducing peanut allergenicity. This hypothesis was verified by evaluating and studying the composition of modified peanuts and their mechanisms of action in the context of allergenicity.

1.6. Objectives

It is evident that novel strategies for better understanding the structure of peanut proteins to advance the treatment of peanut allergy are needed. The goal of this research project was **to evaluate potential methods to decrease peanut allergenicity**. Particularly, our approach was to characterize the structure of the peanut and its proteins in order to **obtain structure-function correlations with allergenicity**.

1. Evaluate high-pressure and temperature autoclaving as a novel thermal processing method and its effect on peanut allergenicity.
2. Develop a means to monitor protein degradation as an indirect signature for allergenicity via Nuclear Magnetic Resonance (NMR) spectroscopy.
3. Evaluate the protein allergen content of peanut seeds harvested at a range of developmental stages and their effects on peanut allergenicity.
4. Correlate allergen-specific IgE levels at baseline with outcomes of oral immunotherapy.

BRIDGING TEXT 1

The previous chapter (Chapter 1) presented an introduction to food allergy with a focus on allergy to peanut and its proteins. The literature review described the peanut, *Arachis hypogaea*, its composition, and the role its protein allergens play in triggering allergic reactions. Different approaches towards reducing allergenicity and treating peanut allergy were discussed, such as peanut oral immunotherapy (OIT). Processing of the peanut prior to consumption, i.e., modifying the peanut and its components in some way, has shown to have promise at reducing allergenicity by decreasing the frequency and severity of allergic reactions.

The following chapter, Chapter 2, will discuss the first novel approach evaluated in this dissertation: high-pressure and temperature autoclaving. Although this thermal process has marginally been assessed in the context of peanut allergy over the past decade leading to few original publications, this work is the first to evaluate a wide range of autoclaving parameters, particularly temperatures and time durations, and their effect on peanut allergens, IgE binding, and thus, peanut allergenicity.

CHAPTER 2: *High-pressure and temperature autoclaving hydrolyzes peanut proteins into peptides and reduces peanut specific-IgE binding*

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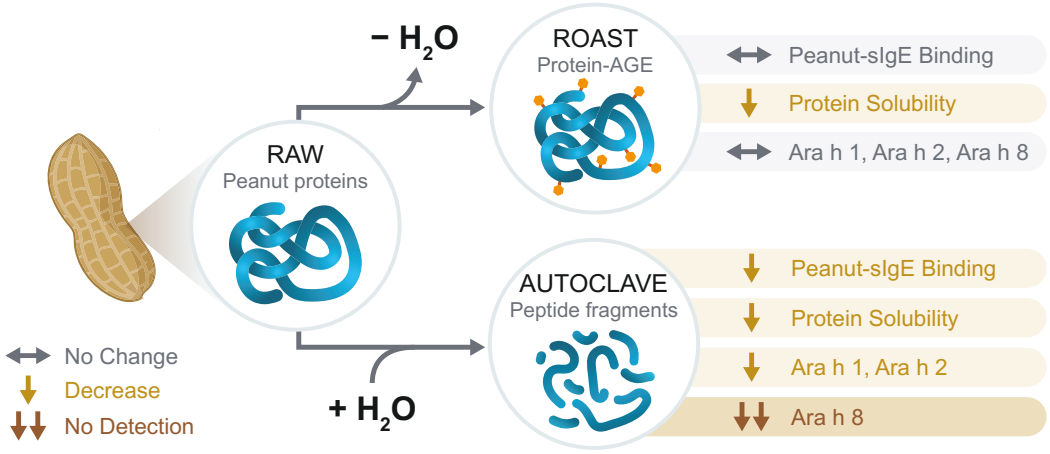
Graphical abstract:



2.1. Abstract

Major peanut protein allergens are not believed to denature under traditional cooking conditions such as roasting. In this study, we evaluated the effects of different high-pressure and temperature autoclaving parameters on peanut proteins with the goal of reducing allergenicity. Raw, roasted, and autoclaved peanuts were evaluated via Western blot and ELISA to detect relative levels of total protein, peanut-specific IgE binding, and specific peanut allergens Ara h 1 and Ara h 2, associated with a risk of anaphylaxis, and Ara h 8, analogous to birch pollen protein, and associated with oral allergy symptoms. Results showed that autoclaving at 130°C for 30 minutes significantly degraded allergens Ara h 1 and Ara h 2, leaving low but detectable levels, and thoroughly degraded Ara h 8 to the point of no detection. Mass spectrometry followed by proteomics analyses of filtered extracts (<10 kilodaltons) showed a greater number and diversity of peptides corresponding to peanut proteins, including major allergens, in the autoclaved extract when compared to raw or roasted. Our findings demonstrate that high-pressure and temperature autoclaving cleaves peanut proteins into short peptides, against which highly peanut-allergic patient sera exhibit a 74% reduction in IgE binding compared to raw. Altogether, autoclaving may reduce allergenicity and create a less potent and potentially safer peanut.

Keywords: Food processing, Peanut allergy, Autoclave, Protein degradation, IgE binding

2.2. Introduction

The common peanut, *Arachis hypogaea*, is an important legume crop worldwide, serving as an affordable source of protein, unsaturated fatty acids, and several vitamins and minerals (Bonku & Yu, 2020). However, for a small, but increasing, percentage of the population in Western societies, allergy to peanuts prevents their consumption and requires constant vigilance to avoid

any accidental exposure. As a leading cause of fatal anaphylaxis, a potentially life-threatening allergic reaction, peanut allergy is often considered the most severe of all food allergies, affecting over 1.5% of children and adults in North America, Australia, and the UK (Ben-Shoshan et al., 2012; Clarke et al., 2020; Poirot et al., 2020; Warren et al., 2021). The allergic reaction to peanut is characterized by immunoglobulin E (IgE) antibodies binding and cross-linking to peanut proteins, which triggers a cascade of symptoms such as hives, swelling, difficulty breathing, and anaphylaxis, which can lead to death (Poirot et al., 2020).

Peanuts are differentially processed prior to consumption throughout the globe (Shah et al., 2019). In North America and Western Europe, peanuts are commonly dry roasted, particularly in the preparation of peanut butter, while in Asia it is common to boil peanuts in briny water. Peanut proteins do not appear to denature under typical cooking conditions. Structural biology analyses have attributed the resistance of peanut denaturation at high temperatures to the three-dimensional structure of the peanut allergens, Ara h 1 to Ara h 18. Recent reports have described their X-ray structures (Chruszcz et al., 2011; Hurlburt et al., 2013; Mueller et al., 2011) and observed that the major peanut allergens are rich in disulfide bridges (Koppelman et al., 2010). Ara h 2 is a member of the 2S albumin protein group, part of the prolamin super-family of proteins, which share a common fold consisting of α-helices bundled together by 4 or 5 conserved disulfide bonds, explaining its high resistance to heat treatment and degradation (Breiteneder & Mills, 2005; Shewry et al., 2002). Additionally, specific IgE binding to Ara h 2 has been suggested as the best predictor for clinical peanut allergy (Agabriel et al., 2014; Kukkonen et al., 2015).

Nevertheless, evidence suggests a change in the allergenic properties of the peanut following thermal processing (Zhou et al., 2021). Glycation via roasting at high temperatures is a proposed mechanism of enhancement of sensitization to peanut primarily resulting from the

Maillard reaction, an addition of amines on reducing sugars to provide Schiff bases that rearrange to form a range of products, of which the advanced glycation end-products (AGE) are believed to play a role in the enhancement of allergenicity (Guillon et al., 2016; Maleki et al., 2000; Mueller et al., 2013; Vissers et al., 2011). However, evidence of IgE antibodies specific for the glycated forms of proteins or glycated structures is lacking, and the debate is ongoing over whether the Maillard reaction plays a significant role in food allergy (Gupta et al., 2018; Toda et al., 2014; Toda et al., 2019).

Previous studies demonstrated a decrease in IgE binding in boiled and fried peanuts when compared with raw, and that boiling results in the transfer of low-molecular-weight proteins from the peanuts into the cooking water, particularly the 2S albumins Ara h 2, Ara h 6, and Ara h 7 (Comstock et al., 2016; Mondoulet et al., 2005). Allergic individuals have demonstrated a greater tolerance to boiled peanut when compared to raw or roasted, and trials are ongoing evaluating its desensitization potential (Grzeskowiak et al., 2023; Turner et al., 2014). Furthermore, Cabanillas et al. (2015 & 2012) reported the effect of autoclaving on peanut allergens and found that autoclaving raw, roasted, and fried peanuts at a pressure of 2.56 atm for 30 minutes produces a significant decrease in IgE binding capacity of peanut allergens and in allergy skin prick test wheal size, accompanied by unfolding of proteins and reduction in overall secondary structure (Cabanillas et al., 2015; Cabanillas et al., 2012). Other processes such as enzymatic hydrolysis or high-moisture extrusion have also shown promise in degrading peanut allergens (Faisal et al., 2022; Mikiashvili & Yu, 2018). These alternative processing methods may be serving as means of reducing peanut allergenicity by decreasing protein levels while keeping the flesh of the seed intact.

Since the substantial findings of Cabanillas et al. (2015) on the effect of thermal processing on peanuts, further research into the topic has been scarce. We hypothesize that high-pressure and temperature autoclaving reduces the proportion of intact allergens, thereby reducing peanut-specific IgE binding and allergenicity. Here, we evaluate the effects of two thermal processing methods – roasting and a range of autoclaving conditions – on peanut proteins, allergens, and specific IgE binding to a serum pool acquired from highly allergic patients.

2.3. Materials and methods

2.3.1. Sample preparation

2.3.1.1. Physical processing

Raw, shelled peanuts were commercially purchased (Marché Victoria Orientale, Montreal, Canada). Peanuts were roasted in a convection oven at 150°C for 30 minutes or autoclaved in a tabletop autoclave (VWR AS12 AccuSterilizer, Radnor, PA) at temperatures ranging from 100°C-130°C (pressures ranging from 1 to 2.5 atm) for 30 minutes. The optimal time duration was then determined by autoclaving at 130°C for 5 minutes, 15 min, 30 min, 45 min, or 60 min. Analyses were performed in triplicate in comparison with raw, unprocessed peanut.

2.3.1.2. Defatting into flour

Twelve peanuts of each processing condition were ground into a paste using a coffee grinder (Hamilton Beach/Proctor-Silex, Belleville, Canada). The paste was then suspended in 40 mL of n-hexane for 2 rounds of 5 minutes each, passing the solution through a vacuum filter between rounds. The resulting defatted peanut flour was collected by filtration under vacuum.

2.3.2. Preparation of protein extracts

Peanut flours were processed into whole protein extracts by dissolving 50 mg of flour in 1.5 mL of 20 mM Tris Buffer (pH 8.5) or 7 M urea (pH 7.4). Samples were vortexed for 30 seconds

and were then left rotating overnight at 4°C. Following 3 rounds of centrifugation for 5 minutes at 12,600 g, the supernatant was collected as the protein extract. Extract concentrations were determined by Bradford assay using known concentrations of bovine serum albumin (BSA; Sigma-Aldrich, Oakville, Canada) to construct a standard curve, and by using the NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, Canada) via quantification of absorbance at 280 nm (Protein A280 method).

2.3.3. Lyophilization and comparison of masses of extraction fractions

Equal masses (700 mg) of defatted raw, roasted, and autoclaved (130°C, 30 minutes) peanut flours were lyophilized for 2 hours to remove any water. Masses were recorded after lyophilizing and were then dissolved in 7 mL of extraction buffer (20 mM Tris, pH 8.5) overnight. Following centrifugation, supernatants and pellets (i.e., soluble extracts and insoluble peanut mass, respectively) were reconstituted in 2 mL of distilled water, frozen at -80°C for 30 minutes, and then lyophilized overnight. The resulting dried solids were weighed and compared across conditions. An equal volume of buffer only was used as a control to quantify the contribution of salts to total mass.

2.3.4. Mass spectrometry (MS) and proteomics analyses

Sample preparation (LC-MSMS)

Whole protein extracts (1.5 mL) created from raw, roasted, and autoclaved peanuts as described above were passed through 2-mL Amicon Ultra-2 centrifugal filters with a 10 kDa cut-off (UFC200324, Millipore Sigma, Canada) by spinning at 3,000 g for 15 minutes. Filtrates were collected and stored at -20 C until used. 5 µL of peanut extract protein filtrate was mixed with 5 µL proteomics grade Trypsin (Promega Gold) diluted to a concentration of 12 ng/µL. The digestion was performed over night at room temperature.

LC-MSMS and bioinformatics

High performance liquid chromatography (HPLC) was conducted on 50% of the protein digest using a 2 cm pre-column (Acclaim PepMap 50 mm × 100 μm inner diameter (ID)), and 25 cm analytical column (Acclaim PepMap, 500 mm × 75 μm diameter; C18; 2 μm; 100 Å, Thermo Fisher Scientific), running a 120 min reversed-phase buffer gradient at 350 nl/min on a Thermo EASY-nLC 1000 pump in-line with a Thermo Q-Exactive HF quadrupole-Orbitrap mass spectrometer. A parent ion scan was performed using a resolving power of 120,000, then up to the 25 most intense peaks were selected for MS/MS (minimum ion count of 1000 for activation) using higher energy collision induced dissociation (HCD) fragmentation. Dynamic exclusion was activated such that MS/MS of the same m/z (within a range of 10 ppm; exclusion list size = 500) were excluded from analysis for 3.5 s.

For protein identification, raw files were converted to mgf format using Mascot Distiller (v3.0), then searched using the Mascot Search engine (Matrix Science Ltd) against the Uniprot Peanut database. Search parameters specified as: parent MS tolerance at 6 ppm and MS/MS fragment ion tolerance at 50 mmu, and with 1 missed cleavage allowed for trypsin. No fixed modifications, but oxidation of methionine was allowed as a variable modification. Data were re-searched using X!Tandem, additionally allowing deamidations of glutamine and asparagine as variable modifications. The combined search data were validated using standard validation software of the Scaffold proteome software platform (Proteome Software Inc.). Proteins identified with an FDR of 1% on the peptide level and protein level were considered and quantified, relative to the other samples, by total spectral counts.

2.3.5. SDS-PAGE and Western blot analysis

Whole protein extracts normalized by flour mass were diluted 1 in 30 (1 mg/mL for raw peanut extract) and separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (2.5% β-mercaptoethanol). Protein gels were either stained with 0.1% Coomassie Brilliant blue to visualize protein bands or were transferred to polyvinylidene difluoride (PVDF) membranes for Western blot analyses. After blocking with 5% BSA for 1 hour at room temperature (1h RT), membranes were incubated overnight at 4°C with rabbit anti-Ara h 1, Ara h 2, or Ara h 8 polyclonal antibodies (1:1,000; PA-AH1, PA-AH2, PA-AH8, Indoor Biotechnologies, VA). Bound antibodies were visualized using horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (1:1,000, 1h RT; #406401, BioLegend, CA) and Clarity/Clarity Max enhanced chemiluminescence (ECL) substrates (Bio-Rad Laboratories, CA). Products were imaged with the ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories).

2.3.6. Determination of specific IgE responses

The specific IgE binding capacity of peanut proteins was analyzed using the enzyme-linked immunosorbent assay (ELISA). Polystyrene 96-well microplates were coated overnight at 4°C with four 100-fold serial dilutions of each protein extract normalized by flour mass (0.0001 to 100 µg/mL for raw extract). Pooled sera from 4 patients with high levels of peanut-specific IgE (median IgE for peanut: 474 kU/L, median age: 15 years old, 75% male) was diluted 1:250 in 1% BSA and used as the primary antibody (50 µL/well, 2h RT). Biotinylated, goat anti-human IgE antibody (1:20,000, 50 µL/well, 1h RT; #A80-108B, Bethyl Laboratories Inc., TX) followed by incubation with HRP-streptavidin (1:3,000, 50 µL/well, 1h RT; BioLegend) were used for detection. After incubation with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (BioLegend), optical density (OD) values were measured at 450 nm with reference at 570 nm. To construct a

standard curve, wells were coated with anti-human IgE capture antibody (1:1,000; #A80-108A, Bethyl Laboratories Inc.) and subsequently incubated with 10-fold serial dilutions of recombinant human IgE antibody starting at 100 ng/ml (ELISA Ready-SET-Go! Kit, #88-50610-77, Thermo Fisher Scientific). All values were averaged over two technical replicates.

2.3.7. Relative quantification of allergens

Relative levels of Ara h 1, Ara h 2, and Ara h 8 in peanut extracts were quantified using a similar ELISA protocol as described above. Following blocking with BSA (1%, 1h RT), rabbit anti-Ara h 1, Ara h 2, or Ara h 8 polyclonal antibody (1:1000, 50 µL/well, 2h RT; Indoor Biotechnologies) was used as the primary antibody and HRP-conjugated donkey anti-rabbit IgG antibody (1:1,000, 50 µL/well, 1h RT; BioLegend) was used for detection. After incubation with TMB substrate (BioLegend), OD values were measured at 450 nm with reference at 570 nm. All values were averaged over two technical replicates.

2.3.8. Statistical analyses

RStudio software was used for statistical and data analyses (v2022.07.2+576 Spotted Wakerobin Release, Boston, MA; R version 4.2.2). Data are expressed as mean ± standard error (SE) of 3 replicates. Paired Student's t test was used to compare measured concentrations for each condition from each of Bradford assay and NanoDrop Protein A280. Analysis of Variance (ANOVA) and Tukey tests were used for multiple comparisons between OD and IgE binding values by ELISA, and between extracted protein concentrations by Bradford assay. Statistical analyses of ELISA data were performed for raw concentration of 1 µg/ml, within the dynamic range of samples of each condition. All analyses were performed in comparison to raw peanut unless otherwise specified. A p-value of ≤ 0.05 was considered statistically significant in all cases.

2.4. Results

2.4.1. Protein profiles via SDS PAGE

Defatted peanut flours derived from raw, roasted, and autoclaved peanuts were used to create protein extracts which were run via SDS-PAGE (Fig. 1A). Raw and roasted peanuts were characterized by multiple protein bands of similar molecular weight. By contrast, autoclaving led to considerably fewer and less intense bands. Roasting the peanut either before or after autoclaving did not affect this result.

Given these differences, we then set out to evaluate a range of autoclaving parameters to find the optimal condition for major protein degradation. Peanuts were autoclaved at a range of temperatures while keeping the time duration constant at 30 minutes and samples of each were run via SDS-PAGE (Fig. 1B). Similar total protein detection levels were observed in peanuts autoclaved at 100°C and 110°C as for raw and roasted. At 120°C, overall band intensity decreased, while at 130°C, distinct bands could no longer be visualized. In parallel, peanuts were autoclaved at a range of time durations at a constant temperature of 130°C. SDS-PAGE analysis revealed a clear decrease in protein detection and disappearance of bands as autoclaving duration increased, with almost no protein detected at 30 minutes or longer (Fig. 1C).

2.4.2. Differences in measured protein concentration across peanut extracts

To quantify total protein extract concentrations for each condition, we used the Bradford assay. Peanuts roasted or autoclaved at 120°C or 130°C for 5 to 60 minutes yielded significantly lower concentrations when compared to raw ($p<0.001$; Fig. 2A, 2B). Interestingly, despite the drop in Bradford concentration following both processing methods, there was a substantial difference observed in the protein band profiles of roasted and autoclaved peanut extracts via SDS PAGE (Fig. 1A).

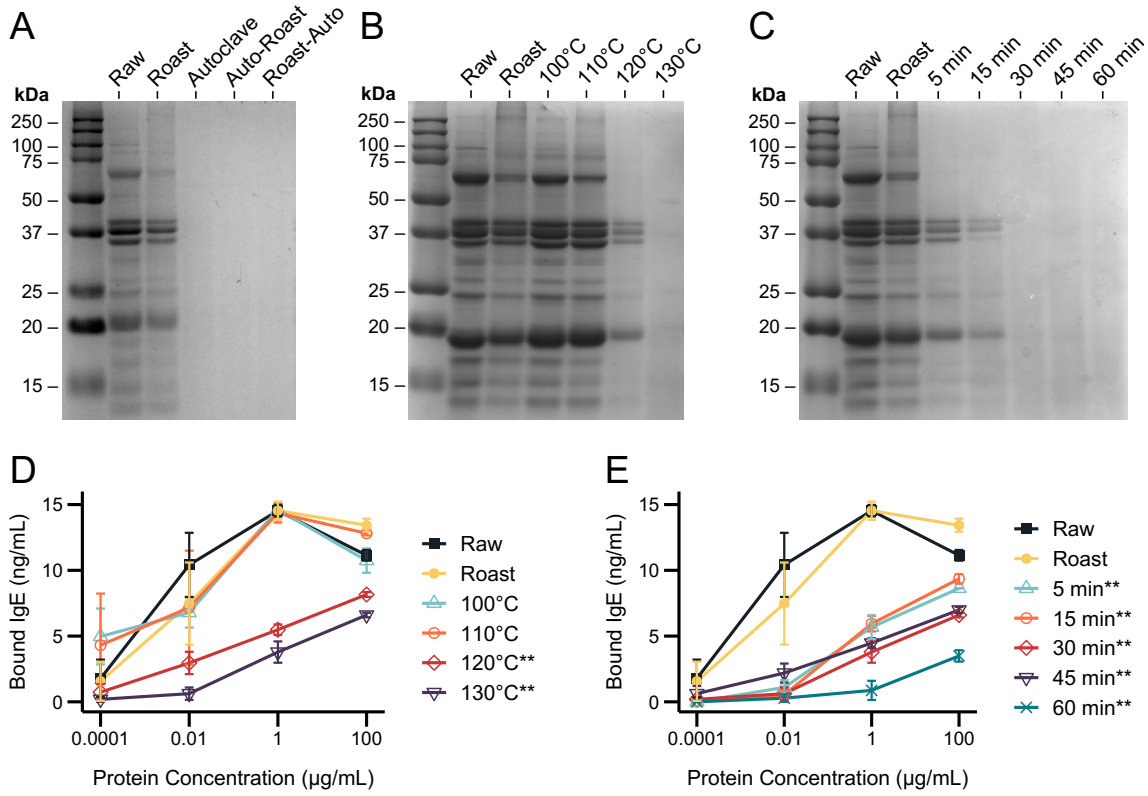


Figure 1. Effect of autoclaving on peanut proteins and IgE binding. **A-C.** Coomassie stain of protein gels following SDS-PAGE analysis comparing (A) raw, roasted, autoclaved (130°C, 30 min), autoclaved then roasted (Auto-Roast), and roasted then autoclaved (Roast-Auto) peanut samples; (B) autoclaved peanut samples at a range of temperatures at constant time duration of 30 minutes; (C) autoclaved peanut samples at a range of time durations at a constant temperature of 130°C. Gels were loaded with 20 µg of protein of each sample. **D, E**. Total peanut-specific IgE ELISA using pooled sera of 4 subjects highly allergic to peanuts evaluating IgE binding to autoclaved peanuts at a range of (D) temperatures and (E) time durations. Plates were coated with peanut extracts at a range of concentrations normalized by total peanut flour mass; x-axis values correspond to raw extract concentrations determined by Bradford assay. **p<0.01, ANOVA & Tukey HSD. Error bars represent standard errors (n = 3).

Two possibilities may contribute to these observed differences: changes in protein solubility and protein fragmentation via hydrolysis. Since the Bradford assay has a lower detection limit of 3 to 5 kDa (Sedmak & Grossberg, 1977), we compared these readouts with protein concentrations determined via absorbance at 280nm using NanoDrop (A280 method), a size-independent alternative technique. Indeed, we detected significantly greater concentrations by Nanodrop in autoclaved extracts compared to the Bradford assay, while raw extracts had similar readouts using both methods ($p<0.05$; Fig 2A, 2B). This suggests that the autoclaved peanut extract

may indeed have more protein matter below the Bradford assay limit of detection. Despite increased protein detection by Nanodrop in autoclaved extracts, concentrations were still approximately 50% of raw values, suggesting changes in protein solubility in addition to fragmentation.

2.4.3. Solubility and protein aggregation

To quantify changes in solubility, equal masses of peanut flour were dissolved in buffer, followed by the separation of soluble and insoluble fractions (Suppl. Fig. 1). We then lyophilized both fractions and compared the relative mass of each fraction across each condition. Indeed, a greater proportion of the total mass of peanut flour was retained in the insoluble fraction from autoclaved peanut flour (130°C, 30 min) compared to raw and roasted, demonstrating autoclave-induced changes in solubility (Table 1, Fig 2C). When comparing masses of peanut protein quantified by Bradford assay, the autoclaved peanut extract yielded approximately half as much detectable protein when compared to raw after adjusting for lyophilized extract mass (44.3% vs. 91.3%, respectively; Table 1, Fig 2D).

Table 1. Relative extracted masses from defatted peanut flours dissolved in extraction buffer (20 mM Tris, pH 8.5).

Condition	Pellet Mass	Extract Mass	Bradford Mass[a]	Bradford % out of extract
Raw	57.2%	42.1%	38.4%	91.3%
Roast	70.8%	28.3%	23.7%	83.7%
Autoclave[b]	80.1%	17.4%	7.7%	44.3%

[a]Calculated based on protein extract concentration determined by Bradford assay.
[b]Peanuts autoclaved at 130°C for 30 minutes.

However, the relative contribution of different flour components (proteins, carbohydrates, etc.) to the insoluble fraction mass is unclear. To determine if protein solubility was a significant contributor, we measured protein concentrations in extracts prepared using standard extraction

buffer (20 mM Tris, pH 8.6) or urea (7 M, pH 7.4). We observed a minimal difference in protein concentrations of raw peanut extracts prepared with Tris or urea as determined by Bradford (1.2-fold increase; Suppl. Fig. 2). Interestingly, when prepared with urea, there were increases in protein concentrations of roasted (2.0-fold) and autoclaved (3.7-fold) peanut extracts. This suggests that processing peanuts, via both roasting and autoclaving, results in some decrease in protein solubility.

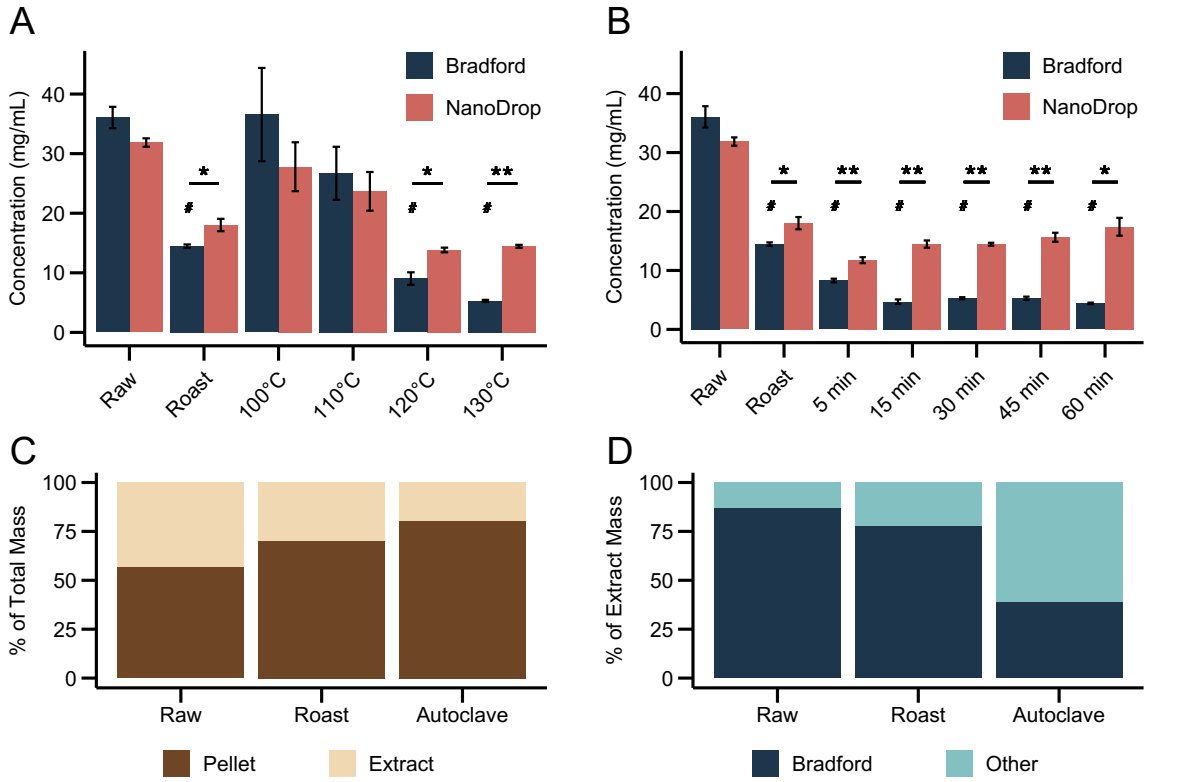


Figure 2. Autoclaving degrades peanut proteins into peptides. **A, B.** Concentration in mg/mL of protein extracts derived from raw, roasted, and autoclaved peanuts measured by Bradford assay and NanoDrop (A280nm). Peanuts were autoclaved (A) at a range of temperatures for a constant 30 minutes, or (B) for a range of time durations at a constant temperature of 130°C. **C.** Mass distribution of soluble (extract) versus insoluble (pellet) fractions, expressed as percentage of total mass for raw, roasted, and autoclaved (130°C, 30 min) peanut samples. **D.** Mass distribution of soluble extract fraction detected by Bradford assay or other (below the Bradford detection limit) expressed as percentage of total mass for raw, roasted, and autoclaved peanut samples. *p<0.05, **p<0.01, paired Student's t test. #p<0.001 (compared to raw), ANOVA & Tukey HSD. Error bars represent standard errors (n = 3).

2.4.4. Protein hydrolysis

To assess protein hydrolysis induced by autoclaving, protein extracts created from raw, roasted, and autoclaved peanuts were passed through centrifugal filters with a cut-off of 10 kDa prior to performing mass spectrometry (MS) followed proteomics analyses on the filtrates. When comparing total spectral counts and total unique spectral counts corresponding to raw, roasted, and autoclaved peanut peptides, we observed a greater number of peptides in the autoclaved sample when compared to either raw or roasted (Fig. 3A, 3C). When looking at specific peanut allergens Ara h 1, Ara h 2, and Ara h 3, all of which are above the 10 kDa cut-off, we observed significantly greater numbers of unique peptides in the autoclaved samples than in raw or roasted (Fig. 3B, 3D). Moreover, upon evaluating the specific peptide sequences of each of these allergens, we found a much greater number and diversity of peptides in the autoclaved sample (Figure 3E-3G). This suggests a random cleavage of proteins into peptides throughout autoclaving, contrasting with raw and roasted filtrates where consistent common peptides are observed.

2.4.5. Antibody binding to peanut extracts

As described previously, a multitude of challenges were associated with determining and normalizing extract concentrations for subsequent experiments. To account for these challenges, we compared IgE binding to raw, roasted, and autoclaved peanut extracts that were normalized to either flour mass or Bradford concentration. Despite inflated concentrations in autoclaved extracts resulting from normalizing by Bradford concentration (due to protein fragmentation below the limit of detection), we still observed decreased IgE binding in autoclaved extracts compared to raw and roasted (Suppl. Fig. 3). Extracts normalized to flour mass were used for remaining experiments.

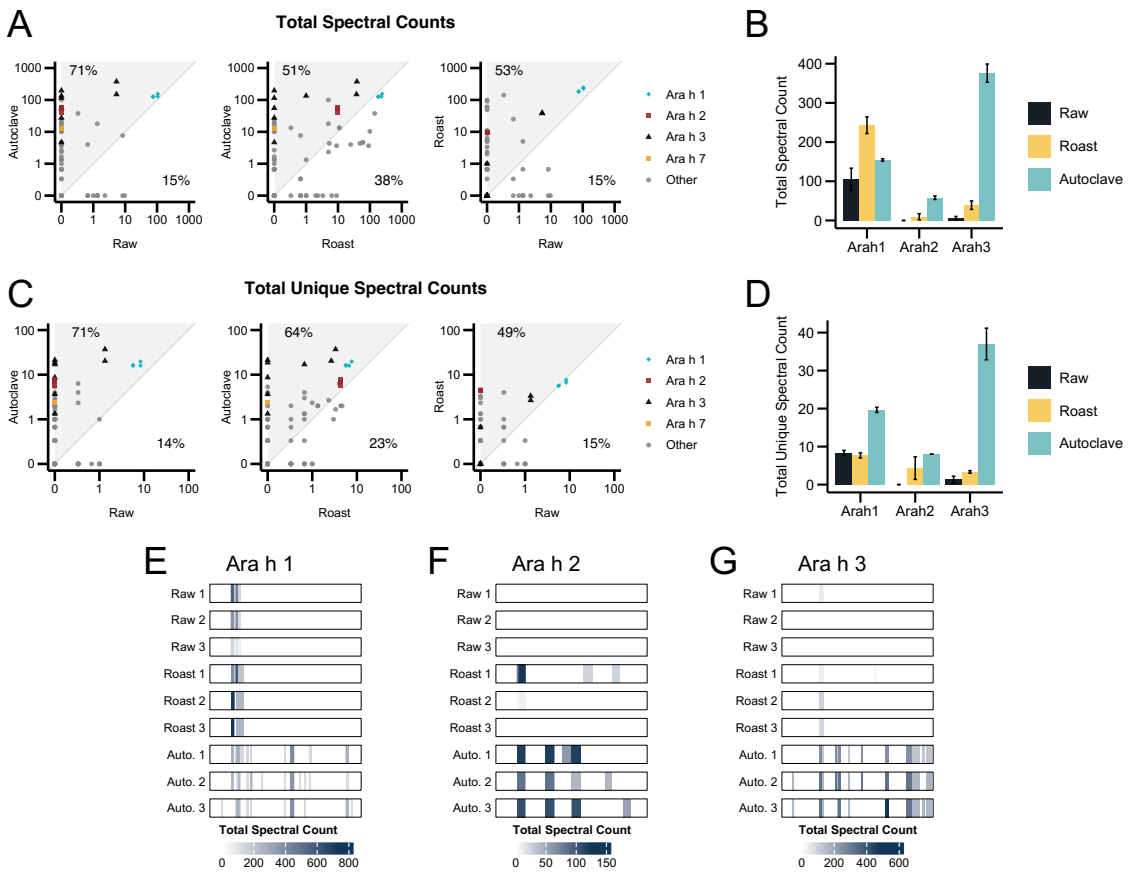


Figure 3. Mass spectrometry followed by proteomics analyses on raw, roasted, and autoclaved (130°C, 30 min) peanut protein extract filtrates (10 kDa cut-off). **A, C.** Scatterplots displaying (A) total spectral counts and (C) total unique spectral counts corresponding to peptides mapped to proteins in the peanut proteome. Percentages represent the proportion of proteins above and below the $y = x$ line. **B, D.** (B) Total spectral counts and (D) total unique spectral counts corresponding to major peanut allergens Ara h 1, Ara h 2, and Ara h 3. **E-G.** Peptide sequence coverage of (E) Ara h 1 (accession: B3IXL2), (F) Ara h 2 (A8VT44), and (G) Ara h 3 (A0A445CPR7) representing detected peptide fragments. Error bars represent standard errors (n = 3).

2.4.6. Peanut-specific IgE binding

We performed ELISA experiments using pooled sera from subjects with high levels of peanut-specific IgE antibodies to evaluate IgE binding to peanuts autoclaved at a range of temperatures and times (Fig. 1D, 1E). Roasting did not significantly affect IgE binding to the pooled sera when compared to raw. However, autoclaving at 120°C or 130°C for as little as 5 minutes resulted in significant decreases in binding to sera that contained high levels of peanut-

specific IgE ($p<0.01$; Fig. 1D, 1E). Autoclaving at 130°C for 30 minutes resulted in 74% (95% CI: [63%, 85%]) reduction in IgE binding. Importantly, when using high concentrations of total protein coated to the plate (100 μg/mL), IgE binding was still detected under all conditions, indicating that some IgE-binding epitopes were intact following autoclaving.

2.4.7. Effect of autoclaving temperature and time on Ara h 1, Ara h 2, and Ara h 8

Commercially available antibodies specific for peanut allergens Ara h 1, Ara h 2, and Ara h 8 were used to quantify relative levels of each allergen under the different autoclaving conditions via Western blot and ELISA. Ara h 1 and Ara h 2 were each substantially detected at raw, roasted, and autoclaved temperatures up to 110°C, while at 120°C and 130°C, their detection was reduced significantly (Fig. 4A). However, while ELISA experiments show similar trends, Ara h 1 and Ara h 2 were still detected at high protein concentrations, indicating only partial degradation (Fig. 4B, 4C). In the case of Ara h 8, similar levels of detection were observed in samples autoclaved at temperatures up to 120°C and in raw and roasted samples. However, at 130°C, no trace of Ara h 8 could be detected by Western blot or by ELISA (Fig. 4A, 4D).

At a constant temperature of 130°C, peanuts were then autoclaved at a range of time durations (0 to 60 min). In as short as 5 minutes of autoclaving, a considerable decrease in band intensity was observed for Ara h 1 and Ara h 2 detection by Western blot, which was no longer distinguishable at 15 minutes or greater (Fig. 5A). Importantly, at high total protein concentrations coated to the plate in ELISA experiments, Ara h 1 and Ara h 2 were still detected after autoclaving even for as long as 60 minutes, again indicating only a partial degradation (Fig. 5B, 5C). By contrast, Ara h 8 detection decreased after 5 and 15 minutes of autoclaving, while after 30 minutes or greater, no Ara h 8 could be detected by Western blot or by ELISA (Fig. 5A, 5D).

Altogether, the data suggest that autoclaving raw peanuts at a temperature of 130°C for at least 30 minutes results in a peanut composition with considerably less intact, soluble protein. In particular, Ara h 1 and Ara h 2 are heavily degraded but still detected, while Ara h 8 is degraded to the point of no detection.

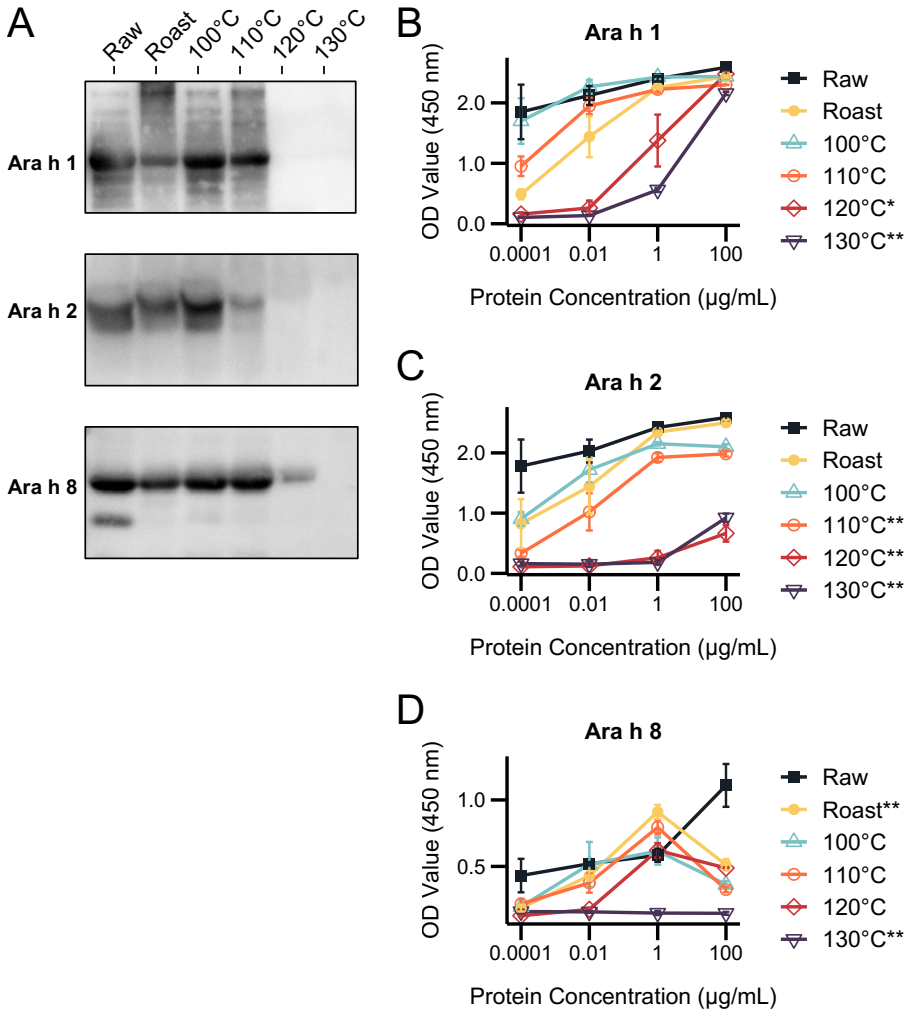


Figure 4. Effect of **temperature** of autoclaving on peanut protein allergens at a constant time duration of 30 minutes. **A.** Western blot analysis following SDS-PAGE using antibodies specific for Ara h 1, Ara h 2, and Ara h 8. **B-D.** Relative quantification of (B) Ara h 1, (C) Ara h 2, and (D) Ara h 8 via ELISA using serial dilutions of total protein concentrations of each condition coated to the plate. *p<0.05, **p<0.01, ANOVA & Tukey HSD. Error bars represent standard errors (n = 3).

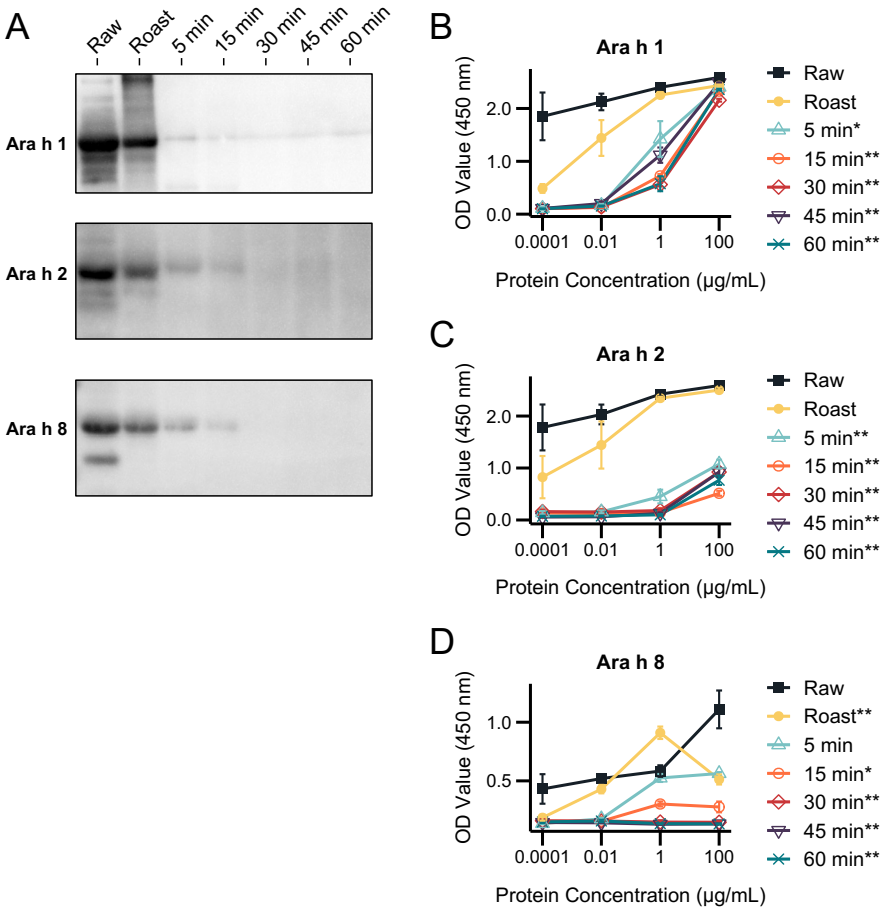


Figure 5. Effect of **time duration** of autoclaving on peanut protein allergens at a constant temperature of 130°C. **A.** Western blot analysis following SDS-PAGE using antibodies specific for Ara h 1, Ara h 2, and Ara h 8. **B-D.** Relative quantification of (B) Ara h 1, (C) Ara h 2, and (D) Ara h 8 via ELISA using serial dilutions of total protein concentrations of each condition coated to the plate. *p<0.05, **p<0.01, ANOVA & Tukey HSD. Error bars represent standard errors (n = 3).

2.5. Discussion

The steady increase in the prevalence of peanut allergy in children has catapulted this topic into intense investigation over recent decades. In this study, we evaluated the effects of roasting and autoclaving on peanut proteins and allergenicity. We demonstrated that high-pressure and temperature autoclaving reduces the detection of intact peanut proteins, including allergens Ara h 1, Ara h 2, and Ara h 8, as well as peanut-specific IgE binding when compared to raw or roasted peanuts. This effect was observed as a function of both temperature and time duration of autoclaving. Autoclaving for as little as 5 minutes at 130°C showed a significant reduction in protein and allergen detection, which continued to decrease with longer times. Overall, the data indicate that of all parameters evaluated, autoclaving at 130°C for 30 minutes or longer may be crossing a threshold of temperature and time duration of autoclaving where proteins are being degraded via hydrolysis, creating a novel peanut formulation with low levels of intact Ara h 1 and Ara h 2 present, but lacking Ara h 8. By contrast, these allergens remain largely intact in raw and roasted peanuts. This is likely due to the limited ability of dry roasting to degrade and hydrolyze peptide bonds in the allergens.

We also observed a decrease in protein solubility following processing via both roasting and autoclaving, in line with previous reports (Cabanillas et al., 2012; Kopper et al., 2005; Meng et al., 2019). However, despite this decrease following autoclaving, our observation of sporadic cleavage of numerous proteins by MS-proteomics analyses implies that proteins throughout the whole peanut are degraded. It cannot be ruled out that other mechanisms influencing protein solubility may be involved throughout processing, such as protein aggregation or crosslinking, which may also affect peanut allergenicity through a mechanism not yet known. We opted to focus on the readily accessible soluble fraction of defatted peanuts for immunological assays and

proteomics analyses because high urea concentrations are known to induce conformational changes to proteins, potentially introducing changes in epitope recognition by allergic patient IgE. Further work is required to better understand differences in allergen composition and IgE binding to extracts prepared with urea.

Autoclaving is a condition where temperature, pressure, and moisture play a significant role. There are currently few studies in the literature addressing the effect of high-pressure treatments on peanut proteins. Cabanillas et al. (2015) showed that peanut-specific IgE binding, as well as the detection of major allergens Ara h 1, Ara h 2, and Ara h 6, can be reduced by autoclaving raw and processed peanuts, demonstrated by a decrease of α-helix content and an increase in random coils and loops as a function of autoclave pressure and duration (Cabanillas et al., 2015; Cabanillas et al., 2012). Although they also reported that none of the seven peanut-allergic patients reacted to autoclaved peanut extracts via skin prick testing, it is important to note that all seven patients had very low peanut-specific IgE levels (range: 0.2-4.0 kU/L). With our pooled sera from patients with high specific IgE to peanut (range: 104-774 kU/L), we found a significant 74% decrease in IgE binding in autoclaved peanut samples when compared to raw and roasted ($p<0.01$). However, IgE binding was not eliminated.

The literature is more extensive on the comparison between the allergenicity of raw versus roasted peanuts (Zhou et al., 2021). Rao et al. (2016) found that roasting the peanut at temperatures greater than 130°C resulted in a reduction of IgE binding to Ara h 1 and Ara h 3, but an increase in binding to major allergens Ara h 2 and Ara h 6 (Rao et al., 2016). Blanc et al. (2011) found no difference in IgE binding between raw and roasted Ara h 1 protein (Blanc et al., 2011), which is in agreement with our findings as we did not observe any significant difference between the allergen detection and IgE binding responses of raw versus roasted peanut. An important potential

limitation to note is that thermal processing of peanut was recently demonstrated to impact detection by current analytical techniques such as the quantitative ELISA, over-detecting raw peanut 3.9-fold and under-detecting roasted peanut 3.5-fold (Marsh et al., 2020).

Our work is the first to report on the complete absence of detection of Ara h 8 from autoclaved peanut extract when using commercially available polyclonal antibodies specific for Ara h 8. This is in line with the fact that Ara h 8 is an allergen deprived of disulfide bonds, thereby leaving its α-helices as the sole barrier to denaturation and subsequent degradation. Ara h 8 is homologous to the birch pollen protein, Bet-v 1, and is known to induce oral symptoms to peanut in individuals with specific IgE exclusively to Ara h 8 (Asarnoj et al., 2012). Given that Ara h 8 cannot be detected in the autoclaved peanut, it is likely that allergic individuals will experience fewer oral symptoms to this modified peanut, making it a potentially safer substrate for oral immunotherapy (OIT) treatments. Further research is required to understand what impact this modified, heavily degraded peanut can have on peanut sensitization, the allergic reaction, or the desensitization potential compared to current standards.

The peanut matrix is tightly organized into membrane-bound organelles referred to as protein bodies (Weber & Neumann, 1980). The presence of high moisture and vapour pressure throughout autoclaving likely plays a significant role in disrupting this complex arrangement of peanut proteins. Our MS-proteomics data suggests autoclaving induces random cleavage of peptide bonds, thereby heavily degrading proteins and allowing the release of peptide fragments. Roasting, on the other hand, is a dry process by which water is driven out of the peanut, potentially strengthening the protein matrix structure, and allowing for other mechanisms such as the Maillard Reaction to proceed. This is believed not to decrease peanut allergenicity (Toda et al., 2019), explaining the similarity of the roasted peanut protein profiles to raw.

This study demonstrates that autoclaving significantly decreased the ability of peanut specific IgE to bind peanut proteins. This decrease may be caused by the partial degradation of Ara h 1 and Ara h 2, affecting the accessibility of epitope regions, and/or by the complete degradation of Ara h 8. These findings suggest autoclaved peanuts have a considerable potential for use in allergy reduction and therefore can be applied to develop new treatments, such as formulations to improve oral immunotherapy (Grzeskowiak et al., 2023), and diagnostic methods which are currently under investigation (pending patent applications, (Cohen et al., 2020)).

2.6. Conclusion

Altogether, the data reported in this study suggest that high-pressure and temperature autoclaving leads to a significant hydrolysis of peanut proteins and a decrease in specific IgE binding. This includes the extensive degradation of allergens Ara h 1 and Ara h 2 along with the complete degradation of Ara h 8. This modified, less potent peanut may imply a decreased risk of allergic reaction upon consumption and may serve as a useful substrate for treatment. Further studies are required to evaluate the clinical potential of the autoclaved peanut.

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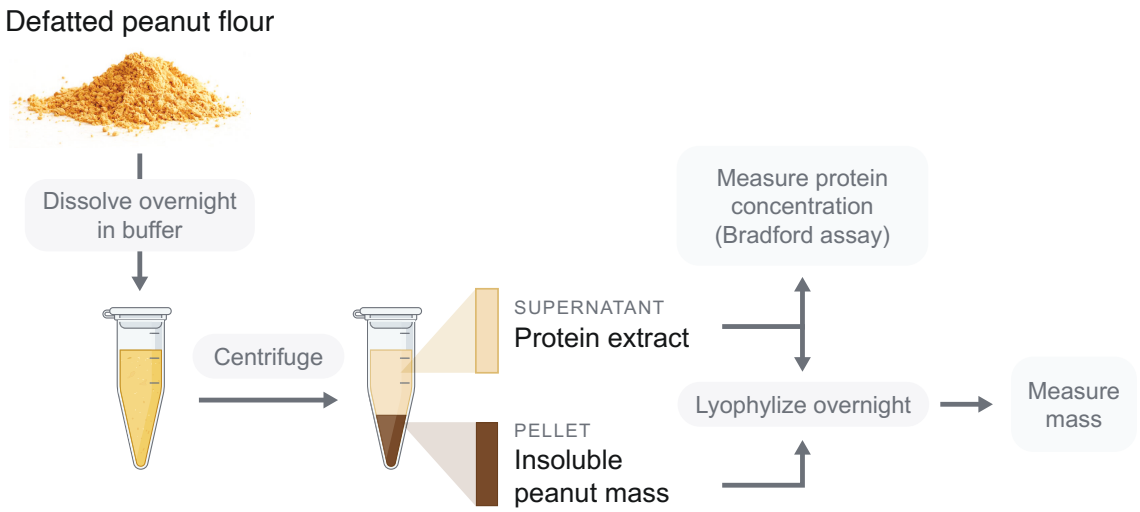
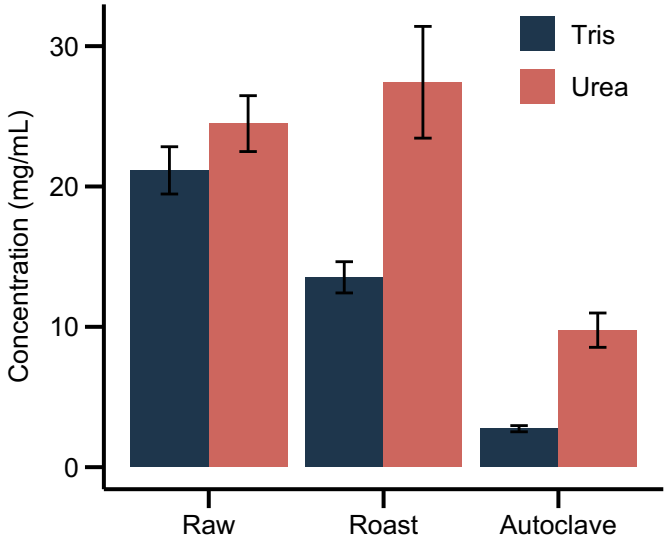
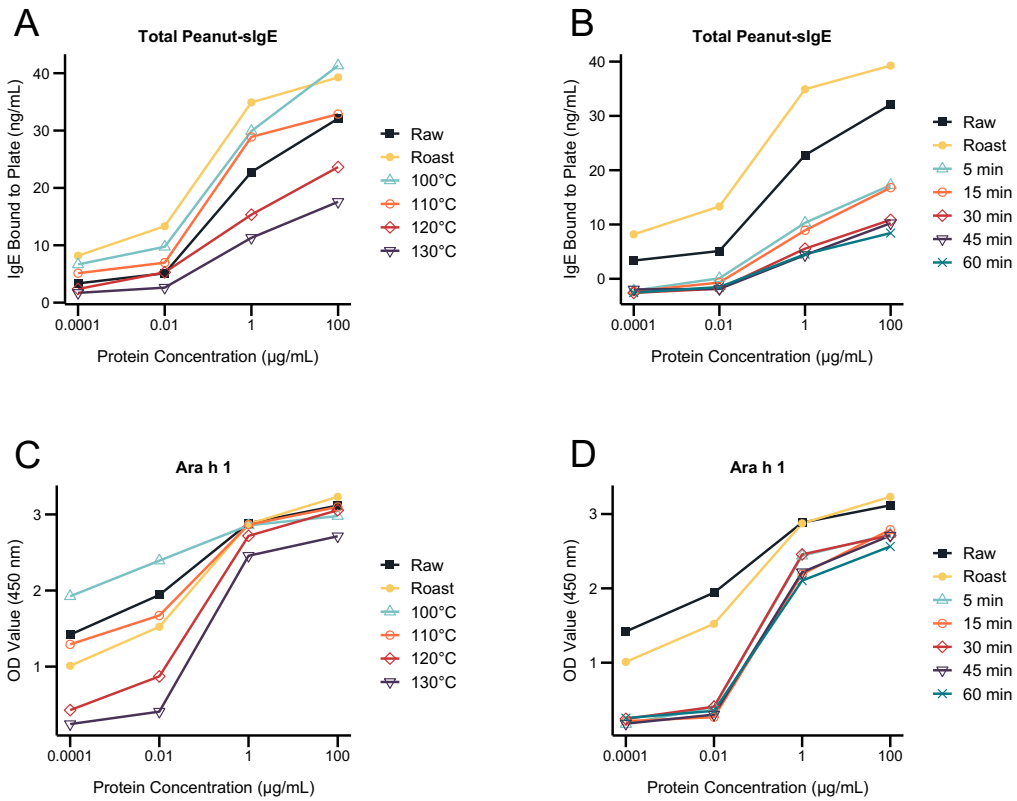


Supplemental Figure 1. Schematic describing protein extraction procedure and lyophilizing experiments. Equal masses of defatted peanut flours of each condition were dissolved in equal volumes of extraction buffer overnight. Following centrifugation, supernatant fractions were collected as protein extracts, and the remaining pellet fractions of insoluble peanut mass were reconstituted in distilled water prior to lyophilizing. Masses were then weighed and compared across conditions. Sample of extract was also used for Bradford assay to measure protein concentration.



Supplemental Figure 2. Raw, roasted, and autoclaved (130°C, 30 min) peanut protein extract concentrations using either Tris buffer (20 mM Tris, pH 8.6) or urea (7 M, pH 7.4) determined by Bradford assay. Error bars represent standard errors (n = 3).



Supplemental Figure 3. Effect of autoclaving **temperature** and **time duration** on antibody binding via ELISA. Plates were coated with serial dilutions of peanut extracts normalized by concentrations determined by **Bradford assay**. **A, B.** Total peanut-specific IgE ELISA using pooled sera of 4 subjects highly allergic to peanuts. **C, D.** Relative quantification of Ara h 1 via ELISA using commercial polyclonal antibodies.

BRIDGING TEXT 2

Chapter 2 presented work evaluating the effect of autoclaving at a range of parameters on peanut allergenicity. The results demonstrated that autoclaving at 130°C for 30 minutes or greater crosses a threshold of protein degradation in the peanut where major allergens Ara h 1 and Ara h 2 are partially intact, but birch pollen protein homolog Ara h 8, largely responsible for oral symptoms to peanut, can no longer be detected. These findings have important potential implications on the improved diagnosis and treatment of peanut allergy, and patent applications protecting this technology have been filed.

In parallel with this work, the following chapter (Chapter 3) describes a novel method to monitor changes in peanut composition throughout processing using ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy. Given that allergenicity was reduced following autoclaving as described in Chapter 2, the objective of Chapter 3 was to establish an indirect signature for allergenicity that can be rapidly obtained and assessed. Both solid forms and solutions derived from soaking of peanuts of each condition were analyzed, each of which provide different, complementary information in the NMR spectra.

CHAPTER 3: *Molecular Profiling of Peanut under Raw, Roasting, and Autoclaving Conditions Using High-Resolution Magic Angle Spinning and Solution ^{1}H NMR Spectroscopy*

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3.1. Abstract

A higher incidence of peanut allergy has been observed in countries that roast peanuts prior to consumption. This has led to investigation of the effects of thermal processing on peanut composition. In addition to high lipid, carbohydrate, and protein contents, dry roasting enhances protein glycation and aggregation, leading to a complex molecular composition. Despite the importance of understanding the role of thermal processing in allergy, studies toward generating signatures that identify the molecular contents following processing are scant. Here, we identified spectral signatures to track changes and differences in the molecular composition of the peanut under raw, roasted, and autoclaved conditions. We analyzed both the solid flesh of the seed and solutions derived from soaking peanuts using High-Resolution Magic Angle Spinning (HR-MAS) and solution ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy, respectively. The NMR spectra of intact peanuts revealed that triglycerides were the dominant species under each condition, and corresponding defatted flours revealed the presence of sugars. Sucrose and triglycerides were the most abundant small molecules observed with little variation between the conditions. Soaked peanut solutions were devoid of lipids and their resulting spectra matched the profiles of defatted peanut. Spectral signatures resulting from autoclaving strikingly differed between those from raw and roasted peanuts, with considerable line-broadening in regions from 0.5 to 2.5 ppm and between 6.5 and 8.5 ppm. Taken together, by using two complementary ^{1}H NMR methods to obtain a full picture of the peanut's molecular components, we demonstrated that autoclaving led to a distinct composition, likely resulting from hydrolytic cleavage of proteins, a major constituent of the peanut matrix.

Keywords: peanut; food processing; autoclaving; nuclear magnetic resonance; molecular profiling

3.2. Introduction

Peanut, Arachis hypogaea, is an important legume crop worldwide, serving as an excellent and affordable source of protein, fat, and several vitamins and minerals [1]. Peanuts are frequently processed prior to consumption. In North America and Western Europe, peanuts are commonly dry roasted, particularly for the preparation of peanut butter. In Asia, it is common for peanuts to be boiled in briny water. Importantly, for a small, but increasing, percentage of the population in Western societies, allergy to peanut is a condition with a high risk of severe allergic reactions, requiring constant vigilance to avoid accidental exposure [2]. The allergic reaction to peanut is characterized by immunoglobulin E (IgE) antibodies binding and cross-linking to specific protein molecules known as allergens [3]. Most peanut allergens play the role of seed storage proteins and are tightly organized into a unique, complex matrix of membrane-bound organelles, referred to as protein bodies [4, 5].

Several studies suggest significant changes in the allergenic properties of the peanuts following thermal processing [6-8]. Protein glycation at high temperature is proposed to enhance antibody binding to allergens, primarily resulting from the Maillard reaction, an addition of amines on reducing sugars that leads to Schiff bases and other intermediates before reacting to form many different advanced glycation end products (AGE's) [9, 24]. Maleki et al. (2000) simulated roasting and the Maillard reaction by incubating allergen extracts in the presence of glucose [10]. They found that glycated peanut proteins bound IgE in sera from peanut-allergic patients approximately 90-fold higher when compared to raw peanuts, though evidence of enhanced IgE binding to commercially roasted peanut proteins is lacking. Despite the ongoing debate over whether roasting and the Maillard reaction play a role in enhancing allergic reactions to foods [11], the effects of other processing methods on allergenicity have shown more promise in reducing allergenicity.

Cabanillas et al. (2012 & 2015) demonstrated that high-pressure and temperature autoclaving of peanuts resulted in decreased major allergen detection and alterations in protein secondary structure [12, 13]. Our laboratory recently showed that autoclaving significantly decreased in IgE binding to peanut allergens via ELISA when compared to raw or roasted peanuts using a pooled sera from highly allergic patients [14]. We endeavoured to define small molecule and protein profiles of the peanut under different conditions in order to establish a signature specific for raw and thermally processed peanuts, particularly roasted and autoclaved.

The chemical composition of peanuts consists primarily of lipids, proteins, and small molecules such as amino acids, reducing and non-reducing sugars [1, 15, 16]. At high temperatures, a myriad of chemical reactions occur, leading to a complex, multifarious mixture of protein products. Accordingly, monitoring changes in molecular profiles of intact seeds or soaked extracts from peanuts requires a technique that can permit capturing a signature of both small molecules and proteins under the different processing conditions.

Given that nuclear magnetic resonance (NMR) spectroscopy is a technique that provides detailed information about the structure, dynamics, and chemical environment of molecules, we used ^{1}H NMR spectroscopy to obtain molecular distribution profiles reflecting the various peanut compositions under raw, roasted, and autoclaving conditions. Furthermore, recent advances in solid state NMR have led to the development of high-resolution magic angle spinning (HR-MAS) approaches that allow the analysis of intact tissues by spinning the samples at a very specific angle to minimize chemical shift anisotropic interactions, resulting in spectra with very high resolution [17, 18]. This makes the novel HR-MAS NMR technology ideal to read the molecular composition of food products in situ [19, 20]. Here, we report our novel results from complementary NMR

analyses, analyzing both intact peanut and aqueous solutions resulting from soaking the seeds, leading to a rapid, complete insight into the molecular composition of each peanut condition.

3.3. Results

3.3.1. Predominance of lipids observed through intact peanut analysis

The HR-MAS ^{1}H NMR profiles demonstrate lipids as the dominant component of peanut in its whole, intact form (Figure 1). The lipids observed are primarily triglycerides with characteristic protons at 4.1 and 4.3 ppm, corresponding to the pair of CH_2's on the glycerol backbone, and overlapping multiplets at 5.3 ppm corresponding to olefinic protons, matching the reported spectrum of peanut oil [21-23]. Analysis of roasted peanuts did not show a noticeable difference in the lipid profile when compared to raw. Lipids are stable at high temperatures and therefore were not expected to degrade or react with other biomolecules in the peanut. Although lipids were the predominant signal in the spectra, when the scale was magnified, peaks could be observed corresponding to other molecules, such as sugars, from 3.5 to 4.0 ppm. This implies that while HR-MAS analysis captures signals corresponding not only to lipids, but also to other small molecules in the peanut. However, due to the high proportion of lipids, peaks corresponding to other small molecules were masked in the spectra.

3.3.2. Sugars revealed through the analysis of peanut flour

To unveil signals corresponding to less abundant molecules masked by lipids, we defatted the peanut samples through suspension and washing in hexanes. HR-MAS analysis of the resulting peanut flour showed a marked decrease in lipid-corresponding peaks and revealed signals suggesting the presence of sucrose (Figure 2). This was possible through the detection of a doublet at 5.4 ppm corresponding to the glucopyranose anomeric proton, as well as multiple carbohydrate-

corresponding peaks from 3.0 to 4.5 ppm, such as the triplet and doublet corresponding to the fructose moiety at 4.1 ppm and 4.2 ppm, respectively. This was in agreement with sucrose being the dominant sugar in peanuts, representing approximately 88% of all sugars [15]. Minor peaks at 5.2 ppm suggested the presence of glucose as well. The roasted and autoclaved spectra showed no major differences compared to raw in the HR-MAS spectra of defatted flour, and sucrose was the dominant sugar detected in each spectrum. Although defatting resulted in the resolution of peaks corresponding to sugars, signals related to triglycerides remained observable and covered the region from 0.5 to 2.5 ppm where protons of side chains of amino acids resonate.

3.3.3. Detecting protein matter through the analysis of soaked peanut solutions

To obtain a full picture of soluble molecules in peanuts and circumvent the interference by lipids, we soaked whole raw seeds in water and analyzed the resulting solution by ^{1}H NMR (Figure 3). As with the analysis of defatted peanut flour, we used the peak of the anomeric protons to assign the presence of sucrose and glucose; small doublets at 5.3 and 4.7 ppm were assigned to the α- and β-anomeric protons of glucose, respectively (Figure 3B, 3C). Sucrose was much more abundant than glucose under all conditions. In contrast to defatted peanut flour, no lipids remained in the soaked solution, revealing peaks in the region from 0.5 to 2.5 ppm, which were assigned to aliphatic protons of free amino acids and protein amino acid side chains. Likewise, peaks in the region from 6.5 to 8.5 ppm were assigned to aromatic and amide protons of free amino acids and protein side chains (Figure 3A, 3D).

3.3.4. Differential signature for autoclaved peanut

A striking difference was observed in the shape and number of signals in the autoclaved spectrum when compared with the raw or roasted peanut spectra, particularly in the regions between 0.5 to 2.5 ppm and from 6.5 to 8.5 ppm corresponding to protons of protein and amino

acid side chains (Figure 4A). This indicates significant molecular changes induced by the high-temperature and pressure environment of the autoclaving process. The observed line-broadening can perhaps be attributed to the abundance of hydrolyzed products (e.g., proteins and peptides). Remarkably, when raw peanut was subjected to roasting, no clear difference was observed in the regions assigned to protons of amino acid side chains. Analysis of the different areas of the spectrum showed that the relative distribution of the sucrose-corresponding peaks did not change. Likewise, under autoclaving conditions, no significant changes were seen in the sucrose peaks.

To further confirm hydrolytic degradation of proteins, an SDS-PAGE analysis was performed to assess the protein content of each soaked solution (Figure 4B). The results showed very little signal in the raw or roasted peanut-soaked solutions, in line with low levels of protein leaching out into solution throughout soaking. However, a broad smear was observed at small molecular weights in the autoclaved peanut-soaked sample, supporting the idea that autoclaving leads to the release of hydrolytically cleaved products, particularly peptides ranging from approximately 12 to 25 kDa in size, indicating protein hydrolysis and/or degradation. This is in agreement with the broad peaks in the regions corresponding to proteins and amino acids in the NMR spectra, and perhaps a greater molecular diversity of the solution when compared to the other two conditions.

3.4. Discussion

The steady increase in peanut allergy incidence over the past three decades has catapulted the topic into intense investigation. Thermal processing of peanuts has been investigated in the context of both elucidating the mechanism of allergenicity and in developing methods that can render them less allergenic. However, little attention has been paid to the analytical methods to characterize these processed products with the purpose of understanding the effect of processing

on composition. Our study specifically sought to monitor molecular differences between the overall composition of raw, roasted, and autoclaved peanuts. Indeed, we demonstrated differential changes between the three processing conditions using complementary NMR techniques, which provided a unique insight into the total molecular composition of the differentially processed peanuts (Table 1).

Table 1. Summary table. Three different forms of peanut were evaluated using two complementary ^{1}H NMR techniques, each enabling the detection of different molecular profiles. Only through the analysis of the peanut-soaked solution could a differential signature across all three conditions be observed: broad peaks exclusively in the autoclaved peanut spectrum, corresponding to greater numbers of proteins and peptide molecules in solution.

Peanut form	**^{1}H NMR technique**	**Molecules detected**			**Signature across conditions (raw, roasted, autoclaved)**
		Lipids (triglycerides)	Sugars (sucrose, glucose)	Proteins (peptides, amino acids	
Whole, intact peanut	HR-MAS	+++	+	–	No major change
Defatted peanut flour	HR-MAS	+	+++	+/–	No major change
Peanut-soaked solution	Solution	–	+++	++	**Distinct for autoclave** (broad peaks)

^{1}H HR-MAS NMR allowed us to capture the major molecules in the chemical composition of peanut in its original matrix. It was found that the spectrum of intact peanuts largely contains lipids as the major molecular constituent, which remained unchanged following processing under the conditions that we evaluated. It is well established that peanuts are composed of approximately 50% lipid, largely dominated by triglycerides [25]. Importantly, following peanut defatting into flour, the ^{1}H HR-MAS NMR spectra revealed water-soluble sugar molecules, mainly sucrose, with low levels of glucose, which, as with the lipids, also did not change significantly by thermal processing.

Since our study demonstrated the relative stability of lipids, sugars, and perhaps other small molecules unaffected by thermal processing of the peanut, we searched for a signature for macromolecules and proteins, which could be dissolved in soaked solutions. Indeed, in a previous report, 1D ^{1}H NMR was used to assess the tertiary structure of purified seed storage peanut globulins Ara h 1 and Ara h 3, generating a fingerprint representing their overall structural and dynamic information [26]. Recombinant Ara h 6, an allergenic 2S albumin from peanut, was evaluated via 2D HSQC NMR and sequence-specific resonance assignments for ^{1}H, ^{15}N, and ^{13}C were successfully identified, helping to understand the particular secondary structures of the protein [27]. Schmitt et al. (2020) discovered an isolated signal at 3.05 ppm corresponding to N-methyl-hydroxyproline in the ^{1}H NMR spectrum of polar peanut extract as an indicator of peanut adulteration and could be used to identify peanut additives and contaminants in foods [28].

The major finding of our study was that the molecular profiles of soaked solutions of autoclaved peanuts were dramatically different than those associated with raw and roasted peanuts. Evident peak broadening was observed in regions corresponding to amino acids and peptides (0.5 – 2.5 ppm and 6.5 – 8.5 ppm), suggesting the presence of multiple small molecules, perhaps resulting from protein hydrolysis. It has been shown that broad peaks around 8.3 ppm are a good indicator of proteins lacking orderly tertiary structures, because this region is characteristic of backbone amides in random-coil configuration [26]. Under the high-temperature and pressure conditions of autoclaving, line broadening may be due to protein and peptide entities released into solution via the denaturation, hydrolytic cleavage, and/or breakdown of protein molecules into short peptides and amino acids [29, 30]. This results in a range of molecular species and conformations, and thus broader peaks in the NMR spectrum. In corroboration, significant protein

degradation was seen in the SDS PAGE analysis of autoclaved peanut-soaked solutions, which showed a smear corresponding to small proteins and peptides between 10 and 25 kDa.

Taken together, the data suggest that the high vapor pressure and temperature throughout autoclaving not only disrupts the tightly organized protein matrix of the peanut, but favors hydrolytic cleavage, allowing the release of peptide fragments [5, 31]. Roasting, on the other hand, is a dry process by which water is driven out of the peanut, potentially strengthening the protein matrix structure, which may explain the similarity of the roasted peanut spectra when compared to raw. Autoclaving may be hydrolyzing the proteins to a point where epitope recognition by peanut-specific antibodies, and thus allergenicity, is greatly affected. These results are in corroboration with recent studies by our group and others, demonstrating that autoclaving results in the lowest peanut-specific IgE binding via ELISA when compared with raw or roasted peanut [12-14]. Upon further work, we ultimately aim to achieve a signature defining molecular structure that correlates with decreased IgE binding, eventually leading to an indirect signature for allergenicity.

In conclusion, ^{1}H HR-MAS analysis of whole peanuts provided a simple and elegant method to obtain a characteristic profile of the overall peanut composition. **In parallel, the ^{1}H NMR spectra of soaked extracts revealed clear, lipid-free spectra that translated the differential effect of processing via autoclaving on peanut proteins.** Soaked peanut solutions, a medium in which all molecules were soluble, was more conducive for the detection of molecular changes resulting from these processes. The extensive degradation caused by autoclaving is captured by NMR, indicating that it may help correlate molecular composition and structure with function. Upon further research, the NMR characterization may well represent an indirect signature of allergenicity through the degradation, fragmentation, and leaching of peptides into

solution. This is being developed into an effective quality control method for detecting allergens in food products (patent pending).

3.4. Materials and Methods

3.4.1. Sample preparation

3.4.1.1. Physical processing

Raw, shelled peanuts were commercially purchased (Marché Victoria Orientale, Montreal, Canada). Peanuts were roasted with their seed coating in a convection oven at 150°C for 30 minutes or were autoclaved in a tabletop autoclave at 130°C, 2.5 atm for 30 minutes. Analyses were performed in comparison with raw, unprocessed peanut.

3.4.1.2. Defatting peanuts

Raw, roasted, and autoclaved peanuts (12 of each) were ground into a paste using a coffee grinder (Proctor Silex Fresh Grind™ Coffee Grinder, Hamilton Beach, Belleville, ON, Canada). The paste was then suspended in hexanes and the defatted peanut flour was collected by filtration under vacuum.

3.4.2. NMR sample preparation

3.4.2.1. Solid preparation

Small pieces (6 mg) of whole, intact peanut or defatted peanut flour (4 mg) collected from raw, roasted, or autoclaved peanuts were loaded into a Kel-F disposable insert and subsequently placed inside a reusable 4 mm rotor.

3.4.2.2. Solution Preparation

Six whole peanuts from each condition were placed in 10 mL of double distilled water in a 15 mL Falcon tube. The peanuts soaked in water at room temperature for 24 hours. Three aliquots per condition (1 mL each) were evaporated under a SpeedVac at 45°C for 1.5 hours and the

resulting residue was reconstituted in 0.6 mL of double distilled water. The three aliquots were combined to give a total volume of 1.8 mL, 450 µl of which was collected for analysis.

3.4.3. HR-MAS and solution ^{1}H NMR spectroscopy

3.4.3.1. HR-MAS ^{1}H NMR analysis of solid peanut

Fifteen (15) µl of 5 mM 3-(Trimethylsilyl-propionic-2,2,3,3-d4) acid (TSP-d4; Sigma-Aldrich, Oakville, ON, Canada) in 100% deuterium oxide (D2O; Sigma-Aldrich) was added to the rotor as an internal standard set to 0.0 ppm prior to the addition of the insert. Analysis was performed in a Bruker 600 MHz NMR spectrometer equipped with an advanced HR-MAS probe using the water suppression pulse sequence, zgpr (Bruker standard sequence). Sixty-four scans were acquired with an acquisition time of 0.97 s and spectral width of 8.4 kHz.

3.4.3.2. Solution ^{1}H NMR analysis of peanut-soaked solutions

Solution ^{1}H NMR spectra were run on a Bruker 400 MHz NMR spectrometer for analysis using the zgpr water suppression pulse sequence. Thirty-two scans were acquired with an acquisition time of 3 s and a spectral width of 12 kHz. The ^{1}H chemical shifts were internally referenced by adding 0.5 mM of TSP-d4 set to 0.0 ppm.

3.4.4. SDS PAGE analysis

Raw, roasted, and autoclaved peanut-soaked solutions were adjusted to protein concentrations of 0.5 mg/mL (determined by Bradford assay) and separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (2.5% β-mercaptoethanol). Protein gels were stained with 0.1% Coomassie Brilliant blue to visualize protein bands.

3.5. Conclusions

Our results demonstrate that NMR spectroscopy can be used to monitor changes in protein composition throughout peanut processing to develop characteristic signatures. Moreover, autoclaving peanuts, but not roasting, results in the widespread cleavage of proteins into peptide fragments. Studies are ongoing to further investigate the signatures associated with and the clinical uses of the autoclaved peanut.

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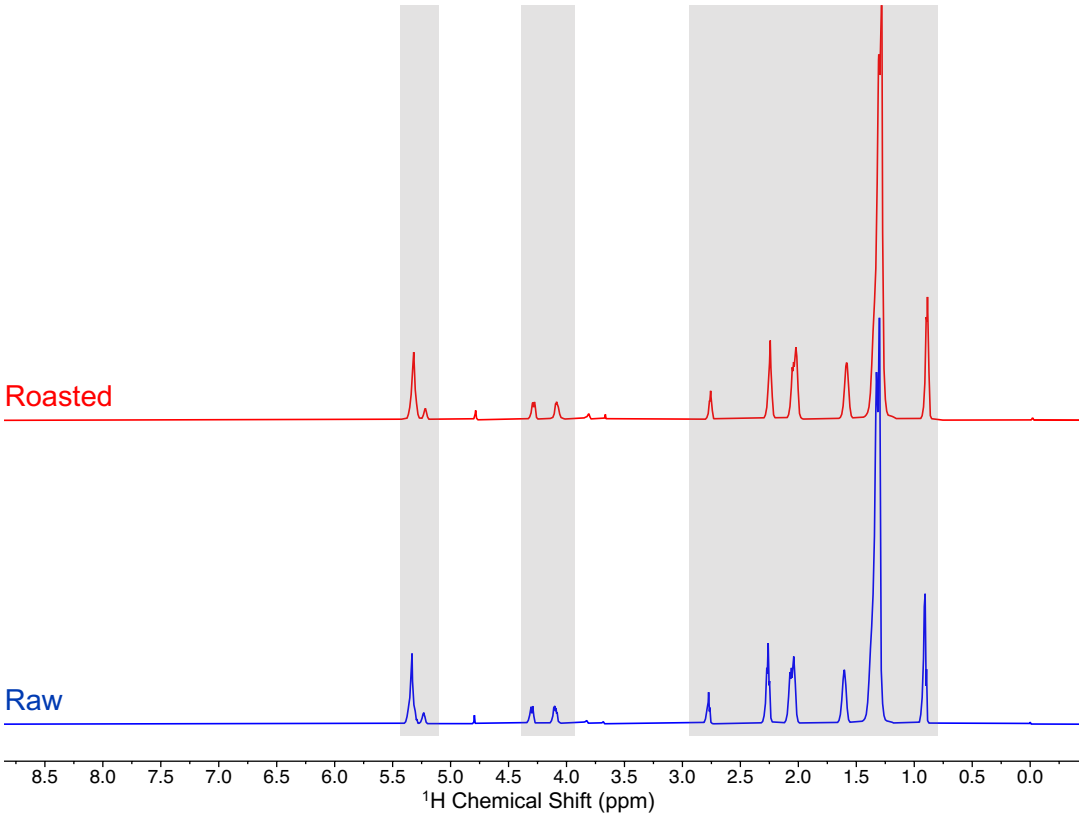

Figure 1. HR-MAS ^{1}H NMR spectra of raw and roasted intact peanut. Shaded boxes highlight peaks corresponding to triglyceride H's, which dominate the spectra.

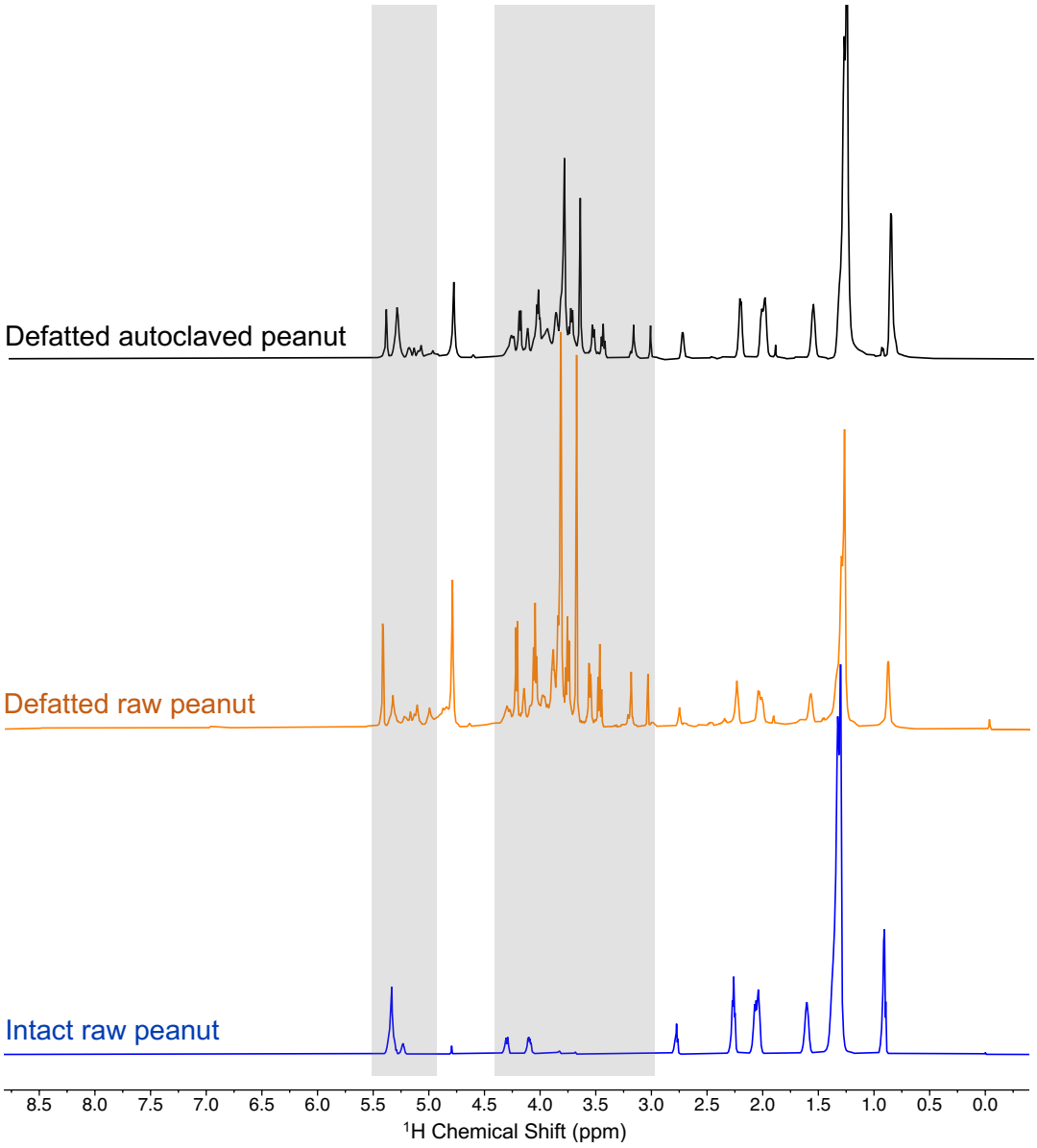

Figure 2. HR-MAS ^{1}H NMR spectra of autoclaved peanut flour, raw peanut flour, and whole, intact raw peanut. Peanut flours were defatted with hexane. Shaded boxes highlight sugar-corresponding peaks, revealed through defatting.

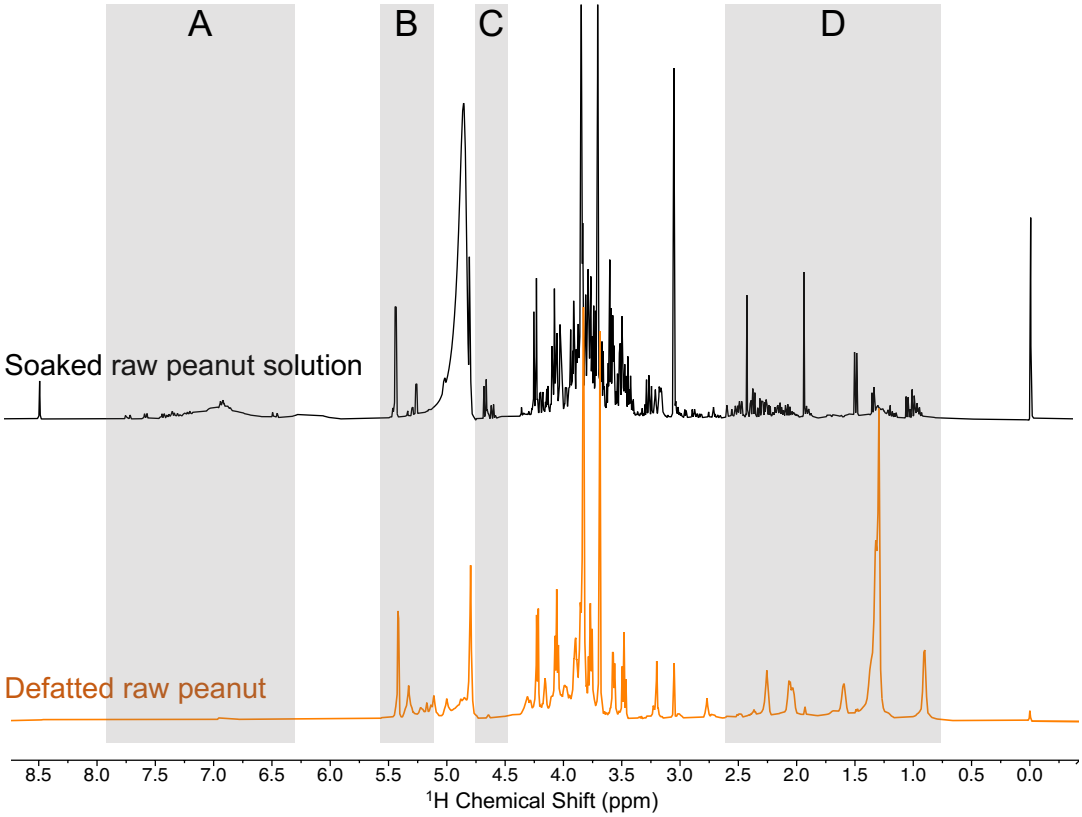

Figure 3. HR-MAS ^{1}H NMR spectrum of defatted raw peanut flour and solution ^{1}H NMR spectrum of a peanut-soaked solution of 6 raw peanuts in distilled water for 24 hours. Shaded boxes highlight: **A, D.** regions containing protons of peptide and amino acid side chains of proteins, uncovered via soaking the peanuts in water and **B, C.** peaks corresponding to the anomeric protons of sucrose and glucose.

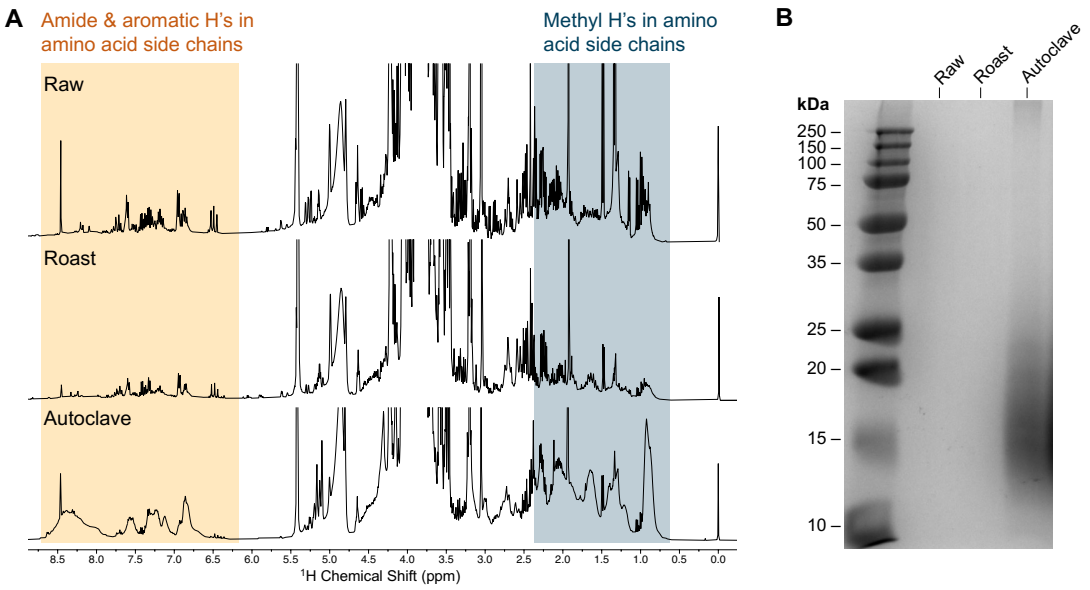

Figure 4. A. Solution ^{1}H NMR spectra of peanut-soaked solutions of 6 whole raw, roasted, or autoclaved peanuts in distilled water for 24 hours. Shaded boxes highlight peaks corresponding to H atoms in peptide and amino acid side chains. **B.** Coomassie stain of proteins following SDS PAGE of the peanut-soaked solutions of each condition showing protein band distributions.

BRIDGING TEXT 3

The third chapter of this dissertation described the establishment of an indirect signature associated with the decreased allergenicity through the autoclaved peanut. As shown in chapter 2, autoclaving the peanut leads to decreased specific IgE binding when using sera from highly allergic patients and this is likely due to the random cleavage of proteins into peptides, disrupting the complex protein matrix of the peanut. By using a complementary approach of two NMR techniques, we were able to obtain molecular profiles for the peanut under different physical states (e.g., intact seed, defatted flour, or soaked solutions) as well as under different conditions. Only following autoclaving, and not roasting, could we identify a distinct molecular signature of soaked solutions, corresponding to the leaching of peptides into solution following soaking, which is in line with protein hydrolysis throughout processing. This concludes the discussion of autoclaving and thermal processing in this thesis.

Next, we discuss another novel approach to reducing peanut allergenicity in the following chapter (Chapter 4) through the evaluation of an immature peanut. This chapter was a collaboration project with peanut geneticist, Ran Hovav, of the Volcani Institute and pediatric allergist, Mona I. Kidon, at the Sheba Medical Centre, both in Israel. The peanut seed undergoes various stages of development as it matures. The rationale behind this approach stems from the idea that the tightly bound, complex protein matrix of the peanut only develops later in maturity and the seed harvested at earlier stages may be composed of fewer, less complex allergenic structures. We were fortunate to receive lyophilized samples from peanuts of each stage of development and I was invited to perform analyses of protein levels, including major allergens, as well as peanut-specific IgE binding to allergic sera. Experiments evaluating gene expression levels, immunohistochemical staining, and SPT studies were performed by our collaborating partners in Israel. Each of these were assessed and the results are described in Chapter 4.

CHAPTER 4: *Allergen Characterization and Allergenicity Throughout Developmental Stages in Peanuts*

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4.1. Abstract

Background: Peanut allergy (PA) in children is a major concern. There is a need for better biological material for both diagnosis and oral immunotherapy (OIT) treatments. The unique state of seeds at early reproductive stages may affect the allergenicity of storage proteins, and impact clinical diagnostic and OIT protocols.

Objective: To study the major allergen content in sequential seed developmental stages and monitor allergenicity via specific IgE binding quantification and skin prick testing.

Methods: Seeds were collected from growing plants and were sorted into five developmental groups. Samples were characterized by RNA-Seq, Western blot, ELISA, and immunohistochemistry. Lyophilized, ground preparations were used for evaluation of skin test responses in sixty challenge-proven PA children.

Results: Gene expression, protein content, and specific IgE binding of allergenic proteins increased throughout seed maturation and development. An expression bias towards the less allergenic A-genome copy of the major allergen Ara h 2 was found in earlier stages, especially in R5. Immunohistochemical staining showed that Ara h 2 is more dispersed in the cell and less accumulated within organized bodies at R5 versus R7. Significant differences were found in mean wheal responses between the commercial peanut extract versus R4 and R5 stages, but not with R7, upon skin prick testing in subjects with PA.

Conclusion: The observed decrease in allergenicity of specific immature peanut seeds may not only be a result of decreased amounts of allergenic proteins, but also of profound changes in seed composition and conformation. This may be significant for developing a safer and more effective peanut OIT protocol.

Key Messages

- Peanut protein allergens show a specific accumulation pattern throughout seed development.
- At equal protein concentrations, immature peanuts demonstrate decreased specific IgE binding and reduced wheal size by skin prick testing.
- R5 peanuts may be used as a safer and more efficacious substrate for oral immunotherapy.

Key Words

Peanut, Food allergy, Seed development, Storage proteins, Skin prick test

4.2. Introduction

Food allergy has emerged as a major health concern in the 21st century. Known as the "second wave" of the allergy epidemic, food allergy is a specific immune response to a particular food that can reproducibly cause adverse health effects upon exposure.[1] Recent studies suggest that in Western countries, the prevalence of clinical food allergy among preschool children may be as high as 10%.[2, 3]

Over the past recent decades, the incidence of peanut allergy has increased, affecting 1.5% to 3% of children worldwide, making it a significant global public health problem.[4, 5] Peanut allergy is particularly dangerous, with accidental exposure leading to life-threatening reactions and even fatalities. Unfortunately, peanut allergy does not spontaneously resolve in most children, and it is usually a lifelong condition with no known cure.[6, 7] The current management approach is strict avoidance of the food and the constant availability of an epinephrine auto-injection in the case of accidental ingestion. In January of 2020, the US FDA approved Palforzia™, the first ever FDA-approved treatment for peanut allergy. This oral medication consists of capsules containing peanut

allergen powders at precise doses and incrementally larger doses are to be ingested daily by allergic individuals, desensitizing them over time.[8, 9] Despite the positive advancements reaching FDA approval, individuals still experience frequent allergic reactions and long-term daily compliance is not always feasible. Peanut allergy severely impacts quality of life of affected individuals and their families, causing heightened anxiety and limiting their participation in school and social events.[10]

Several peanut proteins have been identified as allergens in various individuals and populations, titled Ara h 1 through Ara h 18 by the WHO/IUIS Allergen Nomenclature Subcommittee.[11, 12] The cupin superfamily includes Ara h 1 and Ara h 3, while the prolamin superfamily includes the conglutins Ara h 2, Ara h 6, and Ara h 7, and are responsible for the majority of life-threatening reactions to peanuts. The non-specific lipid transfer protein, Ara h 9, the profilin, Ara h 5, and the birch pollen protein Bet v 1 homolog, Ara h 8, can cause allergic reactions that may be related to inhalation allergy to pollen due to the similarity in sequential and/or conformational molecules.[13-15] The relatively large number of allergenic peanut proteins presents a challenge for the scientific and medical communities in discovering solutions to this serious problem.

Most seed-based allergies, including peanuts, are based on immune-mediated reactions to storage proteins, which are relatively stable to heat denaturation.[16] In some cases, such as dry roasting of peanuts, there is evidence to suggest the allergenicity of the major allergens is enhanced.[17, 18] Boiling can reduce the solubility of major peanut allergens and result in their loss into the cooking water, but it does not produce hypoallergenic material from peanuts.[19, 20] The stability of peanut allergens to heat denaturation is at least partly due to the state of these storage proteins in the mature seed, where they accumulate in large and rigid protein bodies of about 5–

10 μm in diameter.[21] These organelles are bound by a single membrane and retain a highly stable homogeneous matrix in which crystalloids and/or globoids may be embedded. Members of these storage proteins, such as Ara h 1 and Ara h 2, are responsible for immediate, life-threatening reactions in peanut-allergic individuals with specific IgE for these allergens.[22, 23]

The peanut seed undergoes several changes during its developmental process. Upon anthesis or flowering, the pod, which is a simple fruit structure that originates from a carpel, grows quickly and develops a large and juicy shell wall or pericarp.[24] Initially, the pericarp occupies most of the fruit volume and serves as a temporary source of nutrients that are transferred to the nascent seed. As development progresses, the seed accumulates more nutrients and grows rapidly, eventually constituting most of the total pod volume at maturity, a process known as "pod filling". Five distinct stages in peanut pod development have been identified: R4 – pods with tiny embryos, R5 – seed growth, R6 – fully expanded but immature seeds, R7 – expanded and fully mature wet seeds, and R8 – mature dry seeds suitable for commercial use.[25] Storage proteins start to accumulate as early as the R4 stage, while later in seed development, oil and fats, along with other storage nutrients, accumulate.[26, 27] Consequently, during the early stages of seed maturation, when fats and fat globules are relatively scarce, the weight per volume concentration of peanut proteins, including major allergens, is relatively high.

The allergenicity of storage proteins in seeds may be influenced by their unique state during developmental stages. However, no studies have investigated the allergenicity of peanut maturational stages in children with peanut allergy. We have recently shown that prick-prick skin tests using a proprietary lyophilized powder from peanuts at the R5 stage was capable of predicting peanut allergy in children with a positive predictive value of over 92%, serving as a far better predictor for the presence of peanut allergy compared to commercially approved diagnostics.[27]

Therefore, this study aimed to explore the changes in allergenicity and protein allergen composition throughout the course of peanut development.

4.3. Methods

4.3.1. Plant material and seed preparation

A late-maturing peanut genotype with large pods and seeds, an indeterminate growth habit, and a relatively long period of seed development was used for the identification and production of sequential maturational stages of peanut seeds. The plants were grown in a net-house on 1.9 m wide beds. Seed production was carried out by manually harvesting the plants at the appropriate number of days post-sowing, followed by washing the pods to remove sand residues and shelling them. The resulting seeds were sorted into four reproductive groups based on their developmental stage as follows: R4, when initial seed development occurs and seeds occupy less than 30% of the total pod volume; R5, when early seed expansion takes place and seeds occupy 40-60% of the total volume; R6, when late seed expansion occurs and seeds occupy 60-70% of the total volume; R7, when seeds reach maturity and occupy over 70% of the total volume, but the pod is still not ready for harvest, as the mesocarp is not yet black; and R8, the fully mature seed.[25] Seeds were immediately flash-frozen after production and kept at -20°C until needed.

4.3.2. RNA expression of peanut allergen gene families

Changes in gene expression of allergenic proteins Ara h 1, Ara h 2, Ara h 3, and Ara h 6 throughout seed development were investigated. Sequences of these allergen genes were obtained from the peanut genome, available at peanutbase.org, using both the local tblastx in the transcript assembly and the KEGG database. To measure the expression levels of these genes, RNA-Seq libraries were constructed for each developmental stage, as previously described.[27] The transcript quantification (i.e., number of reads per gene) was done by mapping the reads to the tetraploid

peanut reference genome, also available on peanutbase.org, using the bowtie2 aligner[29] and the Expectation-Maximization method (RSEM), which estimates the maximum likelihood expression levels by handling the read mapping of uncertainty with a statistical model. To calculate the expression of each allergenic gene family, the Reads Per Kilobase Million (RPKM) values were summed for all homologous genes of that family. Data for the RNA-Seq analysis can be found using the NCBI Database project number: PRJNA982443.

4.3.3. Protein extraction

Following harvest, whole peanuts of each reproductive stage were lyophilized and ground into a dry powder. In addition, a mature seed sample (R8) was homogenized, and samples of all five stages were suspended in n-hexane for 2 rounds, passing the solution through a vacuum filter between rounds. The resulting defatted peanut flours were processed into whole protein extracts by dissolving 100 mg of flour in 1.5 mL of 20 mM Tris buffer (pH 8.5). Samples were vortexed for 30 seconds before rotating overnight at 4°C. Following 3 rounds of centrifugation for 5 minutes at 12,600 g, the supernatant was collected as the protein extract. Extract concentrations were determined by Bradford assay using known concentrations of bovine serum albumin (BSA; Sigma-Aldrich, Oakville, Canada) to construct a standard curve.

4.3.4. Western blot analyses

Protein extracts were normalized by concentration (20 µg protein), separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (2.5% *β*-mercaptoethanol), and were then transferred to polyvinylidene difluoride (PVDF) membranes for Western blot analyses. After blocking with 5% BSA for 1 hour at room temperature (1h RT), membranes were incubated overnight at 4°C with rabbit anti-Ara h 1, Ara h 2, or Ara h 8 polyclonal antibodies (1:1,000; PA-AH1, PA-AH2, PA-AH8, Indoor Biotechnologies, VA). Bound

antibodies were visualized using horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (1:1,000, 1h RT; #406401, BioLegend, CA) and Clarity/Clarity Max enhanced chemiluminescence (ECL) substrates (Bio-Rad Laboratories, CA). Products were imaged with the ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories).

4.3.5. Relative quantification of Ara h 1, Ara h 2, and Ara h 8

Relative levels of allergens Ara h 1, Ara h 2, and Ara h 8 were quantified using the enzyme-linked immunosorbent assay (ELISA). Polystyrene 96-well microplates were coated overnight at 4°C with four 100-fold serial dilutions of each protein extract, R4 to R8 (0.0001 to 100 μg/mL). Following blocking with BSA (1%, 1h RT), rabbit anti-Ara h 1, Ara h 2, or Ara h 8 polyclonal antibody (1:1000, 50 μL/well, 2h RT; Indoor Biotechnologies) was used as the primary antibody and HRP-conjugated donkey anti-rabbit IgG antibody (1:1,000, 50 μL/well, 1h RT; BioLegend) was used for detection. After incubation with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (BioLegend), optical density (OD) values were measured at 450 nm with reference at 570 nm. All values were averaged over two technical replicates.

4.3.6. Determination of specific IgE responses

The specific IgE binding capacity of peanut proteins was analyzed using a similar ELISA protocol as described above. Pooled sera from 4 patients with high levels of peanut-specific IgE (median IgE for peanut: 474 kU/L, median age: 15 years old, 75% male) was diluted 1:250 in 1% BSA and used as the primary antibody (50 μL/well, 2h RT). Biotinylated, goat anti-human IgE antibody (1:20,000, 50 μL/well, 1h RT; #A80-108B, Bethyl Laboratories Inc., TX) followed by incubation with HRP-streptavidin (1:3,000, 50 μL/well, 1h RT; BioLegend) were used for detection. To construct a standard curve, wells were coated with anti-human IgE capture antibody (1:1,000; #A80-108A, Bethyl Laboratories Inc.) and subsequently incubated with 10-fold serial

dilutions of recombinant human IgE antibody starting at 100 ng/ml (ELISA Ready-SET-Go! Kit, #88-50610-77, Thermo Fisher Scientific).

4.3.7. Immunohistochemical staining

Ara h 2-specific staining was performed on seed sections taken from R5 and R7 developmental stages. The immunostaining process followed a protocol reported by Tsuda & Chuck (2019) with modifications.[30] To prepare the tissue, the seeds were fixed in formalin, acetic acid, and ethanol (FAA) in a 5:5:90 ratio, gradually dehydrated in ethanol, fixed in paraffin, and dissected by microtome to obtain 10 µm-thick slices. For immunolocalization analysis, the fixed samples were initially incubated with 1% BSA for 1h RT for blocking and then washed in PBST (1X PBS + 0.5% Tween20). The samples were then incubated with chicken anti-Ara h 2 (provided by Dr. Soheila Maleki) as the primary antibody (1:100) and rabbit anti-chicken IgY (IgG, H+L, conjugated with Alexa Fluor-488; 303-545-003 Jackson Immunoresearch Laboratories) as the secondary antibody (1:200). The developing signal was detected by the Leica DNLB microscope and documented using a Nicon ds-fi 1 camera. For counterstaining, propidium iodide (red color) and calcofluor white (blue color) were used to stain the nucleus and cell wall, respectively.

4.3.8. Study population

Children aged 1 to 18 years old with challenge-proven peanut allergy at the pediatric allergy clinic of the Safra Children's Hospital were evaluated between January 2017 and July 2019. Prior to study entry, parents and guardians received thorough counseling on the potential risks and signed informed consent forms approved by the Institutional Review Board (IRB) of the Sheba Medical Center and the national IRB, as required for any study involving children in Israel. After informed consent was obtained, all children underwent a full diagnostic workup, including a standardized open oral food challenge (OFC) to confirm the presence of peanut allergy.

4.3.9. Skin prick tests (SPTs)

SPTs were conducted on the forearms of children using single-head lancets. A positive control with histamine (1 mg/ml) and a negative control with glycerinated saline were used. In addition, commercial whole peanut extract (ALK-Abelló, Denmark) and lyophilized R4, R5, and R7 peanut powders were used. After 15 minutes, the widest diameter of wheal and flare were measured. To avoid any interference, patients were instructed to abstain from using antihistamine-containing medications for at least one week before the procedure.

4.3.10. Statistical analyses

Statistical and data analyses were performed using SPSS (version 25, 2020, SPSS Inc., Chicago, IL, USA) and RStudio software (R version 4.2.2; v2022.07.2+576 Spotted Wakerobin Release, Boston, MA). Gene expression and ELISA values were expressed as mean ± standard error (SE) of 3 replicates. Analysis of Variance (ANOVA) and Tukey tests were used for multiple comparisons between OD and IgE binding values by ELISA. Statistical analyses of ELISA data were performed for an extract concentration of 1 μg/ml, within the dynamic range of samples of each condition. Repeated measures ANOVA was used for the comparison of SPT wheal sizes across the various developmental stages. A p-value of <0.05 was considered statistically significant in all cases.

4.4. Results

4.4.1. Molecular & genetic analysis of peanut seeds throughout development

Expression levels of genes that encode peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6 during seed development are presented in Fig. 1A. The gene expression of all evaluated allergenic proteins was detectable at an early stage (R4) and increased throughout development. In contrast, the RNA levels of Ara h 3 were consistent across the R5, R6, and R7 stages.

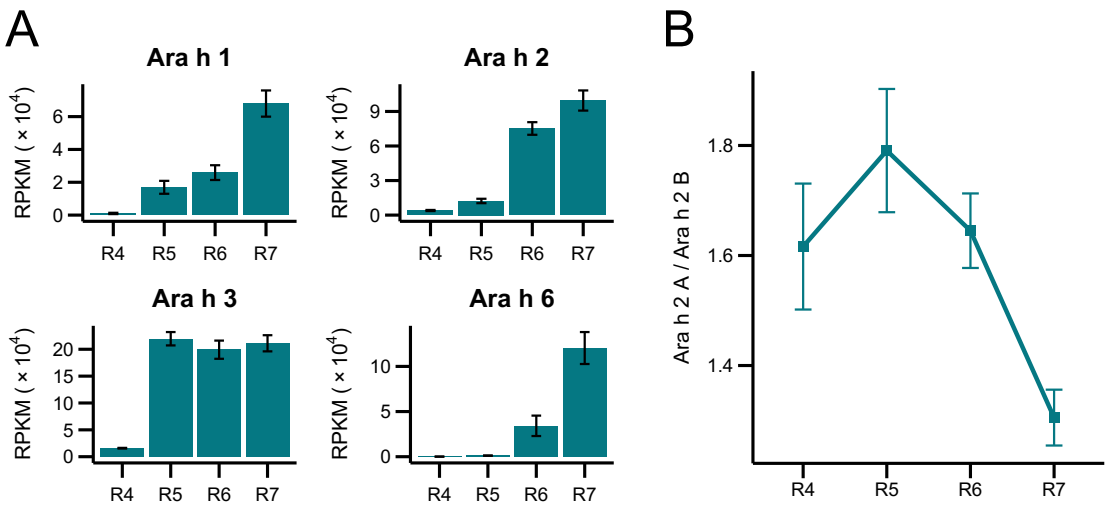

Figure 1. RNA expression throughout peanut seed development. **(A)** Expression levels of gene families encoding allergenic proteins Ara h 1, Ara h 2, Ara h 3, and Ara h 6 across the stages of seed development represented as reads per kilobase million (RPKM). **(B)** Relative genome expression of the homoeologous genes encoding Ara h 2 in each stage of development given as the ratio between the A- and B-Ara h 2 sub-genome expression within the polyploid peanut genome.

The Ara h 2 protein is encoded by 2 copies of homoeologous genes, one from the A-genome and the other from the B-genome of the AB allopolyploid peanut. Homoeologs are pairs of genes that originated by speciation and were brought back together in the same genome by allopolyploidization (i.e., multiple genomes duplicated via polyploidy in a single nucleus).[31] Previous research has suggested that the B-genome homoeolog may be more allergenic than the A-genome homoeolog because of a 12-amino acid insertion of a hypersesnsitve epitope.[32] The relative expression levels of the specific copies of the Ara h 2 gene were measured and we found a significant bias towards the less allergenic A-genome homoeolog in the early stages of seed development, particularly in stage R5 (Fig. 1B). No significant differences in the expression bias of the other three gene families were observed (data not shown).

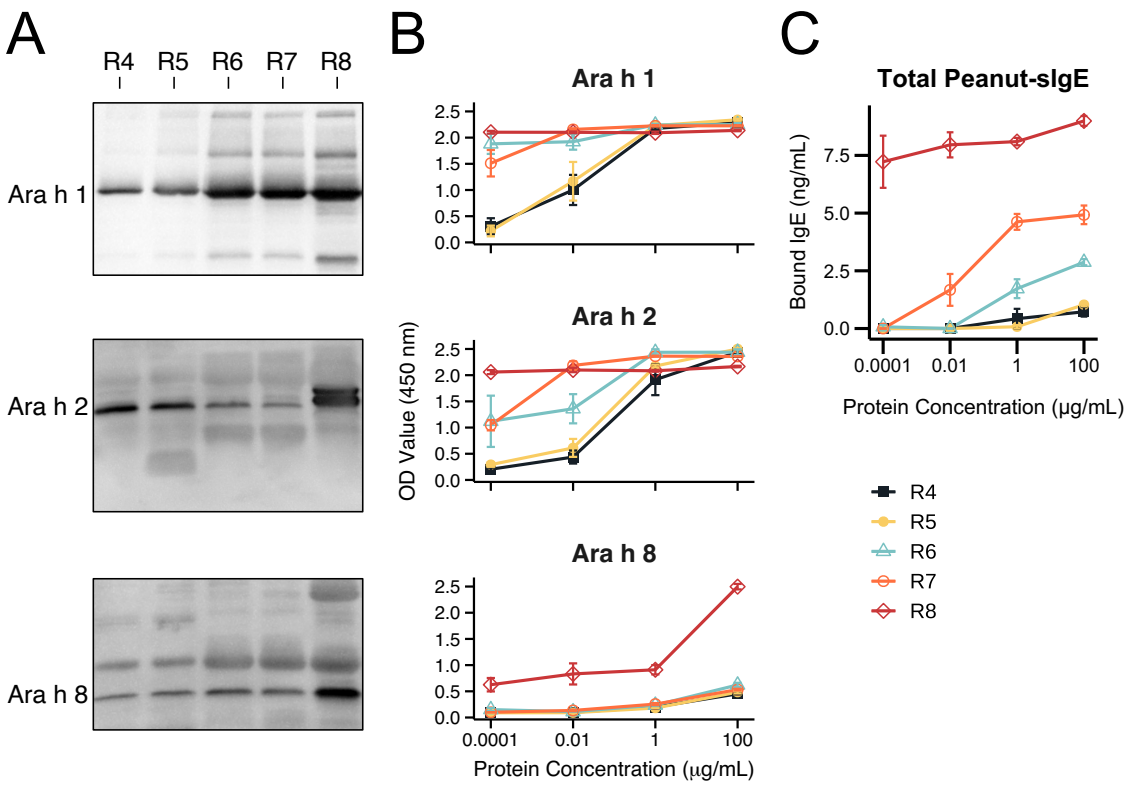

Figure 2. Relative allergen quantification and peanut-specific IgE binding**. (A)** Western blots following SDS PAGE separating extracts created from seeds of each developmental stage (R4, R5, R6, R7, R8) using antibodies specific for Ara h 1, Ara h 2, and Ara h 8. **(B)** Relative Ara h 1, Ara h 2, and Ara h 8 quantification in R4, R5, R6, R7, and R8 peanuts by ELISA using allergen-specific antibodies. **(C)** Total peanut-specific IgE quantification by ELISA using a pooled serum of 4 highly allergic subjects. Plates were coated with a maximum concentration of 100 µg/mL of each developmental stage. Optical density (OD) values were measured at 450 nm and referenced at 570 nm. Peanut-specific IgE levels were converted to concentration values using a standard curve constructed using known concentrations of human recombinant IgE. Error bars represent standard errors (n = 3).

4.4.2. Relative allergen quantification and peanut-specific IgE binding

Western blots were performed using antibodies specific for allergens Ara h 1, Ara h 2, and Ara h 8 (Fig. 2A). The results showed maximal detection of each allergen component at the mature R8 stage compared to earlier stages. Interestingly, for Ara h 2 in R8 peanuts, two distinct and intense bands were observed, while earlier reproductive stages showed only a single band of much lower intensity, corresponding to the isoform of smaller molecular weight (i.e., the A-genome homoeolog).

In Fig. 2B, ELISA experiments were performed to quantify the relative amount of Ara h 1, Ara h 2, and Ara h 8 present in whole protein extracts. The results showed that the detection levels of Ara h 1, Ara h 2, and Ara h 8 increased with maturity, demonstrating relatively low levels of detection in the earlier stages and the highest level of detection in the R8 stage, in line with the results obtained by Western blot.

Peanut-specific IgE binding to peanut proteins was quantified using a pooled serum of 4 patients with high specific IgE to peanuts via the ELISA assay (Fig. 2C). The results demonstrated an increase in IgE binding with peanut maturation, with the highest degree of binding to the mature, R8 peanut, suggesting decreased allergenicity at earlier stages of peanut development.

4.4.3. Specific immunohistochemical staining of Ara h 2 proteins in seeds

Given that Ara h 2 has been established as a potent peanut allergen, we conducted a cellular-level analysis to gain further insights. Specifically, we examined anatomical sections of peanuts in the R5 and R7 stages to trace the development and formation of peanut protein bodies (Fig. 3). Our findings indicate that Ara h 2 protein content is lower in R5 compared to R7. Additionally, we observed that Ara h 2 is more widely distributed within the cell in R5 and accumulates less within the tightly organized protein bodies than in R7.

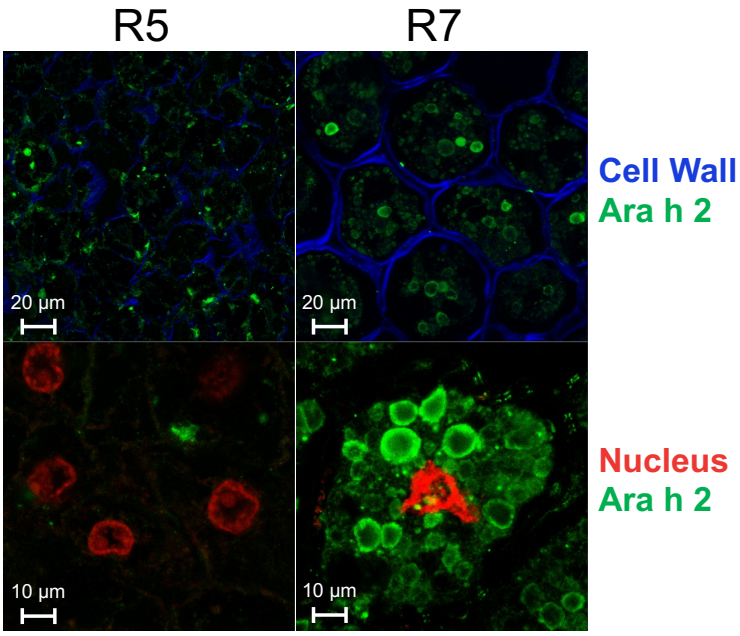

Figure 3. Specific immunohistochemical staining of Ara h 2 proteins in seeds. Immunolocalization of 12 µm-thick peanut seed sections of the R5 and R7 stages using a primary chicken antibody specific for Ara h 2 and a secondary rabbit anti-chicken antibody coupled to Alexa Fluor-488 (green). Cell wall was stained with calcofluor white (blue) and nucleus was stained with propidium iodide (red). Scale of top panels: 20 µm; scale of bottom panels: 10 µm.

4.4.4. Skin test responses to immature peanut seeds versus commercial extracts

Table 1 displays the demographics and clinical characteristics of 60 peanut-allergic children enrolled in the study, of which 67% were male and 85% were under six years of age (interquartile range: 2.6 – 5.4). The children included were highly atopic, with many suffering from additional allergic comorbidities such as atopic dermatitis (50%), asthma (40%), allergic rhinitis (10%), and other food allergies (50%). The mean maximum amount of peanut protein that could be tolerated during oral food challenges before symptoms appeared was 168 mg (95% confidence interval [CI] 106 – 230 mg).

TABLE I. Demographics and clinical characteristics of children enrolled for skin prick testing.

Characteristic	N = 60 (%) or Mean (95% CI)
Age (y)	3.5 (2.6 – 5.4)[†]
Sex: male	40 (67%)
Atopic dermatitis	30 (50%)
Asthma	24 (40%)
Allergic rhinitis	6 (10%)
Other food allergy	30 (50%)
Maximum tolerated peanut dose upon OFC (mg)	168 (106, 230)
Skin prick test wheal diameter (mm)	
R4	2.1 (1.7, 2.6)
R5	7.5 (6.6, 8.4)
R7	11.4 (10.2, 12.6)
Commercial Peanut Extract	10.4 (9.3, 11.6)

CI, Confidence interval; *OFC*, Oral food challenge.
[†]Values for age are represented by median (interquartile range).

Mean skin wheal diameters for immature peanut stages R4, R5, and R7 in comparison to the commercial peanut extract (CPE) are presented in Fig. 4. Initially, the R6 stage was also used, but the results were highly similar to R5, and thus R6 was excluded for the remainder of the experiment to manage patient discomfort. When using the R4 peanut, the mean wheal diameter was 2.1 mm (95% CI 1.7 – 2.6), while for R5, it was 7.5 mm (95% CI 6.6 – 8.4), for R7, it was 11.4 mm (95% CI 10.2 – 12.6), and for the CPE, it was 10.4 mm (95% CI 9.3 – 11.6). Repeated measures ANOVA showed significant differences between mean SPT wheal diameters to peanut developmental stages R5 and R7 with mean difference of 3.9 mm ($p < 0.001$), and between R4 and R7 with mean difference of 5.7 mm ($p < 0.001$).

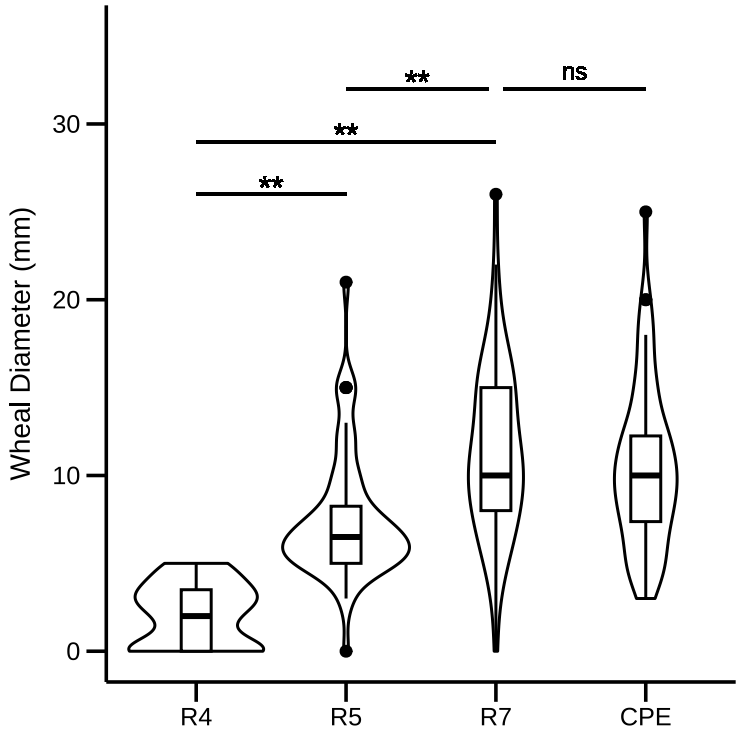

Figure 4. Skin test responses to seed reproductive stages R4, R5, R7, and to commercial peanut extract (CPE; ALK-Abello) in 60 peanut allergic children. Values are reported as wheal diameters in millimeters (mm). Repeated measures ANOVA followed by pairwise Student's *t* tests (Bonferroni correction) were performed to assess group differences. **: $p < 0.001$; ns: not significant ($p \geq 0.05$).

4.5. Discussion

This study aimed to investigate the maturational changes in the allergenicity of peanuts throughout sequential developmental stages, including relative allergen levels, specific IgE binding, and allergen location across stages. We observed lower expression levels of genes encoding major peanut allergens, decreased relative allergen levels, and reduced specific IgE binding levels to pooled sera in earlier developmental stages. We also observed a significant bias at earlier stages for the less allergenic A-genome homoeolog of Ara h 2, the peanut allergen most closely linked to anaphylaxis.[33] Moreover, total Ara h 2 content was lower, was more widely distributed in peanut cells, and accumulated less in protein bodies in the R5 stage than in R7.

The *in vivo* measurement of allergenicity of immature peanut seeds, SPT wheal size, was significantly lower than that of mature peanuts in children with challenge-proven peanut allergy.

Moreover, the allergenicity of peanuts appears to increase with peanut seed maturation. This study is the first to systematically explore the sequential development of allergenicity in immature peanut seeds.

The reduced allergenicity of immature peanuts can be explained by several mechanisms. Firstly, while total protein concentrations were similar and Ara h 3 expression levels were consistent across reproductive stages, the relative expression and protein detection levels of two of the most potent allergenic proteins, Ara h 1 and Ara h 2, were significantly lower in early seed developmental stages. This finding is supported by reduced IgE-binding to protein extracts of early peanut stages when compared to mature peanuts.

Secondly, the RNA and protein expression of Ara h 2 in the R4 and R5 stages is biased towards the production of its less allergenic isoform, rendering these seeds naturally hypoallergenic. Changes in expression bias between homoeologous genes in allopolyploid plants is a well-known phenomenon and can have significant evolutional outcomes, such as alternate or novel functions.[34] This phenomenon of homoeolog-specific gene expressions can vary vastly between organs, seed development stages, and even in the same tissue due to environmental stresses.[27, 35, 36] Therefore, it is not surprising that changes in the relative expression between the two Ara h 2 homoeologs occur during seed development, even between the R7 and R8 stages, which are two forms of mature peanuts (i.e., before and after pod drying).

Another possible explanation for the lower allergenicity of immature seeds could be the organization and composition of the protein bodies, the cellular organelles responsible for the long-term storage and accumulation of proteins within peanut cells. In the R5 maturational stage, these protein bodies are smaller and highly dispersed throughout the cell. Additionally, immature seeds have relatively higher water contents and carbohydrate levels, and lower oil and fat levels, which

may render the allergenic proteins more amenable to digestion or to thermally induced denaturation.

It is important to note that while the use of an immature peanut with reduced allergenicity may lead to fewer adverse reactions upon consumption, further studies are necessary to evaluate the risk of initial sensitization to peanut and the ability of immature peanuts to induce desensitization via OIT.

4.6. Conclusions

The observed decrease in allergenicity of immature peanut seeds may not only be a result of decreased amounts of allergenic proteins, but also of profound changes in the composition, conformation, glycosylation, and/or conglomeration of the antigens presented to the immune system. Using these naturally hypoallergenic peanuts may enable novel, safer pathways for the treatment of life-threatening peanut allergy.

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Ethics approval and consent to participate. This study was ethically approved by the Sheba Medical Center Institutional and the Israel Ministry of Health's Review Boards (Approval number: 20161653). **Trial registration:** Retrospectively registered.

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BRIDGING TEXT 4

Chapter 4 described the second approach to reducing peanut allergy evaluated in this dissertation: using an immature form of the peanut. We demonstrated that peanut proteins, including major allergens, are less abundant at earlier stages of development and that they accumulate into complex structures, protein bodies, later in the life cycle. Additionally, we observed that allergic individuals experienced significantly smaller wheal diameters upon skin prick testing when using lyophilized R4 or R5 immature peanuts when compared to mature peanut or commercial extracts. These results indicate that immature forms of the peanut may serve as safer and potentially still efficacious substrates for treatment of peanut allergy via oral immunotherapy (OIT).

The following chapter (Chapter 5) consists of a manuscript published in the *Journal of Allergy and Clinical Immunology: In Practice* in 2022. After optimizing an ELISA method for quantifying specific IgE levels to food allergens in patient serum samples, we expanded the analysis from peanuts to cow's milk. Serum samples from a cohort of cow's milk OIT participants from a multi-center study across Canada were analyzed via ELISA. This article reported the associations of cow's milk-specific IgE levels and other factors at baseline with outcomes of OIT. This report was the first to find that allergic individuals with elevated cow's milk component-specific IgE levels at baseline have a decreased likelihood of reaching the maintenance dose of OIT. These findings have important clinical implications for food allergy treatment and management and can be relevant for future studies on peanut allergy.

CHAPTER 5: *Elevated cow's milk-specific IgE levels prior to oral immunotherapy decreases the likelihood of reaching the maintenance dose*

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5.1. Abstract

BACKGROUND

Food desensitization via oral immunotherapy (OIT) is gaining higher acceptance in clinical practice. Due to adverse reactions, the duration of the build-up phase until a maintenance dose is achieved may be prolonged, and in a minority of cases, OIT is stopped.

OBJECTIVE

We aimed to assess factors associated with the probability of reaching the maintenance dose in cow's milk (CM) OIT.

METHODS

Data was collected from patients undergoing CM OIT at the Montreal Children's Hospital, BC Children's Hospital, and Hospital for Sick Children. We compared uni- and multivariable Cox regressions to evaluate sociodemographic factors, co-morbidities, clinical characteristics and biomarkers at study entry associated with the likelihood of reaching a maintenance dose of 200 mL of CM.

RESULTS

Among 69 children who reached 4 mL of milk, the median age was 12 years (Interquartile Range [IQR] 9-15) and 59% were male. The median duration of build-up phase from 4 mL to 200 mL was 24.0 weeks (IQR 17.7-33.4). After adjusting for age and sex, higher baseline levels of specific IgE (sIgE) antibodies for α-lactalbumin (ALA, hazard ratio [HR] 0.80, 95% confidence interval [CI] 0.67-0.95), β-lactoglobulin (BLG, HR 0.86, 95% CI 0.76-0.98), casein (HR 0.82, 95% CI 0.72-0.94), and total CM (HR 0.79, 95% CI 0.65-0.97), were associated with a decreased probability of reaching maintenance. Additionally, for every increase of 10 mL CM tolerated at entry challenge, the probability of reaching maintenance increased by 10%.

CONCLUSION

The data suggest that higher levels of CM-sIgE decreased the likelihood of reaching maintenance, while an increased cumulative CM dose at entry challenge increased the likelihood. Assessing these factors prior to therapy may assist in predicting the success of CM OIT.

Highlights box

What is already known about this topic?

Due to adverse reactions throughout treatment, the duration of cow's milk oral immunotherapy (CM OIT) until a maintenance dose is achieved varies considerably across patients. It is unclear what clinical factors are affecting this variability.

What does this article add to our knowledge?

This study assesses what effect sociodemographic factors, co-morbidities, clinical characteristics, and biomarkers at study entry may have on the likelihood of reaching maintenance in CM OIT.

How does this study impact current management guidelines?

The specific IgE profile and cumulative dose of CM tolerated at entry challenge affect the likelihood of reaching maintenance. Assessing these factors prior to therapy can contribute to properly counselling families and allocating adequate resources.

Key words: *Cow's milk allergy; oral immunotherapy; α-lactalbumin; β-lactoglobulin; casein; specific Immunoglobulin E*

5.2. Introduction

Currently, IgE-mediated food allergy affects approximately 6% of all children in North America and Europe.[1-3] Cow's milk allergy (CMA) affects 0.2% to 0.6% of children[2, 3], is the most common cause of inadvertent reactions[4] and a common trigger of fatal anaphylactic reactions.[5, 6] It is reported that up to 42% of children with CMA outgrow their allergy by the age of 8 years old.[7] However, recent studies reveal a lower resolution rate.[8, 9] Given that milk is ubiquitously present in numerous food products and given the nutritional benefits of milk, milk allergy presents a unique health problem.

The current treatment for CMA is strict avoidance of any foods containing cow's milk, with baked milk occasionally being an exception.[10] More recently, clinical trials in milk oral immunotherapy (OIT), exposing the allergic individual to small incremental doses of milk, have shown promising results in reducing the risk of accidental ingestion and achieving desensitization.[11] However, concerns regarding safety and efficacy of OIT treatment continue to exist, and the debate is ongoing over whether therapy should be provided outside the context of well-controlled trials.[12, 13]

Quantifying the levels of certain biomarkers, such as specific immunoglobulin E (sIgE) for the major protein allergens, has aided in predicting clinical outcomes like CMA persistence or OIT success.[14, 15] Elevated sIgE levels for α-lactalbumin (ALA), β-lactoglobulin (BLG) and casein before the start of OIT were associated with lower maintenance dose reached.[16] Additionally, those with increased levels and a broader diversity of sIgE binding to CM peptides were more likely to discontinue OIT due to frequent adverse reactions.[17]

In the present study, the duration of the build-up phase of CM OIT, defined as the time between the 4 mL dose of CM and achieving a maintenance dose of 200 mL, varies considerably

across patients.[18] This time period is highly dependent on the occurrence of adverse reactions throughout the build-up phase, and in a minority of cases, OIT is stopped.[18] To date, it is unclear what factors are associated with longer duration of build-up phase. Assessing such factors prior to the start of treatment is crucial in order to counsel families and to allocate adequate resources for OIT.

The objective of this study was to assess factors affecting the probability of reaching the maintenance dose of 200 mL of CM. More specifically, we aimed to evaluate the association between the likelihood of reaching the maintenance dose with sociodemographic factors, co-morbidities, clinical characteristics, and biomarkers at study entry.

5.3. Materials & Methods

This is a cohort study expansion of the original clinical trial (NCT03644381) following children undergoing OIT at the Montreal Children's Hospital (MCH), the British Columbia Children's Hospital (BCCH) and the Hospital for Sick Children (SKH) in Toronto, ON between April 2013 and December 2020. This study received approval from the Research Ethics Boards of each of the respective sites and written informed consent was obtained from every participant. Children with physician diagnosed IgE-mediated CMA, aged 6 to 18 years old, were recruited at the allergy clinics of the MCH, BCCH and SKH. Eligible children had a suggestive clinical history of IgE-mediated CMA and the presence of at least one of the following confirmatory tests: positive skin prick test (SPT) defined by a wheal diameter ≥3 mm than normal saline negative control, and/or sIgE >0.35 kU/L to CM protein. We excluded children with uncontrolled asthma, malignancies, autoimmune diseases and/or severe primary and/or secondary immune deficiencies, treatment with β-blockers, and the presence of cardiovascular disease or severe hypertension. In addition, children who tolerated baked forms of milk were excluded.

The CM used for all participants in this study consisted of 40 mg of protein per millilitre of volume. All participants had a single-blinded milk challenge at study entry (total cumulative dose of 150 mL or 6000 mg of CM protein) to establish the presence of CMA. Children who were able to tolerate a cumulative dose of 150 mL were not eligible for the study. Those who did not tolerate milk at challenge were offered OIT and at a subsequent visit underwent a two-day escalation phase to 2.5 mL of milk followed by a build-up phase consisting of periodic up-doses from 4 mL to 200 mL[18] following a protocol adapted from Martorell Aragones et al. (2007)[19] Children unable to tolerate 2.5 mL of milk at entry challenge followed a modified protocol with milk diluted 10-fold and continued desensitization from the highest dose tolerated over the two-day period. The up-dosing protocol of the build-up phase is described in Table E1.

Given the range of starting doses across participants, for this study we defined the duration of the build-up phase as the time from the 4 mL dose of CM until a maintenance dose of 200 mL was achieved. Once children reached maintenance, they were instructed to continue to consume 200 mL of CM daily for 1 month, followed by an open oral challenge with the maximum dose of 300 mL of CM. If well tolerated, they were recommended to consume dairy products without restriction with a minimum of 200 mL of milk twice a week. Children and their families attended regular follow-up visits at their respective research unit for 12 months post-therapy.

For the present analysis, we analyzed a cohort of 69 children who reached 4 mL of milk, 28 of which began treatment following a 1-year observation period after entry challenge, as they were initially randomly assigned to the control group. Figure 1 depicts a consort diagram describing the study population and those included in this analysis.

CM doses increased weekly if tolerated. If moderate to severe allergic reactions occurred throughout therapy (such as abdominal pain, vomiting or difficulty breathing), CM doses were

reduced to a previous tolerated dose. Mild reactions localized to the mouth, throat, or skin (such as pruritus or urticaria) did not result in the reduction of CM doses. Specific allergic reactions categorized as mild, moderate, or severe are summarized in the supplementary information (Table E2). Allergic reactions were registered in the medical chart including timing and dose at which the reaction occurred, symptoms, and treatment given. Two patients that reached 200 mL of milk had slightly longer up-dosing intervals towards the end of their build-up phase due to scheduling challenges related to the COVID-19 pandemic. Six patients did not follow the up-dosing protocol and were thus excluded from the analysis.

5.3.1. Quantification of specific IgE (sIgE)

sIgE antibodies for ALA, BLG and casein were quantified using the enzyme-linked immunosorbent assay (ELISA). 96-well polystyrene plates were coated overnight at 4°C with 20 µg/mL solutions of each protein. Plates were then washed with 0.1% Tween 20 in PBS 1X and blocked with 100 µL per well of 1% BSA for 1 hour at room temperature.

Patient serum samples obtained at baseline challenge were then added as the primary antibody at a range of dilutions in 1% BSA (50 µL per well, 2 hours at room temperature). Biotinylated polyclonal goat anti-human IgE antibody (1:20,000, 50 µL/well, 1 hour at room temperature; Bethyl Laboratories Inc., TX, USA) followed by incubation with HRP-streptavidin (1:3,000, 50 µL/well, 1 hour at room temperature; BioLegend, CA, USA) were used for detection. Following incubation with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (BioLegend), optical density (OD) values were measured at 450 nm with reference at 570 nm.

A serial dilution of recombinant human IgE antibody at 50 ng/ml (ELISA Ready-SET-Go! Kit, Thermo Fisher Scientific, ON, Canada) binding to goat anti-human IgE capture antibody (1:1,000; Bethyl Laboratories Inc.) coated to the plate was used to construct a standard curve by

plotting the known concentrations versus the OD values at 450 nm. Values were converted from ng/mL to kU/L by dividing by a factor of 2.4.[20] Total serum CM-sIgE at study entry was quantified via ImmunoCAP (Uppsala, Sweden) for 67 of the 69 subjects.

5.3.2. Statistics

Descriptive statistics were used to represent demographics, comorbidities, clinical characteristics, and biomarker levels. Kaplan-Meier curves were used to depict the probability of reaching 200 mL of CM stratified by high and low sIgE based on the median estimate for each major CM allergen (ALA, BLG and casein). Univariate and multivariable Cox regression models were compared to evaluate factors associated with longer OIT build-up phase duration, assessed as a continuous variable measured in weeks. sIgE values were log-transformed due to the geometric increase and positively skewed nature of the data[21]. All statistical analyses were performed using R version 4.0.3 (R Foundation, Vienna, Austria; 2019).

5.4. Results

This analysis focuses on the duration of build-up phases in 69 children who reached 4 mL of milk (N = 58, 7 and 4 at MCH, BCCH and SKH, respectively). The median age at study entry was 12 years (Interquartile Range (IQR) 9, 15), and just over half were males (59%). The majority of the children had other allergic conditions including well-controlled asthma (83%), eczema (41%) and pollen allergy (39%). During treatment, almost a third (29%) were engaged in regular exercise, defined as vigorous physical activity at least 3 times a week (more than 2 hours daily) such as basketball, hockey, or dancing.

At study entry, the median cow's milk SPT was 7.0 mm (IQR 5.0, 9.3) and the median sIgE for ALA, BLG, casein, and total CM was 21 kU/L (IQR 6.2, 52), 20 kU/L (IQR 1.6, 80), 23 kU/L (IQR 2.1, 119), and 26.1 kU/L (IQR 12.0, 94.3), respectively. The median cumulative dose

of milk causing a reaction upon entry challenge was 14 mL (IQR 1.4, 44.4) and 23% received more than 1 dose of epinephrine. The median duration of build-up phase from 4 mL of milk to reach 200 mL or to withdrawal from the study was 23 weeks (IQR 17, 33). Among those in the treatment group, 73.8% reached 200 mL of CM compared to 0% of those in the control group during the observation year. Demographics and clinical characteristics are outlined in Table I. Reasons for withdrawal from OIT protocol are displayed in Table E3.

5.4.1. Factors affecting duration of cow's milk OIT

Univariable Cox regression models for sociodemographic factors (age at entry challenge, sex) and the presence of co-morbidities (asthma, eczema, other food allergies) did not affect the duration of OIT (Table II). At the univariate level, higher levels of the study entry variables SPT wheal size and number of epinephrine doses at entry challenge were associated with a decreased probability of reaching the goal of 200 mL of CM, while a higher cumulative reactive dose of milk at entry was associated with an increased probability of reaching the goal (Table II). However, in the multivariate regression analysis, only the cumulative reactive dose of milk affected the duration after adjusting for age, sex and CM-sIgE (ALA-, BLG-, casein-, or total CM-sIgE; Table III, HR1.01, 95% CI 1.00-1.02). For every increase of 10 mL of CM tolerated at entry challenge, the probability of reaching the maintenance dose of 200 mL increased by 10%.

5.4.2. Specific IgE responses

IgE levels specific for ALA, BLG and casein were quantified at baseline challenge for each participant using the ELISA assay and baseline sIgE levels for total CM were quantified by ImmunoCAP. The univariate analysis showed that higher sIgE levels for each of ALA, BLG, casein, and total CM were associated with a reduced probability of reaching 200 mL of CM (Figure 2 and Table II). In the multivariate analysis, while adjusting for age, sex, and cumulative reactive

dose at baseline challenge, we were able to demonstrate that elevated sIgE levels for ALA, BLG, casein, and total CM were still associated with a reduced probability of reaching the goal (Table III). For every unit increase of log-transformed ALA-, BLG-, casein-, or total CM-sIgE, the likelihood of success of achieving the maintenance dose of 200 mL of CM decreased by 20% (HR 0.80, 95% CI 0.67-0.95), 14% (HR 0.86, 95% CI 0.76-0.98), 18% (HR 0.82, 95% CI 0.72-0.94), and 21% (HR 0.79, 95% CI 0.65, 0.97), respectively.

5.5. Discussion

In this study, we evaluated a variety of potential risk factors affecting the likelihood of reaching the maintenance dose of 200 mL in CM OIT. The duration of the build-up phase ranged from 14.7 to 93.7 weeks in the 53 patients who reached 200 mL of CM. We found that elevated baseline IgE levels specific for ALA, BLG, casein and total CM were associated with reduced probability of reaching the maintenance dose, while an increased cumulative CM dose tolerated at study entry was associated with an increase in probability, in both the univariate and multivariate analysis after adjusting for age and sex.

Baseline specific IgE values for other foods such as egg or peanut have previously shown to be useful in predicting the safety of OIT in allergic children.[22, 23] Correspondingly, high CM-sIgE levels have been previously associated with the persistence of CMA.[15, 24] More specifically, elevated levels of casein-sIgE antibodies have been reported to be strongly associated with milk allergy in children and are useful in predicting reactivity to baked milk.[25, 26] This is likely due to the fact that casein is a more stable allergen and retains its conformation following thermal processing more than the whey proteins, ALA and BLG, which have lower stability under heating conditions, resulting in a higher tolerance to heated milk in children with CMA.[10, 27]

Our results reported here suggest that individuals with greater total CM- and component-sIgE levels are more likely to experience longer durations of build-up phase to 200 mL of milk. Others have previously studied the association of measurable biomarkers before treatment as predictors for outcomes of OIT.[17] High IgE levels to ALA, BLG and casein before the start of OIT have previously been associated with achieving lower maintenance doses.[16] Furthermore, Martinez-Botas et al. (2015) found two sets of IgE-binding peptides that can be used as biomarkers to predict the safety and efficacy of CM OIT prior to the start of treatment.[28] These reports are in line with the present study in that assessing the IgE profiles of allergic individuals before therapy is beneficial in predicting certain outcomes such as the likelihood of reaching maintenance.

Another major factor affecting adverse reactions throughout CM OIT is asthma status. Elizur et al. (2015) found patients with asthma are at risk for more severe reactions and are less likely to reach full desensitization during OIT in a study of almost 200 Israeli children.[29] Another study concluded that teenagers with persistent asthma and high CM-sIgE levels should be considered a high-risk group of OIT.[30] In the current analysis, the presence of asthma was not found to be associated with an increase in adverse reactions and did not affect the likelihood of reaching the goal of 200 mL of milk.

A recent CM OIT study on 42 Turkish children found no differences in the cumulative reactive doses at entry challenge nor in CM-sIgE levels between the two groups of patients with and without adverse reactions.[31] However, similar to the current analysis, a larger study spanning 11 years including 296 children in Finland found that baseline CM-sIgE level and reactivity during the early treatment stages strongly predicted the long-term outcome and safety of milk OIT.[32]

Our study has some potential limitations. Only children aged 6 to 18 years old were included in the study, limiting the relevance of the findings to this age range. Moreover, the patient

cohort analyzed in this study followed one specific protocol of milk OIT, and the results may differ had other protocols been assessed. Further studies are required to confirm if the observed associations still hold for different age ranges and/or using different protocol parameters. Additionally, six patients did not follow the protocol and were thus excluded from our analysis, which raises another potential limitation. However, given their demographics and clinical characteristics were similar to the remainder of the study population (Table E4), we do not expect this would have affected our findings.

5.6. Conclusion

This study aimed to assess factors affecting the likelihood of reaching the maintenance dose in CM OIT. We found that high ALA-, BLG-, casein-, and total CM-sIgE antibody levels are associated with a decreased probability of reaching the goal of 200 mL of CM, while a higher cumulative tolerated dose of CM at entry challenge is associated with an increased probability. Assessing CM-sIgE levels and performing an oral food challenge prior to the start of treatment may contribute to identifying individuals that are more likely to have a longer duration of OIT, and hence may require more resources.

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TABLE I. Demographics and clinical characteristics of children who reached a minimum of 4 mL of milk during the build-up phase of milk OIT.

Characteristics	Median (IQR) N = 69 (%)
Age at challenge (y)	12 (9, 15)
Sex: male	41 (59%)
Known asthma	57 (83%)
Known eczema	28 (41%)
Known pollen allergy	27 (39%)
Regular exercise	20 (29%)
Other food allergy	49 (71%)
Skin prick test (mm)	7.0 (5.0, 9.3)
sIgE α-lactalbumin (kU/L)	20.8 (6.2, 51.9)
sIgE β-lactoglobulin (kU/L)	20.2 (1.6, 79.8)
sIgE casein (kU/L)	23.2 (2.1, 118.6)
sIgE total CM (kU/L)	26.1 (12.0, 94.3)
Cumulative reactive dose at baseline challenge (mL)	14.4 (1.4, 44.4)
Received more than 1 epinephrine dose at baseline challenge	16 (23%)
Duration of build-up phase from 4mL to 200 mL (wk, N = 53)	24.0 (17.7, 33.4)
Duration of build-up phase from 4mL to withdrawal (wk, N = 16)	18.4 (9.1, 28.1)

CM, cow's milk; *OIT*, Oral immunotherapy; *sIgE*, specific IgE.

TABLE II. Factors associated with achieving the maintenance dose of CM OIT assessed by univariate Cox regressions.

Risk factors for increased OIT duration (N = 69)	Hazard ratio (95% CI)
Age at challenge	1.05 (0.97, 1.14)
Male	0.80 (0.46, 1.39)
Active Asthma	0.70 (0.34, 1.45)
Active Eczema	1.14 (0.65, 2.01)
Other Food Allergies	1.87 (0.94, 3.69)
Skin prick test at baseline*	**0.90 (0.82, 0.99)**
Log(sIgE α-lactalbumin)*	**0.79 (0.67, 0.93)**
Log(sIgE β-lactoglobulin)*	**0.85 (0.76, 0.96)**
Log(sIgE casein)*	**0.86 (0.76, 0.96)**
Log(sIgE total CM)*	**0.72 (0.60, 0.87)**
Cumulative reactive dose at baseline challenge*	**1.01 (1.01, 1.02)**
Number of epinephrine doses at challenge*	**0.69 (0.49, 0.98)**

CI, Confidence interval; *CM,* cow's milk; *OIT*, Oral immunotherapy; *sIgE*, specific IgE.

*Values in **bold** are associated with the likelihood of achieving the maintenance dose.

TABLE III. Factors associated with achieving the maintenance dose of CM OIT assessed by multivariate Cox regressions.

Risk factors for increased OIT duration (N = 69)	**Hazard Ratio (95% CI)**			
	Log(sIgE α-lactalbumin)	Log(sIgE β-lactoglobulin)	Log(sIgE casein)	Log(sIgE total CM)
Age at challenge	1.05 (0.97, 1.15)	1.07 (0.98, 1.16)	1.07 (0.98, 1.17)	1.04 (0.95, 1.14)
Male	0.74 (0.41, 1.33)	0.69 (0.39, 1.22)	0.54 (0.29, 0.99)	0.68 (0.38, 1.23)
Cumulative reactive dose at baseline challenge*	**1.01 (1.00, 1.02)**	**1.01 (1.00, 1.02)**	**1.01 (1.00, 1.02)**	**1.01 (1.00, 1.02)**
Log(sIgE α-lactalbumin)*	**0.80 (0.67, 0.95)**	NA	NA	NA
Log(sIgE β-lactoglobulin)*	NA	**0.86 (0.76, 0.98)**	NA	NA
Log(sIgE casein)*	NA	NA	**0.82 (0.72, 0.94)**	NA
Log(sIgE total CM)*	NA	NA	NA	**0.79 (0.65, 0.97)**

CI, Confidence interval; *CM,* cow's milk; *OIT*, Oral immunotherapy; *sIgE*, specific IgE.

*Values in **bold** are associated with the likelihood of achieving the maintenance dose.

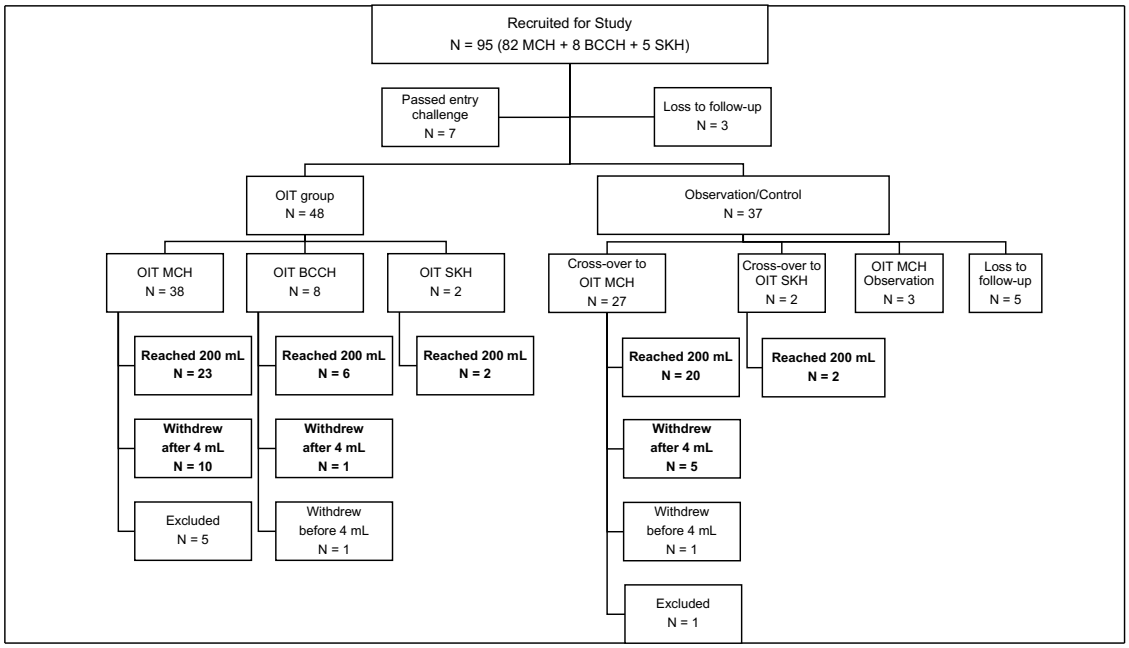

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) diagram of the study population. A total of 95 children were recruited for this study: 82 at the Montreal Children's Hospital (MCH), eight at the British Columbia Children's Hospital (BCCH), and five at the Hospital for Sick Children (SKH). Among them, seven passed the entry milk challenge and were ineligible for the study, whereas three were lost to follow-up shortly after entry challenge. The remaining 85 children were eligible and were randomized to either milk oral immunotherapy (OIT) (n = 48) or observation (n = 37). Among the children in the OIT group, 31 reached 200 mL of cow's milk (CM) and 11 withdrew from OIT after reaching 4 mL. Among children in the observation group, 29 children crossed over to the treatment group after 1 year of observation, 22 of whom reached 200 mL of CM and five of whom withdrew from OIT after reaching 4 mL. This analysis focuses on the duration of the buildup phase of milk OIT in 69 children with challenge-established CM allergy who reached a dose of at least 4 mL (shown in **bold**: n = 42 in the OIT group; n = 27 in the control group and crossed over to OIT). Six patients did not follow the up-dosing protocol and were excluded from the analysis.

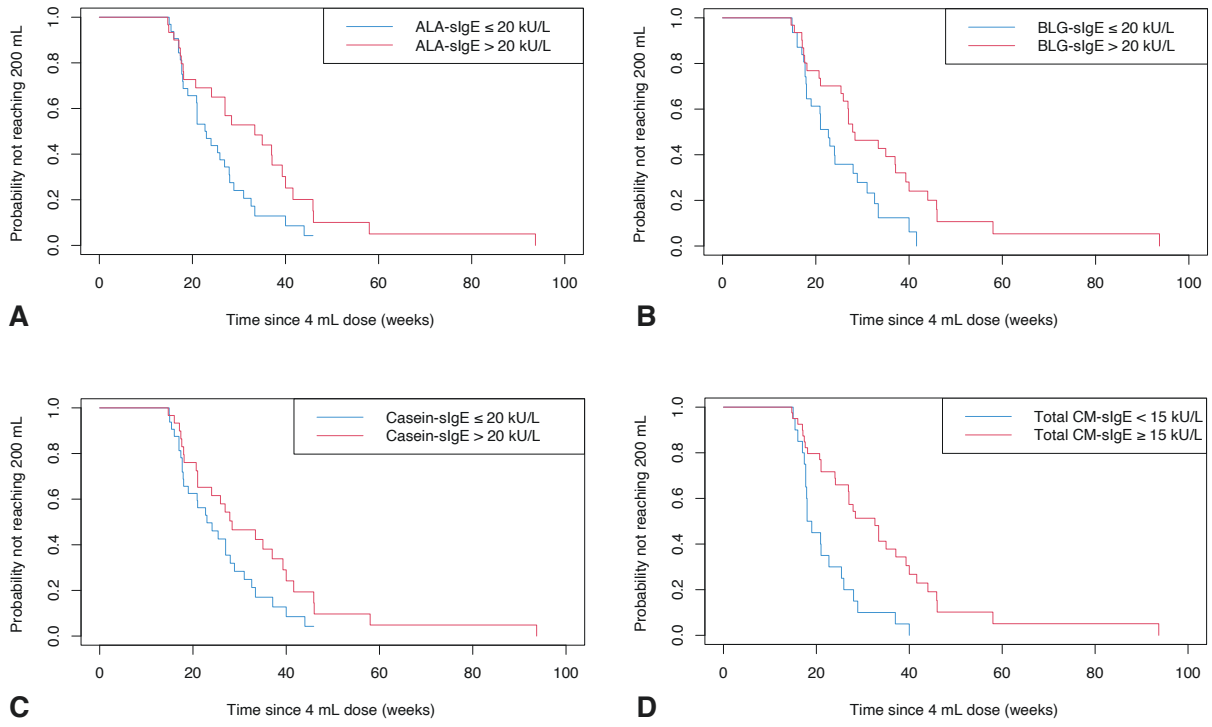

Figure 2. Kaplan-Meier curves displaying the number of weeks since the 4-mL dose of cow's milk versus the probability of not reaching the maintenance of dose of 200 mL, stratified by high and low specific IgE for (**A**) α-lactalbumin (ALA), (**B**) β-lactoglobulin (BLG), (**C**) casein, and (**D**) total cow's milk. Stratification was based on values above or below the median value for A, B, and C, and 15 kU/L for D based on a previously published ImmunoCAP decision point.[33]

Table E1. CM-OIT up-dosing protocol

Study Time Point	Milk Dilution	Volume (ml)	Total Protein (mg)
Day 1	1/100	1	0.4
	1/100	2	0.8
	1/100	4	1.6
	1/100	8	3.2
Day 2	1/10	1.6	6.4
	1/10	1.6	6.4
	1/10	3.2	12.8
	1/10	6.4	25.6
	1/10	12	48
	Undiluted	2.5	100
Week 2	Undiluted	4	160
Week 3	Undiluted	6	240
Week 4	Undiluted	8	320
Week 5	Undiluted	10	400
Week 6	Undiluted	12	480
Week 7	Undiluted	15	600
Week 8	Undiluted	20	800
Week 9	Undiluted	25	1000
Week 10	Undiluted	30	1200
Week 11	Undiluted	40	1600
Week 12	Undiluted	50	2000
Week 13	Undiluted	75	3000
Week 14	Undiluted	100	4000
Week 15	Undiluted	125	5000
Week 16	Undiluted	150	6000
Week 17-52	Undiluted	200	8000

Table E2. Scale for grading reaction severity

Score	Symptoms	Actions
Mild	• Pruritus • Urticaria • Flushing • Rhinoconjunctivitis	• Observe • May give antihistamine (e.g. Benadryl or Reactine, as prescribed) • Call research team • Research team will evaluate if dose adjustment is needed and if next dose will be given at home or in hospital. • For localized symptoms (e.g. pruritus around the mouth), no dose adjustment is required
Moderate	• Angioedema • Throat tightness • Gastrointestinal complaints (cramping, pain, vomiting, diarrhea) • Respiratory symptoms (cough, mucous production)	• Give epinephrine IM, as per protocol • Give antihistamine (e.g. Benadryl or Reactine, as prescribed) • Seek urgent care (hospital ER) • Call research team • To give next adjusted dose in hospital research unit (CIM)
Severe	• Wheeze • Respiratory distress • Hypoxia • Cyanosis • Hypotension • Circulatory collapse (shock)	• Give epinephrine IM, as per protocol • Give antihistamine (e.g. Benadryl or Reactine, as prescribed) • Seek urgent care (Call 911, transfer to hospital ER) • Call research team • If symptoms do not improve within 10 minutes of first dose, instructions will be given from the team regarding use of a second dose of epinephrine. • Give next adjusted in hospital research unit (CIM)

Table E3. Reasons for withdrawal from CM OIT participants

Reason for Withdrawal	Number of Patients
Frequent adverse reactions (vomiting, nausea, abdominal pain, sneezing, pruritus, wheezing)	9
Anxiety	3
Reactions requiring epinephrine	2
Family unable to continue and support process	1
Parents concerned about poor appetite and food aversion	1
TOTAL:	16

Table E4. Demographics and clinical characteristics comparing the 69 included subjects with the 6 excluded subjects not following the up-dosing protocol and have not reached 200 mL of milk (extension of Table 1 in manuscript).

Characteristics	**Median (IQR) N = 69 (%)**	**Median (IQR) N = 6 (%)**
	INCLUDED	EXCLUDED
Age at challenge (y)	12 (9, 15)	7 (6, 9)
Sex: male	41 (59%)	4 (67%)
Known asthma	57 (83%)	5 (83%)
Known eczema	28 (41%)	4 (67%)
Known pollen allergy	27 (39%)	5 (83%)
Regular exercise	20 (29%)	3 (50%)
Other food allergy	49 (71%)	4 (67%)
Skin prick test (mm)	7.0 (5.0, 9.3)	9.5 (6.8, 13.0)
sIgE α-lactalbumin (kU/L)	20.8 (6.2, 51.9)	9.0 (4.1, 10.6)
sIgE β-lactoglobulin (kU/L)	20.2 (1.6, 79.8)	8.0 (1.4, 41.7)
sIgE casein (kU/L)	23.2 (2.1, 118.6)	15.3 (3.5, 17.8)
sIgE total CM (kU/L)	26.1 (12.0, 94.3)	45.9 (15.9, 73.8)
Cumulative reactive dose at baseline challenge (mL)	14.4 (1.4, 44.4)	4.4 (2.2, 11.9)
Received more than 1 epinephrine dose at baseline challenge	16 (23%)	2 (33%)
Duration of build-up phase from 4mL to 200 mL (wk, N = 53)	24.0 (17.7, 33.4)	-
Duration of build-up phase from 4mL to withdrawal (wk, N = 16)	18.4 (9.1, 28.1)	-

CM, cow's milk; *OIT*, Oral immunotherapy; *sIgE*, specific IgE.

CHAPTER 6: *Comprehensive Discussion*

The global prevalence of atopy and allergic diseases, including food allergy, is on the rise. However, there are promising treatments on the horizon, particularly in the form of oral immunotherapy (OIT). Particularly, the US FDA approved Palforzia in 2021, the first ever OIT treatment for peanut allergy. With current OIT protocols using native, unprocessed forms of food allergens as substrates, allergic individuals can increase their margin of safety by increasing the dose of allergen that will cause a reaction. However, adverse reactions throughout treatment are common and, in many cases, OIT is stopped.[179, 180] This dissertation describes the research into novel strategies and mechanisms towards reducing peanut allergenicity initiated at McGill University from 2017 to 2023 (Figure 6.1).

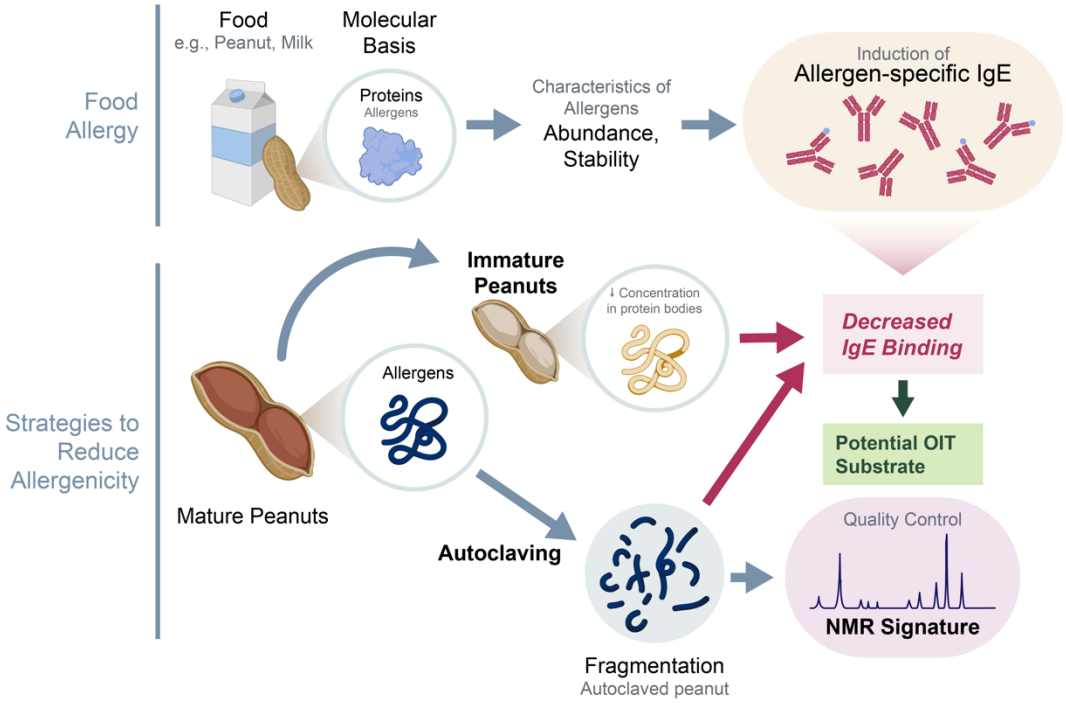

Figure 6.1. Summary Figure. The allergic reaction foods is triggered by immunoglobulin (Ig) E antibodies binding to allergens, which are generally abundant and stable protein molecules. This thesis aimed to develop novel strategies to reducing allergenicity through the development of a **modified peanut**, via processing (high-pressure and temperature autoclaving) or an immature form of the seed. Significant decreases in peanut-specific IgE binding using allergic patient sera were observed with protein extracts created from each modified peanut, demonstrating their potential as safer substrates for future oral immunotherapy (OIT) treatments.

A recent approach to improving current OIT treatments is to replace the current substrate, which is often the commonly consumed form of the food (e.g., roasted peanut), with a modified form of the food. A novel substrate can contain lower doses, altered structures, or any other variation of the protein allergens. It has been previously shown that with certain foods such as egg or milk, individuals who are allergic to these foods can often tolerate baked forms of their allergen due to the temperature-dependent denaturation of allergens.[189, 191] In some cases, frequent consumption of baked egg or milk can accelerate tolerance to the natural, unprocessed foods.[192, 194, 240] The principle is that the amount of intact allergen available to trigger IgE crosslinking and the allergic reaction is greatly reduced, while sufficient allergen matter (e.g., peptides and relevant epitopes) is still available to induce tolerance over time.

One major approach is via thermal processing, like in the case of baked egg or milk products. Peanut proteins, however, do not denature through dry roasting at elevated temperatures. In Chapter 2, the approach of high-temperature and pressure autoclaving as a novel processing method was evaluated at a range of temperatures and time durations in the context of peanut proteins and allergenicity. We found that autoclaving peanuts at 130°C for 30 minutes or greater reduced IgE binding to serum samples from highly allergic patients and decreases relative major allergen levels when compared to raw or roasted peanuts. Specifically, the heat-labile birch pollen protein Bet v 1 homolog in peanuts, Ara h 8, could not be detected in autoclaved peanut extracts, while major allergens Ara h 1 and Ara h 2 could still be detected intact at low levels. These observations can be explained by the high moisture content and vapour pressure throughout autoclaving inducing great levels of protein cleavage, disrupting the complex matrix of the peanut composition, and thus considerably reducing the proportion of intact allergens in the peanut. These

results were confirmed using mass spectrometry followed by proteomics analyses of protein extracts of each condition passed through 10-kDa filters to detect short peptides.

These findings have significant potential implications on peanut allergy diagnosis and treatment. Given the fact that autoclaved peanuts possess undetectable levels of Ara h 8 but detectable levels of Ara h 1 and Ara h 2, and given that individuals with specific IgE for only Ara h 8 are at extremely low risk of anaphylaxis to peanut,[153] we presumed that a protein extract created from autoclaved peanuts can improve peanut allergy diagnosis. As an addition to the current standard SPT extract created from raw or roasted peanuts, the autoclaved peanut extract, devoid of any detectable Ara h 8, may be used to distinguish between peanut-allergic individuals at risk of anaphylaxis (with Ara h 1- and Ara h 2-specific IgE) from peanut-allergic individuals likely at risk of experiencing only oral symptoms to peanut (with only Ara h 8-specific IgE). This led to the development of a clinical study at our research clinic evaluating the effectiveness of both protein extracts used in tandem in an improved PA diagnosis, correlating the SPT wheal diameters with total peanut- and component-specific IgE levels. These findings will be discussed in depth below.

While evaluating the allergenic potential of the autoclaved peanut, we sought to characterize the differentially processed peanuts based on their molecular profiles. This was done using Nuclear Magnetic Resonance (NMR) spectroscopy, a method that allows the analysis of intact samples *in situ* by acquiring a signature of the molecular composition. Chapter 3 describes the results observed using two complementary ^{1}H NMR spectroscopy methods. We found that upon analysis of whole, intact peanuts via ^{1}H HR-MAS NMR, lipids in the form of triglyceride molecules dominated the spectra, reflecting the major component of peanut composition,[241, 242] which remained largely unchanged following processing. We then defatted the peanuts into flour by suspending ground pastes in hexanes, removing the vast majority of the lipids. NMR analysis

of defatted flours revealed considerable information in the sugar regions of the spectra, demonstrating sucrose as the dominant sugar in peanut,[243] whose proportion and composition is also not changing significantly throughout processing. The small proportion of lipids remaining in the flours still produced considerable triglyceride peaks in the spectra, masking certain regions corresponding to protein and peptide side chains.

To circumvent this masking effect, we soaked the peanuts of each condition in water and analyzed the resulting soaked solutions by ^{1}H NMR. In this case, lipids could no longer be detected in the spectra, revealing signals corresponding to protein molecules, particularly protons (H atoms) of methyl and alkyl side chains (from 0.5 to 2.0 ppm), and aromatic and amide protons (from 6.5 to 8.5 ppm). In these specific regions, we observed significantly broader peaks in the autoclaved spectra when compared to raw or roasted soaked solutions. Broad peaks indicate a greater molecular diversity in the sample, including larger molecules such as proteins and peptides.[244] We showed that this difference once again implied that the autoclaving process is disrupting the tight, complex protein matrix of the peanut and hydrolyzing protein molecules into peptides to a point where they readily leach out into solution upon soaking. We did not observe this phenomenon in raw or roasted peanuts, but rather very sharp peaks in those regions, corresponding to far fewer varieties of peptides or amino acids in solution. This was the first indication by NMR of the marked molecular modifications induced by autoclaving when compared with raw or roasted.

Given our findings from Chapter 2 regarding the autoclaved peanut and its reduced peanut-specific IgE binging, the characteristic NMR signature observed for the autoclaved peanut can be further developed as a quality control method for food processing in the context of allergenicity. Importantly, this can also be helpful in defining mechanisms relating food processing with allergenicity, e.g., protein hydrolysis and degradation. Chapter 3 demonstrates the power and

usefulness of qualitative NMR studies on peanut analysis, which can be expanded to other foods in the future upon further research.

Another approach evaluated in parallel to processing is harvesting peanut seeds earlier in development, described in Chapter 4. This project was performed in collaboration with Ran Hovav, peanut agriculturist at the Volcani Center, and Mona I. Kidon, pediatric allergist at the Sheba Medical Centre, both in Israel. Peanuts were grown in a controlled environment and harvested at specific stages of peanut development. We received lyophilized peanut samples of each developmental stage in Montreal, Canada where my role was to characterize relative protein content, major allergen content, and peanut-specific IgE binding levels for each stage of maturity. We found that at the R5 stage (beginning seed), the peanut contains significantly lower levels of allergens Ara h 1 and Ara h 2, and specific IgE binding to sera from highly allergic patients. We also observed that these levels increased throughout peanut development. Our collaborators in Israel observed via immunohistochemical staining that Ara h 2 is more diffusely located in the cell at the R5 stage, whereas in the R7 stage, Ara h 2 is more abundant and more concentrated in protein bodies. They also used immature peanuts in SPT trials in young allergic children (median age: 3.5 years old) and observed significantly reduced wheal diameters using the R5 peanut when compared to R7 or commercial extract. Altogether, these findings suggest that harvesting peanuts earlier in development, particularly at the R5 stage, may provide another route to a safer, more efficacious OIT substrate in addition to the autoclaved peanut. Studies initiated at the at the Sheba Medical Centre in Israel are ongoing to evaluate the efficacy of the R5 peanut in peanut allergy diagnosis, tolerance induction, and sustained unresponsiveness in allergic individuals.

After optimizing the ELISA assay to quantify specific IgE (sIgE) levels in serum samples of peanut-allergic patients in Chapter 2, the assay was expanded to quantify cow's milk-sIgE levels

in our cohort of patients undergoing cow's milk OIT. Chapter 5 describes the correlations between cow's milk-sIgE levels (α-Lactalbumin (ALA), β-Lactoglobulin (BLG), casein, and total cow's milk), sociodemographic factors, comorbidities, and other clinical characteristics at baseline associated with reaching the maintenance dose of 200 mL of cow's milk. We found that elevated ALA-, BLG-, casein-, and total cow's milk-sIgE levels at baseline were associated with a decreased probability of reaching the maintenance dose of OIT, as demonstrated by Cox regression models controlling for age and sex. Additionally, an increased cumulative reactive dose at entry challenge was associated with an increased probability of reaching 200 mL. These findings have important clinical implications, providing crucial information for the allergic individual before starting therapy on how long treatment may take, the likelihood of reaching the maintenance dose, and allowing the proper allocating of resources by physicians for OIT treatments to any foods, such as cow's milk or peanut.

6.1. Future Directions

This section will discuss potential follow-up directions towards exploring and developing the outcomes of this thesis described in the previous chapters. The novel ideas and results discussed have great potential to improve the quality of life of individuals with peanut allergy in the near future upon continued research.

Firstly, it is important to highlight one of the major drawbacks of current *in vitro* experiments in food allergy, particularly in the case of peanut, which is the **solubility** of proteins and allergens. Chapters 2, 3, and 4 describe the analyses of soluble fractions of peanuts, using either protein extracts or peanut-soaked solutions. Additionally, the standard first line of diagnosis of food allergy, the skin prick test, uses soluble protein extracts, which do in fact contain multiple major allergens. However, in Chapter 2, we demonstrated a decrease in protein solubility following

processing, via both roasting and autoclaving. While we showed that the use of a strong denaturing buffer such as 7 M urea can extract more protein from processed peanuts into solution, there was still a pellet of insoluble material left over extraction, of which we cannot confirm what proportion is protein, let alone allergenic material. Moreover, the thermal processing of peanut was recently demonstrated to influence detection by analytical techniques such as the ELISA, over-detecting raw peanut 3.9-fold and under-detecting roasted peanut 3.5-fold compared with the true values.[245] When an allergic individual ingests their allergen in reality, there are no soluble or insoluble fractions, but rather all components are consumed. Thus, a method to analyze the insoluble fraction of protein extracts effectively and reliably is needed to confirm whether or not insoluble material resulting from processing can contribute to allergic reactions.

This thesis evaluates in depth the processing method of high-temperature and pressure autoclaving to decrease peanut allergenicity. One could extend the idea of processing out to other methods, either conventional or not, to disrupt the complex peanut matrix. As suggested by thesis committee member and food scientist Dr. Ashraf Ismail, an interesting novel method to evaluate is treatment by microwave heating. Indeed, the use of microwave heating treatment in the context of allergenicity has been reported for almond and cow's milk allergens.[246, 247] Microwaves are believed to enhance the rate of enzymatic hydrolysis and the combination of microwave treatment with peptic hydrolysis denatured cow's milk protein β-lactoglobulin and generated peptides with low IgE immunoreactivities.[248, 249] Given the complex protein structure of the peanut and its allergens, microwave heating treatment has the potential to disrupt the peanut matrix and is a rapid, simple processing technique worth exploring.

In parallel, the development of signatures associated with processed forms of peanut is an important avenue to explore towards understanding food molecular properties. Chapter 3 described

the establishment of a novel ^{1}H NMR profile specifically associated with the autoclaved peanut, and not with raw or roasted, via the detection of cleaved proteins into peptides in solution. Given the fact that autoclaving peanuts is also associated with reduced specific IgE binding using highly allergic patient sera, this can be further developed into a quality control method to quickly predict the presence of protein hydrolysis. Eventually, the presence of hydrolysis may act as an indirect signature for allergenicity (or the lack thereof) of food products. For example, performing similar NMR experiments on peanuts autoclaved at a range of parameters such as temperatures and time durations could reveal a threshold of autoclaving intensity that could be visualized in the NMR spectra; crossing this threshold would be indirectly correlated with reduced allergenicity of the peanut sample. Indeed, NMR spectroscopy has recently been used to detect peanut adulteration in food samples through the discovery of an isolated signal at 3.05 ppm in the ^{1}H spectrum of peanut extract corresponding to N-methyl-4-hydroxy-L-proline.[250] This could be analyzed in further depth under the context of various processing conditions, different peanut maturational stages, or other peanut variations as a marker for peanut and/or allergenicity.

The immature, R5 stage peanut is currently being evaluated clinically at the Sheba Medical Centre in Israel for both the diagnosis and the treatment of peanut allergy. Dr. Mona I. Kidon and her team at the Sheba Medical Center found that performing skin prick tests with the R5 peanut in the form of a lyophilized powder in allergic children considerably improved the positive predictive value of the outcomes of oral challenge to peanut, the current gold standard for food allergy diagnosis.[251] They are also currently conducting clinical studies evaluating the desensitization potential of the immature peanut in the context of peanut OIT and they are seeing promising results. It would be interesting to evaluate the effects of processing on these immature peanuts as

well as to establish molecular profiles associated with them through NMR spectroscopy or through other methods.

Another interesting avenue of research to pursue is through the mass spectrometry (MS) and proteomics analyses of peanuts under all the various conditions described. Along with the fact that the peanut genome and its corresponding proteome is publicly available, the power of MS allows for an accurate and quantitative measurement of peptides present in a sample. This will help researchers understand the status of proteins, including relevant allergens, under any condition evaluated, which is instrumental to developing any treatments or diagnostics in the clinic.

6.2 Clinical Studies

Ultimately, to truly make an impact on the lives of individuals suffering with peanut allergy, any of the above research must eventually be transitioned to the clinic. Following promising results with the autoclaved peanut, we performed a clinical study performing skin prick tests (SPT) on allergic subjects comparing a protein extract made from autoclaved peanuts with the current standard commercial peanut extract. Forty-five peanut-allergic subjects were recruited to our research clinic where they underwent an SPT and a blood draw for total peanut- and component-specific IgE analyses. We observed significantly lower wheal diameters using the autoclaved peanut extract (mean ± SD = 6.1 ± 5.5mm) when compared to the commercial standard (11.3 ± 6.4mm; $p<0.001$). Upon stratifying the participants into two groups, one that experienced smaller wheal sizes when using the autoclaved peanut extract (≥ 3mm less) and another with no significant decrease, those with smaller wheal sizes to the autoclaved extract demonstrated significantly lower sIgE levels to total peanut ($p=0.029$) and allergen components Ara h 1 ($p=0.015$), Ara h 2 ($p=0.007$), and Ara h 8 ($p=0.017$).

These results placed us in a position to begin a new clinical pilot study evaluating oral food challenges (OFC) with the autoclaved peanut compared to roasted. Institutional REB approval was obtained (REB study number: 2024-9682) in August 2023 to test 14 peanut-allergic subjects through double-blind OFC study where they will consume small incremental doses of autoclaved or roasted peanut, waiting 30 minutes between each dose while monitoring symptoms. Once objective signs of a reaction are observed, the challenge is stopped, and the cumulative dose of peanut is recorded. Each challenge day will be separated by a period of 3 to 6 weeks while subjects, nor the nurse providing the dose, will be aware of which form of peanut is being consumed on any given day. Based on our results described in Chapter 2, we expect that allergic individuals will be able to tolerate greater amounts of autoclaved peanut when compared to roasted.

Depending on the results obtained throughout this pilot study, once complete, we hope to be able to begin the first ever clinical trial evaluating the desensitization potential of the autoclaved peanut for the treatment of peanut allergy via oral immunotherapy (OIT). There are clinical trials ongoing evaluating peanut OIT under different protocols, such as using boiled peanut as a substrate instead of raw or roasted (ClinicalTrials.gov Identifier: NCT03937726). Boiling has shown to result in a relatively hypoallergenic product due to the loss of major allergenic components such as Ara h 2 into the cooking water.[210, 211]

Our results demonstrate that the effect of autoclaving differs from that of boiling. Autoclaving degrades peanut proteins into a wide diversity and number of peptides, as shown by MS-proteomics experiments, while boiling decreases the concentration of proteins in the peanut through their leaching out into the water throughout cooking. Therefore, while boiling may produce a “diluted” peanut, autoclaving seems to produce a differentially modified peanut where there are not solely less proteins present, but they are also in a cleaved, “pre-digested” state. This

peptide-containing peanut may have beneficial effects in terms of inducing tolerance to standard peanut food products over time by presenting different, novel peptides and epitopes to the immune system. The future of peanut allergy research is exciting.

FINAL CONCLUSION AND SUMMARY

In this dissertation, novel strategies for the treatment and diagnosis of peanut allergy were assessed. We set out to establish structure-function correlations with allergenicity using two parallel methods to improve the safety and efficacy of current peanut OIT protocols: thermal processing via autoclaving and harvesting peanut seeds at an earlier stage of development. We successfully demonstrated through laboratory experiments that both methods have the potential to create a novel OIT substrate with reduced numbers of adverse reactions throughout treatment, including anaphylaxis, as well as increased doses that can be tolerated following treatment. This, in turn, would drastically improve the quality of life of thousands of allergic individuals and their families across the globe.

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