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THE EFFECT OF EXTENDED BOILING ON PEANUT ALLERGENICITY AND ITS CLINICAL IMPLICATIONS

by

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DEDICATION

To my family: for the unwavering faith, love and support from my wife and children. I was inspired by the PhD of my first son, who achieved it when he was 20, and that of my second son, who got it despite being diagnosed with autism at the age of 2. They have made me feel young at 72, the year I completed my PhD.

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Executive Summary

Peanut allergy is the leading cause of allergy-related emergency attendances at Western world hospitals and its prevalence is still rising. While desensitisation is not a cure, it is nevertheless the only form of proactive and disease-modifying therapeutic approach that we know of in the treatment of peanut allergy. Oral immunotherapy (OIT) is one of several desensitisation methods, and a promising one, but is still not approved for general use other than in a research setting mainly because of its high rate of adverse events (45-93%). An enhanced safety profile is therefore likely to improve its acceptability.

This thesis began with a simple idea of boiling peanuts to make them hypoallergenic. It then provided the scientific evidence to underpin the idea. SDS-PAGE images confirmed progressive fragmentation of peanut proteins and leaching into cooking water as a consequence of increasing boiling time, while Western blot demonstrated corresponding reduction in human IgE allergenicity. Inhibition ELISA, which was capable of detecting conformational (B-cell) epitopes far better than Western blot, demonstrated that 12-hour boiled peanut resulted in a 19-fold reduction of allergenicity while 2-hour boiled resulted in an 8-fold reduction. Mass spectrometry confirmed the fragmentation of peanut proteins as a result of boiling, but also showed that many known T-cell epitopes were actually retained in significant quantities in boiled peanuts, with 12-hour boiled peanut containing 42 times more peptides than raw peanut. Following that, flow cytometry was used to show that T-cell reactivity did not change after extended boiling. This implied that while boiling could reduce allergenicity by altering conformational epitopes, it did not actually affect T-cell epitopes which were responsible for tolerance induction. Such a strategy of differential modification of B- and T-cell epitopes in peanut (and therefore the clinical effect) would be superior to, and more sophisticated than, simple dose-reduction as a means of minimising adverse events during oral immunotherapy. Finally, *in vivo* skin prick tests were used to demonstrate reduced skin-sensitisation with boiled peanut extracts. The results were published in *Clinical and Experimental Allergy* in July 2016.

The thesis then made a translation from scientific evidence to a proof-of-concept pilot study, using a novel oral immunotherapy protocol in which hypoallergenic boiled and then roasted peanuts were ingested in a sequential manner. Fourteen subjects were enrolled, two withdrew

due to non-compliance and social issues (both had no adverse events during treatment), but 12 were able to ingest 8-10 roasted peanuts at end of desensitisation. Three of 12 subjects showed mild adverse events in the early stages of boiled peanut desensitisation, and two of 12 subjects showed mild adverse event in the first week of roasted peanut desensitisation. This figure was considerably less than those reported in other OIT studies.

The most important message from the pilot study is that up-dosing in oral immunotherapy can be safely performed at home using the sequential method, making hospital-based supervision redundant. This is an important finding and is unique from all other OIT studies. The report was published in *Clinical and Experimental Allergy* in November 2017.

DECLARATION

This work contains no material that has been accepted for the award of any other degree or diploma to me at any previous time in any university or tertiary institution.

To the best of my knowledge and belief this work contains no material previously published or written by another person, except where due reference has been made in the text. All substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

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Billy Tao, September 2018

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ABBREVIATIONS

%	percentage
x g	x gravity
x s	x sedimentary rate (one way to characterise a protein)
ºC	degree Celsius
µg	microgram
µl	microliter
µM	micromolar
1-DE	one-dimensional electrophoresis
2-DE	two-dimensional electrophoresis
2D	two-dimensional
ABO	blood groups A, B and O
AE	adverse events
Ana o n	cashew allergens *Anacardium occidentale* number 1-3
APC	antigen-presenting cells
Ara h n	peanut allergens *Arachis hypogaea* number 1-17
BAT	basophil activation test
B-cell	lymphocyte which matures in bone marrow
BCG	bacilli Calmatte-Guerin attenuated form of tuberculosis
BCR	B-cell receptor
BP	blood pressure
BSA	bovine serum albumin
CDn	cluster of differentiation, usually followed by a number and then + or - sign
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate
CI	confidence interval
Cm	centimetre
CRD	component resolved diagnostics
CSF	colony stimulating factors
CTLA4	cytotoxic T lymphocyte antigen 4
Da	dalton
DBPCFC	double-blind, placebo-controlled food challenge
DC	dendritic cells when used in immunology

DC	detergent compatible when used in protein assay
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPIT	epicutaneous immunotherapy
FA	formic acid
FcεRI	Fc Epsilon Receptor One
FcεRII	Fc Epsilon Receptor Two
FPIES	food protein-induced enterocolitis syndrome
GE	General Electric
H_2O	water
HCl	hydrochloric acid
Hg	mercury (used as mmHg in measuring blood pressure)
HPLC	high performance liquid chromatography
IDDM	insulin-dependent diabetes mellitus
IEF	isoelectric focusing
IFNα	α-interferon
Ig	immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG4	Immunoglobulin G4
IgM	Immunoglobulin M
IL	Interleukin, usually followed by a number, e.g. IL-2
INFγ	Interferon-γ (can also be α or β)
IPG	immobilised pH gradient
iTreg	inducible or adaptive Treg
kDa	kilodalton
kU/L	kilo-unit per litre
LATS	long acting thyroid stimulator
LC-MS	liquid chromatography mass spectrometry
LEAP	Learning Early About Peanut Allergy
M	molar

mA	milliampere
MALT	mucosa-associated lymphoid tissue
M cells	Microfold cells in the gut
MHC	major histocompatibility complex (can be I or II)
ml	millimetre
mm	millimetre
mM	millimolar
MS	mass spectrometry
MS/MS	Tandem mass spectrometry
MW	molecular weight
m/Z	mass-to-charge ratio
ng	nanogram
NK	natural killer cell
nL	nanolitre
NPV	negative predictive value
nTreg	natural or thymic-derived Tregs
OFC	oral food challenge
OIT	oral immunotherapy
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PEF	peak expiratory flow
pH	hydrogen ion concentration
pI	isoelectric point
PPOIT	probiotic-peanut oral immunotherapy
PPV	positive predictive value
psIgE	peanut specific IgE
QoL	quality of life
RAST	radioallergosorbent test
RNA	ribonucleic acid
rpm	revolutions per minute
SAE	Serious adverse events
SCD	successfully consumed dose
SCIT	subcutaneous immunotherapy
SDS	sodium dodecyl sulphate

SEB	staphylococcal enterotoxin B
sIgE	antigen-specific IgE
SLIT	sublingual immunotherapy
SPT	skin prick test
TBS	tris buffered saline
TBST	tris buffered saline with Tween-20
Tc	cytotoxic T-cell
T-cells	thymus-derived lymphocyte
TCR	T-cell receptor
TGFβ	transforming growth factor-β
Th cells	T helper cells, often followed by a number, e.g. Th 2
TNF	tumour necrosis factor
Treg	regulatory T-cells
Tris	hydroxymethyl aminomethane
TT	Tetanus toxoid
V	volts
VP	Viaskin peanut skin patches
v/v	volume per volume
W	watts
w/v	weight per volume

Chapter 1

Literature Review

1.1 INTRODUCTION AND OVERVIEW

1.1.1 The beginning of immunology and allergy

In the late nineteenth and early twentieth centuries a group of scientists led by Louis Pasteur proposed "a novel biological system whose function was to defend the body from attacks by microorganisms", and named it the *immune system* (literally meaning a "system that exempts from diseases") [1]. This idea of "immune protection provided by host antibodies" was quickly embraced by their contemporary doctors and scientists, and subsequent research and development would establish Immunology as one of the fundamental pillars of modern medicine. In those early days, prior to the discovery of antibiotics, a new method of treatment for some of the notorious "killer infections" such as scarlet fever, diphtheria, pneumococcal pneumonia and tetanus had emerged as a result of this new concept. The method was to transfuse patients with passive "antitoxin serum" (or "anti-serum" in short), derived from vaccinated horses, as patients themselves could not produce these antibodies fast enough. This method became so popular that special wards in hospitals were established just to provide such treatment.

Like all medical treatments before it, complications would follow. It was soon observed that patients receiving such treatment (called serotherapy in those days) could develop unexpected reactions, which were initially attributed to non-immunological factors and later to a state of "hypersensitivity", or "super-sensitivity" (which was then a more favoured word), developed by the host to the horse serum. This complication was later called *serum sickness*, but for a long time no immediate explanation for its mechanism came to light. The symptoms were fever, skin rashes, joint pain and lymph node swelling, which usually occurred 8-12 days after the serum was administered.

In 1906 Clemens von Pirquet (1874 – 1929), then a paediatrician specialising in serotherapy and working at the Imperial and Royal Paediatric Clinic of the University of Vienna, made a radical proposition (he was also the same person who originally named this condition serum sickness) [2]. He noted that serotherapy appeared to have produced both initial *immunity* (protection), and subsequent *hypersensitivity* (harm). The prevalent consensus of opinions from doctors and scientists at the time was that the immune system was programmed only to protect, and not to harm, the host, so the complications in serum sickness must have come from the

"alien" horse serum and not from the host's immune system itself. Von Pirquet proposed that the immune system was in fact capable of doing both: on the one hand it could protect the host and fight off infections, while on the other it could also react against the serum, and then do unexpected harm to its host at the same time. He proposed the term *Allergy* (from the Greek *allos*, meaning "different", and *ergia*, meaning "action", in the sense of a "change in reactivity or capacity to react") to describe the second type of reaction from the immune system [2]. He also suggested a new word "allergen" to describe the second role of horse serum, which had now induced an altered reactivity in the host, after the initial exposure [3].

Von Pirquet's proposal was deemed too radical, and had the potential of undermining the principle of immunology prevailing at the time, so it was not well-accepted in 1906. Instead, another phenomenon called *anaphylaxis*, first proposed by Charles Richet in 1902, was easier to understand, and as a result was more readily accepted as an explanation for serum sickness. Richet was a French physiologist studying the pathological effect of toxins from marine animals (mainly the poisonous jelly fish *Portuguese man-of-war*, and sea anemone) to dogs. In order to cut cost, he and a fellow physiologist, Paul Portier, re-administered the toxins to the same dogs that had survived the first injection (in an attempt to re-use instead of to euthanize and "waste" them). They then observed some sudden and impressive deaths when these dogs were injected with the second, but only sub-lethal, doses of the toxin, with the outcome of the reaction completely "out of proportion to the second dose". Richet coined these reactions *anaphylaxis*, (against or contrary to "*phylaxis*", which means protection from infection) because the first dose, instead of protecting the dog as it should be, actually made the dog <u>hypersensitive</u> to the second injection, and as a result the dog died, even after an otherwise sub-lethal dose. However, they also observed that this would only happen if the second injection was administered <u>2-3 weeks after</u> the first one. Richet was among the many prominent scientists who had rejected Von Pirquet's "allergy" proposal when it was first published, calling it "unnecessary" (unnecessary because they thought they had already got the answer to the cause of serum sickness), and there were many similarly derisive criticisms from other influential people, including Paul Ehrlich, partly because von Pirquet did not have a good "track record" at the time. Unfortunately, these unfavourable comments sunk-in and did irreparable damage to von Pirquet's reputation: Richet received the Nobel Prize for Physiology or Medicine in 1913 but von Pirquet never got a mention. It seems ironic that nowadays Allergy is a full medical discipline while Anaphylaxis is just one type of allergic reaction, equal in status to serum sickness. To be fair, neither von Pirquet nor Richet had accurately described the complex nature

of allergic reactions (von Perquet's serum sickness is an immune-complex type reaction, while Richet's anaphylaxis is an immediate type IgE-mediated reaction), but at least von Pirquet can be credited for making an attempt to rationalise the function of the immune system, by saying that allergy is in fact an altered state of biological response, by the host, to an allergen. Nowadays von Pirquet is recognised as the "father of allergy".

When von Pirquet first coined the term *Allergy* more than 100 years ago, his original intention was to propose a fundamental explanation of an unexpected immunological reaction. However, since then the meaning of allergy has been "corrupted or perverted" according to the "purists", especially by the "lay people on the street", and would include all kinds of hypersensitivity and intolerance reactions [1]. Historically though, the major contribution of von Pirquet was not so much a word, but a fundamental biological rule which, arguably, marked the modern approach to immunology [3].

1.1.2 The classification of allergic (hypersensitivity) reactions

In 1963 Philip Gell and Robin Coombs co-edited and published a seminal textbook called *Clinical Aspects of Immunology*, and in Chapter 13 of the book they jointly wrote "The classification of allergic reactions underlying diseases" [4]. It needs to be understood that in those days, and even now, the words *allergy* and *hypersensitivity* have been used in a loosely interchangeable way. Gell and Coombs did not like to mince the word hypersensitivity with allergy, and set out to "restore the word *allergy* to its original sense, expressed with the greatest precision in the (Pirquet) paper published in 1906" [1]. Consequently, in their book they had strongly advocated to stick to the word *allergy*, and divided its reactions into types I to IV, based on their contemporary understanding of the immunological mechanisms. Ironically, with the passage of time, and increasing knowledge of immune mechanisms, their approach to the classification of "allergic" reactions has now been overwhelmingly reverted back to that of "hypersensitivity". However, their original idea of separating allergic reactions into four types is still widely accepted today. More recently, a fifth type has been "added" (*Stimulatory or Type V Hypersensitivity*), in which the antibody binds to a non-immunological receptor of a biologically active cell (e.g. the same binding-site for Thyroid Stimulating Hormone on a thyroid follicle cell), and produces a biologically positive, or stimulatory, effect - just like that of a hormone - but strictly it was not an actual "immunological" effect [5]. The best example of such an antibody is the long acting thyroid stimulator or LATS (which is an IgG antibody)

in Graves' disease, which causes over-stimulation and then excessive production of "effector" thyroid hormones. The following is a brief description of the first 4 types of hypersensitivity.

1.1.2.1 Type I Hypersensitivity

Type I Hypersensitivity is also known as Immediate or Anaphylactic Hypersensitivity. In this type of reaction, the response usually takes place within 30-60 minutes after allergen exposure, but can be even quicker (within minutes), and sometimes quite dramatic as in the case of anaphylaxis. On the other hand, in some situations a late onset reaction may occur, at about 3 to 4 hours after exposure. This late phase reaction generally peaks at approximately 6-12 hours, and subsides by about 24 hours. Sometimes the patient may have both kinds: an initial response and then a late phase one (when this happens it is called a *biphasic* reaction). Type I reactions can affect a single organ, such as the skin (urticaria, angioedema, eczema flare-up), eyes (conjunctivitis), nasopharynx (allergic rhinitis, hoarse voice), respiratory system (coughs, wheezes, breathing difficulties), gastrointestinal tract (vomiting, abdominal cramps, diarrhoea), or multiple organs, culminating in anaphylaxis or even death. Type I hypersensitivity is mediated through the interaction between an allergen and its allergen-specific IgE antibody, which is anchored to high affinity FcεRI receptors at the surface of mast cells and basophils. Cross-linking of antigens and adjacent IgE antibodies would then *amplify* the reaction, causing degranulation and release of both preformed and newly-formed inflammatory mediators from these cells [6, 7]. Examples of *preformed mediators* are histamine, tryptase, proteoglycans and chemotactic factors; and examples of *newly formed mediators* are leukotrienes, platelet activating factor PAF, bradykinin and prostaglandins. Low affinity FcεRII receptors (as different from high affinity FcεRI receptors) are expressed in macrophages, monocytes, lymphocytes and platelets, and together they play a minor role in the overall picture of Type I allergic reactions.

1.1.2.2 Type II Hypersensitivity

Type II Hypersensitivity is also known as Cytotoxic Hypersensitivity or Antibody-dependent Cytotoxicity. In this kind of immune reaction, an antigen (either *endogenous* such as the Rhesus antigen, or *exogenous* such as a bacterial antigen, or drug) is bound to the surface of a target cell, and interaction between the antigen and a circulating antigen-specific antibody (usually an IgG, or IgM) would lead to the demise of the target host cell. Classical examples of Type II Hypersensitivity are autoimmune haemolytic anaemia,

idiopathic thrombocytopenic purpura, haemolytic disease of the newborn (due to either ABO or Rhesus incompatibility), rheumatic heart disease, and some examples of drug reactions.

1.1.2.3 Type III Hypersensitivity

Type III Hypersensitivity, also called Immune Complex Hypersensitivity, is mediated by soluble immune complexes formed by the union of antigens and antibodies (mostly IgG or IgM). Deposition of the immune complexes in tissues results in a tissue-reaction that is initiated by complement activation and followed by mast cell degranulation, leukocyte chemotaxis, and further influx of immunogenic cells. After exposure, reactions may develop against antigens that are *endogenous*, as in DNA/anti-DNA/complement deposits in the kidneys of patients with systemic lupus erythematosus, or in blood vessel wall of polyarteritis nodosa, OR *exogenous* as in post-streptococcal glomerulonephritis. Von Pirquet's serum sickness is an example of exogenous Type III reaction. Such reactions usually take about 1-2 weeks to happen, because of the time it requires to gradually build up the antibody level in the blood until a critical level is reached, to form the causative immune complexes. The Arthus reaction, first discovered by Nicholas Maurice Arthus who repeatedly injected horse serum subcutaneously into rabbits and observed local vasculitis and eventually necrosis, is a localised form of Type III reaction. Also interestingly, drug treatment of a parasite infestation or leprosy can sometimes release so much microbial antigens into circulation suddenly and all at once that they then cause a Type III reaction with the existing antibodies in circulation.

1.1.2.4 Type IV Hypersensitivity

Type IV Hypersensitivity is also known as Cell-mediated or Delayed-type Hypersensitivity. Unlike types I, II, and III hypersensitivities which all involve the interaction of antigens with antibodies, type IV hypersensitivity reactions are exclusively cell-mediated, involving in particular antigen-specific effector T-cells. The term "delayed" refers to the timing of cellular response, which generally becomes apparent 48-72 hours after the antigen exposure, which distinguishes it from type I or immediate type reactions, often appearing within minutes. Type IV hypersensitivity is not represented by a single reaction; rather, it comprises a number of related responses which, at first sight, do not seem to have a lot in common, except for their cellular immune-mechanism base. So apart from the prototypic tuberculin (Mantoux) test, these different reactions may also include

cellular responses to intracellular pathogens such as mycobacteria, fungi and parasites, *graft rejection* and *graft versus host* reactions in organ transplant, *granulomatous inflammations* as seen in Crohn's disease and sarcoidosis, *tumour immunity,* some *autoimmune reactions*, and in *contact allergy* such as allergic contact dermatitis.

1.1.3 The spectrum of clinical reactions related to food hypersensitivity

An adverse reaction to food, or food hypersensitivity**,** is a broad term embracing all abnormal clinical responses following - and thought to be related to - the ingestion of a food. It can be further divided into either *food intolerance* or *food allergy*, depending on the pathophysiological mechanism of the reaction [8].

Food intolerance refers to an adverse physiological response to a food, and may be due to some inherent properties of the *food* (e.g. toxic contaminants such as scombroid or ciguatera fish poisoning, pharmacologically active components such as caffeine or tyramine), or related to the specific characteristics of the *host* (e.g. some metabolic disorders such as lactase deficiency, idiosyncratic responses to a food, psychological stress situations such as a consequence of a panic attack) [9]. Artificial food chemicals are actually not foods themselves, but are added to foods for a special purpose (e.g. to add a colour or enhance a flavour, or to preserve the food), and as a result can cause adverse food intolerance reactions after ingestion.

Food allergy, on the other hand, is an adverse reaction to food mediated by a known immunological mechanism, involving either allergen-specific IgE (IgE-mediated), or immune cells (non-IgE-mediated) mechanisms, or both (mixed IgE- and non-IgE-mediated) [10]. An *allergen* is defined as any substance that can stimulate the production of immunoglobulin IgE or a cellular immune response, and is usually a protein [11].

IgE-mediated food allergies account for most of the allergen-specific food allergic reactions that we commonly see, and the most prevalent ones are due to peanut, milk, egg and seafood. They also include some less well-known conditions such as the oral allergy syndrome (pollen food allergy syndrome) and food-dependent exercise-induced anaphylaxis (the best-known example is wheat-dependent exercise-induced anaphylaxis, mainly due to sensitivity to ω5 gliadin) [12]. Examples of *cell-mediated food allergies* are cow's milk protein-sensitive enteropathy, dietary protein-induced proctitis/proctocolitis, and food protein-induced

enterocolitis syndrome (FPIES). Examples of *mixed IgE- and cell-mediated food allergies* are atopic eczema and eosinophilic oesophagitis.

1.1.4 An introduction to the biology of peanut

Peanut (*Arachis hypogaea*) is a species in the legume family, which is known scientifically as either *Fabaceae* or *Leguminosae*. Botanically, it is similar to peas and beans but different from tree nuts. The definition of a true nut in the botanical sense is an indehiscent fruit that has a hard shell containing one or more seeds (indehiscent means that the fruit does not split naturally to release the seeds, so it has to be either ingested, partially digested, and then excreted from the host, or be left to decay in the wild under natural conditions). Because of the restriction imposed by this definition, only a handful of nuts are "true nuts" (hazelnut, chestnut, acorns), but in the culinary sense, peanuts and tree nuts are all referred to as "nuts" in common English, and peanuts are sometimes also called earthnuts. Interestingly, in China peanuts are never called nuts or fruits, but "flower born" or the more descriptive "flower born rice", referring to the kernels being a source of food, being "born" from the flowers on the plant, and also to their appearance that mimics enlarged rice. In contrast, tree nuts in China are unequivocally called "fruit kernels", or more descriptively "kernels from hard fruits".

The word *hypogaea* means "under the earth", which actually describes how peanuts are propagated. The peanut plant is an annual, herbaceous plant that grows to about 45 cm in height. The lower portion produces yellow-coloured flowers that self-pollinate, and soon after pollination the flower stalks elongate and bend downwards towards the soil, until they touch the ground. Continued stalk growth then pushes the fertilised ovaries underground, where the mature fruits develop into individual legume pods, each containing 2-4 seeds, or kernels, in general. The pods can ripen 3 to 4 months after the seeds are planted.

The four most popular peanut cultivars in Australia are Runner, Virginia, Spanish and Valencia. *Runners* are popular because they have relatively high yield, so they are often used for making peanut butter. *Virginias* have the largest kernels and account for most of the "premium-grade" peanuts sold in Australia. *Spanish* peanuts have smaller kernels covered with a reddish-brown skin, and are used predominantly in peanut candy or snack (such as peanut M&M), but significant quantities are also used for salted nuts and peanut butter. Because of their higher oil content than the other types of peanuts, they are also preferred for making peanut oil. *Valencia* are small, very sweet peanuts usually roasted or sold in the shell, or boiled but seldom used in

processed foods. The peanuts used in this study are provided by *Charlesworth Nuts* of Adelaide, which in turn sources its nuts from the *Peanut Company of Australia* (PCA). The peanuts used in this thesis for both the laboratory investigations and the clinical trial are large ("jumbo") premium grade peanuts, selected from either Runner or Virginia cultivars because of their large kernel sizes (the average weight of each nut is about 1 gram). The peanuts are supplied to Charlesworth by PCA in bulk, consisting of stacks of 25 kg bags which are then stored in a large refrigerated cold room.

Boiled peanuts are popular snacks in Southern United States, India, China, many other Asian countries, and West Africa. In many places these peanuts are simply boiled in brine with intact shells, but in China they are often boiled without shells for long periods of time and with other foods, such as in soups, cooked foods and rice porridge (congee). The average boiling time is about 2 hours.

1.1.5 The first scientific paper that linked boiling to reduced peanut allergenicity

In 2001 Beyer et al. published a paper on the effect of boiling on peanut allergenicity [13], and noted that the prevalence of peanut allergy in China was significantly lower than that in the United States despite high consumption. The authors hypothesised that the disparity could be because of different methods of cooking peanuts before consumption in the two countries (peanuts in China were usually boiled while peanuts in US were usually roasted, including the making of peanut butter). They demonstrated that peanuts that had been boiled for 20 minutes at 100˚C were less allergenic than roasted peanuts for similar period at 170˚C. Their results were later confirmed by several other studies, but none of the papers had boiled peanuts for longer than 60 minutes (for more detailed discussion see Section 1.6).

1.2 CLINICAL ASPECTS OF IgE-MEDIATED FOOD ALLERGIES

1.2.1 The prevalence of food allergies

As recently as 2010, a comprehensive review of food allergy literature up to that time stated that "food allergy affects more than 1% to 2%, but less than 10%, of the population, and it

remains unclear whether the prevalence is increasing" [14]. However, in the ensuing years an ever-enlarging body of evidence has emerged and provided higher estimates of food allergy prevalence as well as an upward trend. Gupta et al. [15] used an electronic US household survey (n=38,480) in 2009-2010 and estimated that 8% of American children had food allergy, 2.4% had multiple food allergies, and approximately 3% experienced severe reactions. Soller et al. [16] surveyed 9667 subjects from 10 Canadian provinces for self-reported food allergy and found an overall rate of 8%. When they excluded adults reporting unlikely allergies, and adjusted for non-responders, the final estimates were 6.7% for the overall population, with 7.1% for children and 6.6% for adults who had reported a food allergy. Cow's milk (2.2%), peanut (1.8%) and tree nuts (1.7%) were the most common allergens in children, and shellfish (1.9%), fruits (1.6%), and vegetables (1.3%) were the most common allergens in adults. Using food allergen-specific IgE results obtained in the National Health and Nutritional Examination Survey (NHANES) in the United States (2005-2006), Liu et al. [17] estimated that allergies to cow's milk, egg and peanut were 1.8% each in children age 1 to 5 years.

Two recent studies have used oral food challenges (OFC) as the definitive diagnostic tool in their prevalence survey. In the first paper, Osborne et al. [18] evaluated a population-based cohort of 2848 (73% participation rate) 1-year-old infants in the capital city of Melbourne in Australia, and reported the following prevalence of food allergies: peanut 3.0%, raw egg 8.9%, and sesame 0.8% (it must be remembered that the participants of this study were 1-year old infants, and not surprisingly there was a disproportionally higher incidence of egg allergy among this age group). In the second paper, which was a UK-based study on early childhood peanut allergy, Nicolaou et al. [19] used a combination of component-resolved diagnostics and OFCs in their methodology, and estimated that peanut allergy prevalence was 2% for children at age 8 years. Taken together, these studies pointed to a prevalence of food allergy at 5% for adults and 8% for children, with peanut allergy prevalence around 2% in children [20].

In a different approach, based on analysing changing demand for specialist food-allergy services as a *surrogate* for changing prevalence of food allergy in children who were aged 0-5 years and living in Australian Capital Territory (ACT or Canberra) between January 1995 and December 2006, Mullins [21] found that 47% of patients (697/1489) seen in a private allergy practice had food allergy, (175 with food-associated anaphylaxis), most commonly to peanut, egg, cow's milk and cashew. Over a 12-year period covering the study, the number of children in this age group, evaluated each year, had increased more than fourfold, from 55 cases in 1995

to 240 in 2006, but the number with food allergy had increased 12-fold, from 11 to 138 patients (and from 20.0% to 57.5% of children seen). The number with food anaphylaxis also increased from 5 to 37 children (9% to 15.4%) over the same period. There were similar trends in age-adjusted all-Australian hospital admission rates for anaphylaxis in children aged 0-4 years, showing that the number had increased from 39.3 to 193.8 per million population between the financial years 1993-94 and 2004-05, which was a substantially greater increase than the population as a whole (36.2 to 80.3 per million population). In a later paper, also by the same author and based on Australian Capital Territory population but focused on peanut allergy [22], the minimum incidence of peanut allergy and peanut sensitisation in ACT residents by age 72 months (6-years) was found to be 0.73% and 0.84% respectively, for those born in 2001, but for children born 6 years later in 2007 the corresponding figures were increased to 1.15% and 1.53% respectively.

There were a number of other studies outside Australia that showed similar rising trend of food allergy prevalence. A US study by Sicherer et al. [23], who conducted repeated surveys of self-reported peanut allergies in children on 3 separate occasions from 1997 to 2008, found that the prevalence rate of peanut allergy had increased significantly from 0.4% to 1.4% over the 10-year period. Two more publications, also on peanut allergy, reported similar increases, with doubling in UK [24] and tripling in the US [25]. Finally, two recently published review papers, more broadly on food allergy, had echoed the trend of a world-wide increase in food allergy prevalence [26, 27].

1.2.2 The natural history of some well-known food allergies

The majority of food allergies tend to resolve naturally, albeit at different speeds and different proportions between different kinds of foods [26, 28]. In general, childhood food allergies to milk, egg, wheat, and soy typically resolve during childhood, while allergies to peanut, tree nuts, fish and shellfish are more likely to persist into adult life.

A **cow's milk allergy** study in 1994 reported a resolution rate of 45 – 50% at age 1, 60-75% at age 2, and 85-90% at age 3 [29]. In a more recent study in 2007, which followed children up to a much older age, the resolution rate was reported as 19% by age 4, 42% by age 8, 64% by age 12, and 79% by age 16 [30], which means that 21% of their 16-years old cohorts were still milk-allergic at the end of study. The authors found that this latter group of children all had increased

concentration of cow's milk IgE at all time or ages during study, suggesting that increased cow's milk IgE level could be an important predictor for unfavourable outcome.

A similar development of tolerance has been recorded in children with **egg allergy**. Resolution was reported to happen in 4% of children by 4 years of age, 12% by 6 years of age, 37% by 10 years of age, and 68% by 16 years of age. Again, children with increased concentration of egg IgE at all ages, and those with egg IgE greater than 50kU/L, were unlikely to develop egg tolerance later in life [31].

Similarly, about 50% of children with **soy allergy** outgrew their allergy by 7 years of age, with a peak IgE concentration greater than 50 kU/L predictive of an 18% rate of resolution [32]. **Wheat allergy** follows the same pattern of tolerance as egg allergy in children, with rates of resolution of 29% by 4 years of age, 56% by 8 years of age, and 65% by 12 years of age [33]. In this population, the median age of resolution was about 6.5 years, with wheat allergy persisting into adolescence only in a few children. Increased concentrations of wheat-specific IgE levels were associated with poor outcomes, although many children with high concentrations of wheat IgE still managed to outgrow their allergy.

In contrast to the above four types of food allergies, those allergies caused by peanut or tree nuts, sesame seed, and seafood are more likely to persist into adulthood. Interestingly, they are also more likely to be associated with severe or even fatal anaphylaxis, especially for peanut and tree nut allergies [34]. Only about 20% of children with **peanut allergy** [35] and less than 10% of those with **tree nut allergy** [36] can become tolerant to these nuts 10 years after diagnosis. Peters et al. of the HealthNuts study [37] recently followed up 156 challenge-confirmed 1-year old infants with peanut allergy, and rechallenged them 3 years later, at the age of 4. They found that 22% of their peanut allergy cases had resolved by that time. In addition to a poor resolution record, peanut allergy is also known to be able to recur after initial tolerance, with a reported recurrence rate of 8% [38, 39]. Allergy to **sesame** is increasingly detected in many countries, and has an 80% rate of persistence [40]. Also, 80% of patients with **Fish and shellfish allergy** are reportedly to be still allergic 10 years after the initial diagnosis [41].

The rates of natural resolution of these food allergies are summarised below, in Figure 1.2.2.

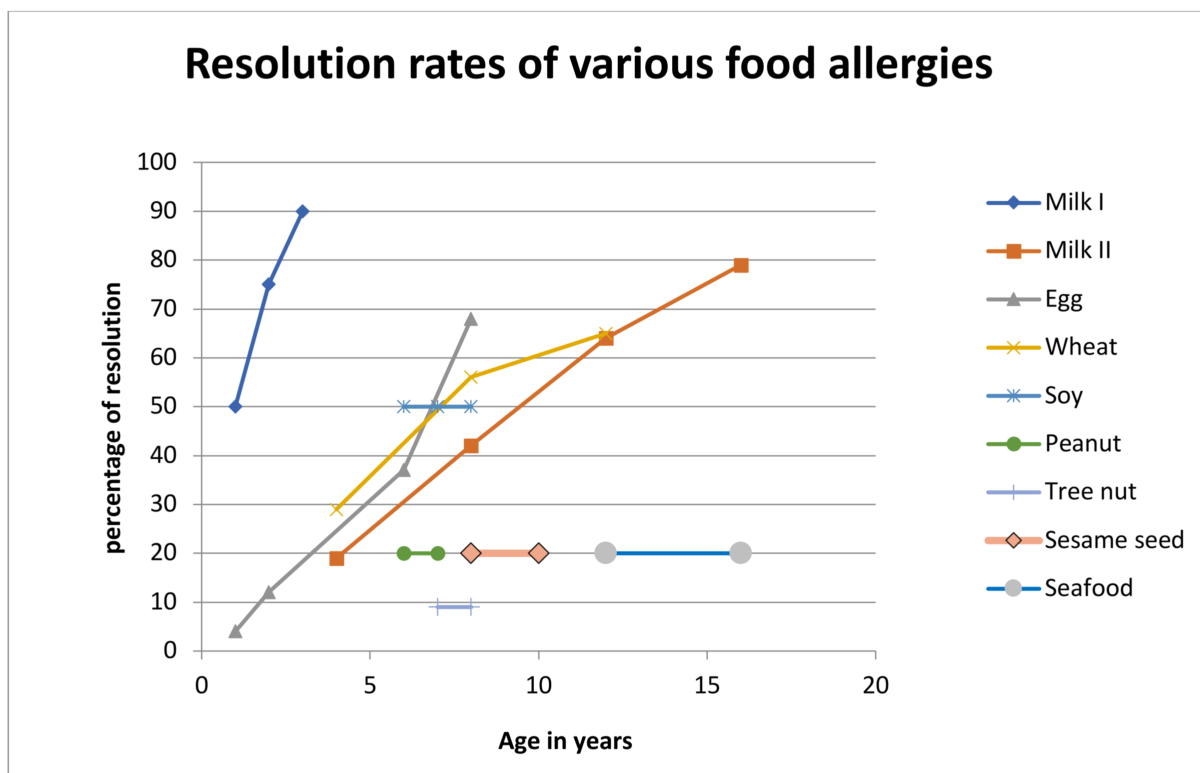


Figure 1.2.2: Milk allergy has the highest natural resolution rate compared to peanut, tree nut, sesame seed and seafood; egg, wheat and soy are in the middle.

1.2.3 Some special features of peanut allergy

For most affected children, the first allergic reaction following their experience of ingesting peanut typically occurred before the age of 2, at a median age of 14-24 months [42, 43]. About 80% of them did not have a known previous exposure, suggesting that they were probably unknowingly sensitised to peanut very early in life. For those children who had experienced more than one accidental exposures to peanut, subsequent reactions could become more severe than the first one [44], but this is not inevitable. In one study to clarify this issue, it was reported that up to a third of patients with initial mild reactions could develop more severe symptoms on subsequent exposures [45], but it also meant that the majority of subsequent reactions in peanut allergic children were actually similar in severity [42, 45].

Many peanut-allergic patients can also be simultaneously *sensitised* (i.e. tested positive but have not actually ingested any) or *allergic* (i.e. having experienced a clinically convincing allergic reaction after an ingestion) to one or more tree nuts, suggesting possible cross-reactivity between the two kinds of nuts, even though they are biologically different. In a study that

followed the progression of nut-allergic children at different ages, Clark et al. [46] found that many children who were initially allergic to a single nut, or only a few nuts, could later become allergic to multiple new nuts. In that study, they prospectively enrolled 784 nut-allergic children with a clear allergic history and presence of either nut-specific IgE or positive SPT ≥3 mm to any of the following nuts: peanut, brazil nut, almond, hazelnut and walnut. The main outcome measures of their study were mono- or multi-sensitisation (i.e. based on a positive SPT), and mono- or multi-allergy (i.e. based on clinical allergy to one or more nut types). At enrolment, about 19% by 2 years of age were multi-sensitised and 2% multi-allergic, but by 14 years 86% were multi-sensitised and 47% multi-allergic.

1.2.4 Tree nut allergies

The prevalence of tree nut allergies, like that of peanut allergy, has also been escalating in recent years [37]. It has been reported that in the United States tree nut allergies affected 1.1% of children younger than 18 years, and 0.5% of adults [23]. However, in a more recent study in Australia, on the prevalence of "clinic-defined" food allergy in early adolescence, based on history, sensitisation data, and OFC results, the prevalence of peanut allergy was found to be 2.7% and that of tree nut allergy 2.3% in their cohort [47]. In USA, walnut was the most common tree nut allergy, closely followed by cashew [48], while in some European countries hazelnut allergy was ranked number 1 among tree nuts [49]. In both Australia and Germany, cashew nut was the leading candidate for tree nut allergy, surpassed only by peanut allergy in prevalence [18, 50].

Severe clinical reactions, including higher incidence of anaphylaxis, seem to occur more frequently in cashew nut than in peanut allergy, according to a case-matched comparison study [51]. It has also been estimated that at least 86% of subjects who are already tree-nut allergic can be also allergic to multiple other tree nuts [46]. Cross-sensitization to pistachio, hazelnut and walnut is common within cashew-allergic subjects, especially to pistachio [52-54] .

As cashew is the most important cause of tree nut allergies in Australia, we shall mention briefly the known cashew nut allergens (*Anacardium occidentale*), but not those of the other tree nuts. So far three major cashew allergens have been reported: *Ana o 1*, a 7s vicilin-like protein [55], *Ana o 2*, an 11s legumin-like protein [56], and *Ana o 3*, a 2s albumin [57]. All three allergens are classified as storage proteins. Currently, no formal clinical trial of oral immunotherapy has been reported on any tree nut allergy, including cashew [58].

1.2.5 The diagnosis of food allergies, in particular peanut allergy

1.2.5.1 Introduction

As is always the case, the most important starting point in making a diagnosis of food allergy is a carefully taken medical history, accompanied by a clinical examination. After the clinical diagnosis, a double-blind, placebo-controlled food challenge (DBPCFC) is considered to be the "gold standard" diagnostic test for all food allergies [59]. However, DBPCFC is a time-consuming and resource-intensive procedure, and has potential risk of inducing anaphylaxis during challenge, partly because of its blinded nature. Consequently, a simplified and less stringent modification of the test is more frequently used in practice, which is called a simple oral food challenge or OFC. OFC is an open challenge and is neither double-blinded nor placebo-controlled, but is still a high-risk procedure that needs to be done in a hospital setting, on selected patients only, or in a research setting. In most cases, patients with a clinical diagnosis of food allergy are not orally challenged, but undergo one or more of the following tests to confirm the diagnosis.

1.2.5.2 Skin Prick Test (SPT)

In Australia, the most commonly used and office-based diagnostic test for food allergy is the skin prick test (SPT), usually done as a confirmation test following a positive clinical history. It involves the application of a food-allergen extract onto the skin, to be followed by a slight prick or puncture to the skin using a small, sterile and single-use implement called a lancet. Allergic patients at the test will develop a local reaction in the form of a wheal, and the size of the wheal is read approximately 15 minutes after the prick. A positive SPT is defined as having a wheal size ≥3mm, and has a positive predictive value or accuracy (PPV) better than 50% in peanut allergy, but the negative predictive value (NPV) is >95% [60]. Using freshly prepared extract from crushed raw peanuts (1:10 wt/vol), Rance et al. showed that the NPV approached 100% [61]. The larger is the size of a wheal on the skin test, the more likely will a patient react to that food being tested [62, 63], so for peanut allergy if the positive SPT cut-off is raised to ≥8mm the corresponding PPV is improved to 78% [64] or 94% [65], and to 96% [66] for tree nut allergy. It becomes 100% for peanut allergy if SPT ≥15mm [64]. Unfortunately, the size of the wheal does not provide specific information on the severity of the patient's allergic reaction [59, 62]. As well, accuracy can be affected by such factors as antihistamine use (false negative) and pre-existing atopic dermatitis (false positive). It has been proposed that titrating allergen

extracts in serial dilutions may increase accuracy by up to 99% [67], but in the same paper the authors also highlighted the fact that SPT readings do not follow a straight linear line, and it would take a 256-fold dilution of the extract (which was hen's egg in that paper) just to achieve a 50% reduction in wheal size.

1.2.5.3 Allergen-specific IgE (sIgE) level in blood

The next popular investigatory tool used for the diagnosis of food allergy is the allergen-specific IgE (sIgE) level in a patient's blood. It is usually performed on serum via a solid-phase ELISA methodology, using a commercial technology called ImmunoCAP® (Phadia AB, Uppsala, Sweden). The values of sIgE have been shown to correlate well with many food allergies in terms of the likelihood of a clinical reaction [68], and in the case of peanut allergy, a sIgE level 15 kU/L is considered to have a PPV of 95% [69]. In patients aged 4-11 months, the measurements of sIgE are actually considered to be more sensitive than SPT [70]. Although some guidelines are predictive of a 95% chance of reaction [63], it is important to note that in 10-25% of clinically positive cases, sIgE levels can still be virtually undetectable [68].

1.2.5.4 Combining SPT with sIgE to achieve better PPV

When results of SPT and sIgE are combined together to help make a decision or interpretation for a case, the accuracy is enhanced. Rance et al. compared the outcome of 363 cases of DBPCFC with combined results of SPT and psIgE readings, and reported that either skin-prick test wheal size ≥16mm or peanut-specific IgE levels ≥57kU/L would achieve 100% specificity and PPV in diagnosing peanut allergy [71], and obviate the need for OFC in these patients. Taking a different approach, in a study on 49 preschool children aged under 5 years with peanut sensitisation (SPT ≥ 2 mm or peanut sIgE ≥ 0.35 kU/L) who were subsequently challenged with an OFC, Johannsen et al. [72] showed that at least half of preschool children with peanut sensitisation (but no antecedent history of peanut ingestion) could actually tolerate peanuts. A SPT of < 7 mm and peanut sIgE < 2 kU/L can identify children most likely to tolerate peanut, with only a 5% likelihood of failing an oral challenge.

In 2013 Peters et al. of the Australian HealthNuts study [73] made a comprehensive analysis of 5276 participants on the values of skin prick test responses and allergen-specific IgE levels as predictors of peanut, egg and sesame allergy in infants, and concluded that

peanut SPT readings of 8 mm or greater (95% CI, 7-9 mm) and peanut sIgE levels of 34 kU/L or greater (95% CI, 14-48 kU/L) had 95% PPVs for challenge-proven peanut allergy.

In Australia, and particularly for patients who have provided a good clinical description of a food-related allergic reaction, SPT is much more commonly used than sIgE (either alone or in combination with the SPT), and most practising allergist would be satisfied with a single SPT result as confirmation of a positive clinical history, rather than performing both. The SPT is also less invasive than a blood test. There are other factors to consider too: e.g. the results of a SPT is instantly available once it is done, while a blood allergen-specific IgE test may take days or weeks before the results are posted to the doctor; a SPT is also much less expensive to perform, and can test against 20-30 foods all at once, while the government-sponsored Medicare Benefit Schedule would only cover the cost of measuring 4 items in a standard sIgE test (still often wrongly called a RAST test in Australia by some of the older generation of medical practitioners).

The combination of SPT and peanut specific IgE (psIgE) can be useful in a large clinical trial when the subject number is high, say over 100. Although OFC is still the gold standard for confirming peanut allergy, it is expensive, time consuming, and not without risk. If one can set the standard of both SPT and psIgE at PPV better than 95% (e.g. SPT >8mm and psIgE >15kU/L), it may be possible to carry out a large clinical trial without the burden of having large numbers of OFC, provided that the number of subjects in the study is sufficiently high.

1.2.5.5 Component Resolved Diagnostics (CRD)

A recent advance in the diagnosis of peanut allergy is the use of Component Resolved Diagnostics (CRD) [19, 68, 74]. In CRD, a pure allergen is generated either from a natural source of food item or through a recombinant expression of allergen-encoding DNA, which is then used for testing in either SPT or sIgE. In the case of peanut allergy, it has been reported that measuring sIgE to Ara h 2 of peanut is much more precise than that to whole peanut allergen, and can be done with a narrower cut-off reading than the latter (97% accuracy in the former using Ara h 2 sIgE >0.35 kU/L, compared to 82% accuracy using peanut sIgE >15 kU/L) [75]. Similar data have been confirmed in a more recent study by Lieberman et al. [76]. Eller et al. similarly reported that the best correlation between component-specific IgE and clinical thresholds following peanut challenges was found for

Ara h 2, and a cut-off for Ara h 2 >1.63 kU/L yielded a specificity = 1.00, with a corresponding sensitivity of 0.70 [77].

In the HealthNuts study of Australia, Dang et al. [78] quantified peanut sIgE and Ara h 2 sIgE in 200 infants who were all tested positive (sensitised) to peanut on SPT. After OFC, 100 of them were found to be allergic to peanut and 100 were actually tolerant. By using a previously published 95% PPV of 15kU/L for peanut sIgE, a corresponding ***specificity*** of 98% (95% CI, 93% - 100%) was found in this study. At the equivalent Ara h 2-specific IgE *specificity* of 98%, the ***sensitivity*** of Ara h 2 sIgE was 60% (95% CI, 50% to 70%), meaning that Ara h 2 sIgE could correctly identify 60% of subjects with true peanut allergy, while peanut sIgE could only identify 26%. The paper also stated that by using a combined approach of plasma sIgE testing for whole peanut followed by Ara h 2 for the diagnosis of peanut allergy, the number of OFC required was reduced by almost two thirds.

CRD can also be proven useful in predicting the persistence and severity of peanut allergy [75, 79], and it has been shown that positive sIgE to Ara h 1, Ara h 2, and Ara h 3 are indicative of severe and persistent peanut allergies, while Ara h 8 binding is associated with allergy in only 17% of patients. However, mono-sensitisation to Ara h 8 can also indicate tolerance, suggesting that CRD could be useful in discriminating between different allergic phenotypes. Sensitisation to Ara h 9 is linked to peanut allergy in the Mediterranean, suggesting that CRD may be useful in investigating regional differences [80].

1.2.5.6 Basophil Activation Test (BAT)

Basophil Activation Test (BAT) is an *ex vivo* cellular test, making use of the fact that cross-linking of an allergen to the corresponding allergen-specific IgE that is bound to the surface of basophils would lead to subsequent activation of these cells, marked by increased expression of CD63 and CD203c when measured using flow-cytometry. It is a worthwhile investigatory tool given that basophil suppression is associated with desensitisation in immunotherapy [81, 82]. However, it is also time- and resource-intensive, and the need for flow-cytometry expertise has limited their use in routine testing, outside that of a research setting. This assay does hold promise for monitoring allergic patients on immunotherapy, but must be standardised to allow for comparison between patients from different research laboratories, and hence different studies [83].

1.2.6 Anaphylaxis: a severe form of acute (Type I) allergic reaction

Anaphylaxis has been dubbed "the latest allergy epidemic" [84], with Australia and USA having some of the highest severe-anaphylaxis rates among developed countries [85]. In an epidemiological study of fatal anaphylaxis in the United States from 1999 to 2010, Jerschow et al. [86] analysed 2458 anaphylaxis-related deaths in that period, and found that medications were the most common cause of such deaths (58.8%), with food allergy fatalities being third in place (after venom anaphylaxis), and accounting for 6.7% of deaths. In an earlier and smaller study in 2007, but exclusively on food-related anaphylactic deaths in US, Bock et al. [87] identified 31 deaths (age 5-50 years) from a registry kept by members of the American Academy of Allergy, Asthma and Immunology and the Food Allergy and Anaphylaxis Network from 2001 to 2006, and found that peanut allergy accounted for 17 of them, tree nuts for 8, milk for 4, and shrimp for 2. Excluding the small percentage of cases that end up fatally, anaphylaxis affects approximately 150,000 Americans a year [88], with food allergy the most frequent cause among children (unlike adults). Pollen allergy and asthma are found to be important risk factors for food-induced anaphylaxis [89]. Based on three European population-based studies, prevalence is estimated at 0.3% (95% CI, 0.1-0.5) and case fatality rate less than 0.001% [89].

Anaphylaxis is defined as a "severe, life-threatening generalised or systemic hypersensitivity reaction" by the Nomenclature Review Committee of the World Allergy Organisation [90], which has also published guidelines for the assessment and management of anaphylaxis from 2011 to 2013 [91]. The European Academy of Allergy and Clinical Immunology, in its own guidelines in 2014, added that "this is characterized by being rapid in onset with life-threatening airway, breathing, or circulatory problems and is usually, although not always, associated with skin and mucosal changes" [92]. However, many lay people, and even some doctors, have a habit of ignoring this fact and calling all acute (Type I) allergic reactions "anaphylaxis", but in truth mild allergic reactions (such as mild urticarial or angioedema, mild conjunctivitis or rhinitis) contradict the meaning of "severe" and should not actually be called anaphylaxis, or even be graded as "mild anaphylaxis". The following clinical criteria for the diagnoses of anaphylaxis were proposed by the *European Academy of Allergy and Clinical Immunology* [92]:

- Anaphylaxis is highly likely when any one of the following three criteria is fulfilled:
  Acute onset of an illness (minutes to several hours) with involvement of the skin, mucosal tissue, or both (e.g., generalized hives, pruritus or flushing, swollen lips–

tongue–uvula AND AT LEAST ONE OF THE FOLLOWING: a. Respiratory compromise (e.g., dyspnoea, wheeze–bronchospasm, stridor, reduced PEF, hypoxaemia); b. Reduced BP or associated symptoms of end-organ dysfunction (e.g., hypotonia, collapse, syncope, incontinence).

- Two or more of the following that occur rapidly after exposure to a likely allergen for that patient (within minutes to several hours): a. Involvement of the skin–mucosal tissue (e.g., generalized hives, itch-flush, swollen lips–tongue–uvula); b. Respiratory compromise (e.g., dyspnoea, wheeze–bronchospasm, stridor, reduced PEF, hypoxemia); c. Reduced BP or associated symptoms (e.g., hypotonia [collapse], syncope, incontinence); d. Persistent gastrointestinal symptoms (e.g., crampy abdominal pain, vomiting).

- Reduced BP after exposure to known allergen for that patient (minutes to several hours): a. Infants and children: low systolic BP (age specific) or >30% decrease in systolic BP*; b. Adults: systolic BP of <90 mmHg or >30% decrease from that person's baseline.

Notes: PEF, peak expiratory flow; BP, blood pressure. *Low systolic blood pressure for children is defined as <70 mmHg from 1 month to 1 year, less than (70 mmHg + 2x age) from 1 to 10 years, and <90 mmHg from 11 to 17 years.

Although the majority of anaphylaxis situations are diagnosed based on clinical grounds, there are times when a diagnosis is uncertain (e.g. asthma symptoms in a known food-allergic person who has just eaten in a restaurant, suspected syncope or cardiovascular collapse, severe nausea or persistent vomiting in a known allergic person who has just ingested a food), and a laboratory test may be required to confirm the diagnosis of anaphylaxis. This is usually done by measuring serum *Tryptase* level of the patient, at the earliest opportunity following presentation [93]. Tryptase is one of the most abundant preformed mediators in mast cells, and is released after activation in an IgE-mediated Type-I allergic reaction. It usually peaks one to two hours after an anaphylactic reaction, so the test must be done within a few hours of an event, and it is still possible that it has already missed the peak. It is generally accepted that a reading >11.4 ng/ml is suggestive of anaphylaxis, but a normal reading does not exclude the diagnosis. A more accurate approach is to measure serial Tryptase levels (at presentation, 1 hour later, and prior

to discharge) [94, 95], but this is relatively time consuming and expensive. More recently, work has been done to evaluate whether basal Tryptase levels may be useful in predicting the likelihood of anaphylaxis in patients with bee venom allergy [96] and food allergy [97], with promising preliminary results.

1.2.7 Oral food challenge (OFC) tests

In 1976 Charles May described for the first time the idea of a double-blind, placebo-controlled food challenge (DBPCFC) test for the objective clinical and laboratory studies of immediate hypersensitivity reactions to food [98], initially in asthmatic children. Since then it has become the gold standard for accurate diagnosis of all food allergies. May had stressed in his paper that a negative challenge result should be followed up with an open feeding of the challenged food, prepared in the usual way, and given in a normal proportion at a later time point, before a patient could be defined as truly non-allergic. This is to ensure that the use of progressive and rapidly increasing challenge doses has not led to the theoretical "*rapid desensitisation*" of the patient, resulting in a "false-negative" challenge. More recently, Niggemann et al. highlighted this potential problem in a study [99], and used lower initiating doses, semi-logarithmic dose increases, and more prolonged periods between doses to avoid rapid desensitisation, as advocated by some investigators. Based on their data, the authors recommended that accurate oral food challenge should include a cumulative challenge dose on a subsequent day if the initial result was negative. Overall, 13% of negative incremental challenge results were subsequently found to be positive when given as a cumulative dose, compared with 1% to 3% reported by an earlier study [68].

There has been some debate about whether simple, non-DBPCFC type of OFCs should be done in an open or double-blind fashion. In an open OFC, the food is given in its natural form. For a single-blind OFC, the food or placebo is given in a vehicle that disguises the appearance and the taste of the food, and the patient is unaware of the nature of the food given, whereas the staff involved in the procedure have this information. For DBPCFCs, none of the parties involved is aware of the composition of the product. Although DBPCFC is generally the preferred scientific research protocol, it is time-consuming, expensive, and troublesome for physician and patients, especially if the latter are very young children. Consequently, open challenge may be indicated in infants and children younger than 3 years of age, according to a review by the European Academy of Allergology and Clinical Immunology in 2004 [100]. In 2010, a US National Institute of Health-sponsored expert panel, who published the "Guidelines

for the diagnosis and management of food allergy", reaffirmed the utility of the DBPCFC for the diagnosing of food allergy after an extensive review of the current literature [101]. However, they also recommended that open or single-blind challenges should be acceptable when the challenge outcome is negative, or when objective symptoms are elicited which exactly recapitulate the history of the reaction. In 2012, a full consensus document on the conduct and interpretation of the DBPCFC was published in the Journal of Allergy and Clinical Immunology [59].

Placebo challenges are indicated if day-to-day variation plays a major role in symptoms, for example in children with atopic dermatitis, or in cases when there are subjective symptoms such as abdominal discomfort, a burning sensation on the tongue, or palpitations [61, 102]. On the other hand, placebo reactions have also been reported even in double-blind, placebo-controlled food challenges in children [103].

1.2.8 Psychosocial impact of food allergy on young patients and their families

It is obvious that food allergy would have a profound psychosocial impact on children, adolescents and their families, on many aspects of daily living and Quality of Life (QoL). In particular, the constant need for vigilance to avoid allergens and daily management of food allergy impacts on family activities and social events have taken their toll. When young patients themselves were asked to report on their own QoL, Avery et al. [104] found that children with peanut allergy reported lower QoL scores than children with insulin-dependent diabetes mellitus (IDDM), and were more afraid of accidentally eating peanuts than children with IDDM being afraid of having a hypoglycaemic event. Sicherer et al. [105] found that parental perception of general health were significantly decreased if their children had food allergy compared to healthy general population norms. Ostblom et al. [106] found that parents of 9-year-old children with food allergy reported that their children had significantly worse physical functioning, more social limitations and poorer general health than children with non-food-related allergic diseases and children with no allergic diseases. Those with high levels of food-specific IgE antibodies also had poorer mental health, or even general health.

A number of studies have reported that family activities were often restricted after having a child with food allergy. Primeau et al. [107] compared adults and children with nut allergy to those with rheumatological disease using the ***Impact on Family Questionnaire***, and found that parents of children with nut allergy reported more disruption to daily activities, and more family

disruption, as a direct consequence of the nut allergy. Interestingly, the simple performance of a food allergy challenge test alone would already cause a favourable impact on the quality of life of patients, regardless of whether the result was positive or negative [108]. In that study, 54 children received oral food challenges, 25 were positive (allergic) and 29 were negative (non-allergic), yet the ***Food Allergy Quality of Life*** (FAQoL) questionnaire improved significantly for both groups, from 2 months pre-challenge to 2 months post-challenge. Unfortunately, the improvement began to decrease at 6 months post-challenge in the allergic patients, while the non-allergic group continued to enjoy improved QoL.

Bollinger et al. [109] investigated the care-giver's perspective of the impact of a child's food allergy on different aspects of daily life, including daily social events, field trips, parties, sleepovers and playing at friends' houses, and half of their families reported significant disruption to these aspects of their lives, and many parents would rather minimise the risk or anxiety induced by such activities by avoiding them altogether. The restrictions that food allergy places upon an individual's social activities is supported by the fact that, following a negative food challenge, the social life of the child and family has been shown to significantly improve as a result [110]. Many parents also found it difficult to separate from their children after the diagnosis of a food allergy. Because of that, parents with allergic children often accompany them in social situations beyond the age at which non-allergic children are accompanied. Although this hyper-vigilance is imposed by parents, they themselves have expressed concern over the effect that such over-protectiveness may have on their children [111].

Eating outside the home has always been a major cause of anxiety in families with children who have significant food allergies, such as peanut allergy. A study of over 200 cases of anaphylactic reactions in the UK showed that most cases of food-induced anaphylaxis occur outside the home [112], and of which 25% have occurred whilst dining at restaurants and 15% have occurred whilst at school or work. In another study of fatalities due to food-induced anaphylaxis, 20 out of the 31 people had their reactions occurring in an away-from-home environment, including restaurants, schools, workplace and friends' houses [87]. Not surprisingly, parents would dread taking their children to eat away from home, and when they do, many would prefer to take them to the same restaurants that they have tried before and known to look after their allergic clients [104].

Food allergy can also have significant direct impact on parents and siblings, who are often forced to share the same restriction put in place because of their relationship with the child. In some cases, it was observed that siblings often avoided the allergenic food themselves [111]. Therefore in terms of food limitations all family members are similarly affected simply by living with the allergic patient [113].

A comprehensive list of previously published papers on the psychosocial impact of food allergy and food hypersensitivity in children, adolescents and their families was reviewed and tabled by Cummings et al. in 2010 [114].

1.3 IMMUNOLOGICAL ASPECTS OF IgE-MEDIATED FOOD ALLERGIES

1.3.1 Introduction

The immune system has four main functions, or abilities, to protect its host from external threats caused by alien pathogens: (1) to recognise the antigens, (2) to activate effector responses to them, (3) to regulate specific immune reactions and (4) to create immune memories. Immunity can be classified as either *innate* or *adaptive*, based on the modus operandi of immune responses. The immune system has two main arms: one formed by effector cells joining forces to become what is called *cellular immunity*, and the other by antibodies and a variety of immuno-active molecules to become what is called *humoral immunity*. The origin of the immune system is created from the pluripotent haematopoietic stem cells. Out of these stem cells arise two important lineages, derived respectively from a common myeloid progenitor and a common lymphoid progenitor. The *myeloid lineage* comprises most of the cells of innate immunity (neutrophils, eosinophils, basophils, mast cells, macrophages), and the *lymphoid lineage* comprises the natural killer cells (of innate immunity), lymphocytes and dendritic cells (of adaptive immunity).

There are two kinds of lymphocytes, namely B lymphocytes (B-cells, or lymphocytes that mature in bone marrow or come from bursa of Fabricius in birds) and T lymphocytes (T-cells, or thymus-derived lymphocytes), each with quite different roles in the immune system, and

distinct types of antigen receptors. After antigen-binding to a B-cell receptor (*BCR*), the B lymphocytes will proliferate and differentiate into plasma cells. *Plasma cells* are the main effector form of B lymphocytes, and their sole function is to produce antibodies. Antibodies are secreted replicates of B-cell receptors, and share identical antigen specificities between them. They can either move freely in circulation (as circulating antibodies), or be bound to the surface receptors of another cell (e.g. an IgE antibody bound to the FcεRI receptor of a mast cell). Antibody molecules as a class are known as *Immunoglobulins* (*Ig*).

The T-cell antigen receptor, or T-cell receptor (*TCR*), is related to an immunoglobulin but is also distinct in its structure and recognition properties, which are very different from a BCR. Also, it cannot directly recognize an antigen, but needs the help of an antigen presenting cell (APC), such as a dendritic cell, which has a Major Histocompatibility Complex (MHC) molecule embedded in its cell membrane. Once activated, the T-lymphocytes proliferate, and differentiate into one of three different functional types of effector cells: *cytotoxic* or killer T-cells, *Helper* T-cells, and *Regulatory* T-cells. Some of the activated B- and T-cells can also differentiate into *memory* cells.

1.3.2 Antigens, Allergens, Epitopes and Paratopes

An *antigen*, which is the abbreviation for "antibody generator", is a substance which, when introduced into the body of a host, can stimulate the production of an antibody. It is important to note that, by itself, the antigen cannot induce an immune response, but once it is bound to an antibody or TCR of a lymphocyte, the combined product can then induce such a response. An *allergen* is a special category of antigen that can initiate an *allergic* reaction, and the same rule for an antigen applies. This explains why the same peanut allergen, for example, does not elicit an allergic reaction in a *non-allergic* person, because there is no corresponding anti-peanut antibody present, but in an *allergic* person it will induce a reaction because the antibody is there.

An *epitope*, also known as an antigenic determinant, is that part of an antigen that is recognised by an antibody, or a receptor from either a B cell or T cell. That part of the antibody that can actually recognise and then bind to the epitope is called a *paratope*. An antigen may have multiple epitopes linked together like a string of keys, and the paratope of the corresponding antibody may be seen as a specific lock, or a series of locks, that can only fit into one particular key. This is how antigen-recognition works.

An epitope can be physically large and retains the full folded-structure of the original tertiary peptide chain that forms it, giving it a specific shape as well as some unique characteristics. Such a large, 3-dimensional epitope is called a *conformational epitope*. Because of its folded nature, the corresponding paratope of an opposing antibody can only recognize *discontinuous* sections of the antigen, brought into close proximity by the three-dimensional folding of the peptide chain. When the conformation is destroyed or modified, for example by a denaturation process such as extended boiling, the paratope may no longer be able to recognise the changed antigen. Most IgE-reactive epitopes come as large and folded conformational epitopes, but some *linear epitopes* can also be large but still recognisable by *B-cell* receptors or related antibodies. However, *T-cell* receptors can only recognise small, segmental liner epitopes (see below).

1.3.3 Epitope recognition by T-cell receptor (TCR)

In contrast to the large conformational epitopes that are recognised by B-cell receptors or related antibodies, T-cell epitopes are always small, linear in shape, and have no conformation. Its antigenicity is purely based on the amino acid sequence, taken up by a molecule called *Major Histocompatibility Complex* (MHC), which is located at the surface of a specialised (or professional) *antigen-presenting cell* (APC), the best-known example of which is the *dendritic cell*. This kind of arrangement means that the T-cell receptors (TCR) themselves cannot recognise these epitopes directly unless the epitopes are presented to them through the APC / MHC combination. MHC can be either class I or class II. *MHC class I* molecules bind to peptides derived from proteins either synthesized *within* a cell or *internalized* by the cell through phagocytosis or pinocytosis, while *MHC class II* molecules bind to peptides derived from proteins made *external* to the cell. Because food allergens are exogenous in origin, MHC class II is the dominant player in food allergy reactions.

Linear epitopes taken up by MHC class I molecules in T cells are typically peptides between 8 and 11 amino acids in length, whereas epitopes for MHC class II are usually 13-17 amino acids in length, sometimes up to 25 in number.

1.3.4 The role of T-helper cells in food allergy: the Th1 / Th2 paradigm

There are three major types of T cells: helper T cells (*Th*), cytotoxic T cells (*Tc* or CTL), and regulatory T cells (*Tregs*). *Helper* T cells can either help the B cells to make antibodies (and therefore become involved in an allergic reaction), or activate the killing function of

macrophages or natural killer (NK) cells (and are therefore cytotoxic and not involved in allergic reactions). *Cytotoxic T cells* are capable of independently engaging and killing virally infected cells without external help, and as a result they are also not directly involved in an allergic reaction. *Regulatory T cells* play a suppressive, or regulatory, role in the immune response, and can therefore modulate an allergic reaction.

Helper T cells can be further subdivided into three types: Th1, Th2 and Th17 cells, depending on the cytokine profiles that they secrete [115], and the three numbers after "Th" refer to the three types of cell-mediated effector immune responses [116]. *Type 1* effector response consists of Interferon-γ (INFγ)-producing NK cells, CD8+ Tc1 cells, and CD4+ Th1 cells, their roles being protection against intracellular microbes through activation of mononuclear phagocytes (and are therefore cytotoxic). *Type 2* effector response consists of Tc2 cells and CD4+ Th2 cells with production of IL-4, IL-5 and IL-13, their role being the induction of mast cell, basophil and eosinophil activation, as well as IgE antibody production (and are therefore involved in allergic reactions). *Type 3* effector response consists of Tc17 and Th17 cells producing IL-17 and IL-22, which not only activate mononuclear phagocytes, but also recruit neutrophils and induce epithelial antimicrobial responses. Typically, a Type 2 bias in immune response is likely to drive more allergic reactions, while a Type 1 bias is likely to do the opposite.

A significant breakthrough in the understanding of operational mechanisms of Th2 cells in allergy is the recent discovery that there is a phenotypically and functionally distinct human Th2 cell subpopulation that is different from conventional Th2 cells and is found specifically in patients with allergic diseases, and the discovery links depletion of these cells with allergen desensitisation immunotherapy [117]. Called *Th2A* cells, they are terminally differentiated CD4+ T cells (CD27- and CD45RB-) characterised by co-expression of CRTh2, CD49d, and CD161, and exhibit numerous functional attributes distinct from conventional Th2 cells. Transcriptome analysis further revealed a distinct pathway in the initiation of pathogenic responses to allergen, and the elimination of these cells is indicative of clinical responses by immunotherapy. The significance of this discovery is that it seems to have identified a human Th2 cell signature in allergic diseases that can be used for response-monitoring in immunotherapy and designing more appropriate immunomodulatory strategies. Further insights and clinical implications of this discovery were addressed in an accompanying paper in the same issue of the journal [118].

1.3.5 The use of CD markers in the identification of leukocyte types

CD molecules are cell surface markers that can be used to identify and characterise leukocytes. For example, all T cells will express the same surface marker called CD3 on their cell membranes, and after that identification further surface markers can be added to differentiate these T cells into more subsets. As a result, the list of CD markers accompanying these cells becomes longer as the cells become more differentiated. Surface markers are usually detected using dedicated monoclonal antibodies, which are specially raised for the sole purpose of finding and binding these surface markers. A system has been developed to "name" these markers with a *CD number*, under a list called the *Cluster of Differentiation* (CD, e.g. all T-cells are CD3+). In 2011 there were 363 designated CD antigens assigned and the list has been growing every year since.

The use of CD markers is indispensable in flow cytometry for the identification and separation of different leucocyte types. Here is a short list of some useful CD markers: CD3 (all T-cells), CD4 (T-helper cells), CD8 (cytotoxic T-cells), CD19-22 (B-cell), CD25 (regulatory T-cells), CD 27 (plasma cells and memory B-cells), CD28 (T cells and activated B-cells), CD 62L and CD 127 (naïve T-cell), CD45RO (memory T-cell), CD63 and CD203c (basophils, as used in BAT test). Foxp3 is not a CD but a transcriptase, but it is highly specific for regulatory T cells (Tregs) when used in conjunction with CD25. Co-expression of CD25 and CD134 (OX40) have been used to demonstrate the state of activated T cells [119], and we have actually employed these markers to demonstrate the activation of T cells after incubating PBMC of peanut allergic individuals with raw and boiled peanut extracts, as described in Chapter 3 of this thesis (see section 3.9).

1.3.6 Cytokines

Cytokines are a large group of structurally diverse polypeptides that are secreted by some specific cells of the immune system. They can also be secreted by non-immune cells such as endothelial cells and fibroblasts, but here we shall limit our discussion to cytokines produced by the immune cells only. Their function is to be a messenger molecule that can communicate signals from one cell-type to another, and as a result can instruct the cells receiving the signal to proliferate, differentiate, secrete additional cytokines, migrate, or even die.

The term "cytokine" is derived from two Greek words – *cyto* meaning "cell" and *kinos* meaning "movement". As mentioned earlier, they are basically a category of "signalling molecules" that can mediate and regulate all things related to immunological activities, but especially in the production of effector cells to induce effector functions. Cytokine is a general name, under which other names can be defined based on their presumed functions, the types of secretions, and the targets of action. For example, cytokines produced by lymphocytes are often referred to as *lymphokines*. Many of the lymphokines are also known as *interleukins (ILs)*, since not only are they secreted by the leukocytes, they can also affect the cellular responses of other leukocytes nearby or even remotely. The cytokines secreted by monocytes or macrophages are termed *monokines*, and *chemokines* are cytokines with chemotactic capabilities.

Cytokines and their corresponding receptors exhibit very high affinity for each other. Because of this high affinity, picomolar or even femtomolar concentrations of cytokines can mediate a biological affect. When stimulated by the presence of infection or inflammation, cytokine levels can increase by 1000-fold. A cytokine can "affect" itself by means of "*autocrine*" action, a neighbouring cell by "*paracrine*" action, or a distant cell by "*endocrine*" action, after travelling through circulation and finding the target cells. T-helper cells are particularly active in producing cytokines.

Cytokines have a number of attributes that can make them so versatile and useful:

- *Pleiotrophy* so that one cytokine can have many different functions.
- *Redundancy* so that several different cytokines can mediate the same or similar functions.
- *Synergism* so that the combined effect of two cytokines on cellular activity is greater than the additive effects of the individuals.
- *Antagonism* so that the effects of one cytokine can inhibit or offset the effects of another cytokine.

Immunologically, the *Interleukin* family is the most important group of cytokines. Members of the family are very diverse, belonging to different structural classes of proteins, mainly because the primary qualification for membership of this family is biological (i.e. evidence of activity on leukocytes) rather than structural homology. To date, approximately 34 interleukins have been described (IL1 to IL35), with the status of IL-14 being an "interleukin in doubt". Other

cytokine family have been established on the basis of their ability to support proliferation of haematopoietic precursors (*colony stimulating factors*, CSF, best known example being GM-CSF), or cytotoxic activity towards transformed cell types (*tumour necrosis factor*, TNFα and β), or the ability to interfere with viral replication (*interferons*, INFα, β and γ).

1.3.7 The role of regulatory T cells in food allergy

Regulatory T-cells, or *Tregs*, are a small group of CD4+ T cells in the same company as the other three known Helper T-cells, but produce a distinctively different set of cytokines from Th1, Th2 and Th17. Their role is mainly suppressive or regulatory, performing a "policing action" on other classes of T-cells. There are two types of such cells, called either natural or inducible Tregs [120-122].

Natural, or *thymic-derived, Tregs* (nTregs or tTreg) are a population of FoxP3+CD25+CD4+ T cells that can suppress auto-reactive T-cell immune responses by mechanisms that are still not entirely understood, but appear to involve several distinct and possibly overlapping strategies. The current view is that they probably achieve this through competition for self-antigens presented by APC, or through CTLA4-mediated (cytotoxic T lymphocyte antigen 4) signals from Treg to APC. They only form about 5% of total CD4+ T cells, and their development is critically dependent on the induction of FoxP3, a transcription factor that can repress the transcription of Th1, Th2 and Th17 type cytokines. This is the mechanism through which Tregs can suppress or regulate other Th cells, and a defect of this factor can lead to many autoimmune diseases.

Inducible, or adaptive, Tregs (iTregs) are immunologically different from nTregs and are generated from naïve CD4+ T cells in the *periphery*, after encounter with an antigen presented to them by DCs. As a result, they are also called *peripherally-derived Tregs* (pTregs). They apparently comprise a diverse group, although it is not yet clear whether these inducible Treg populations are truly distinct from each other. The most important sub-group is *Th3* cells, which are found in mucosa, and secrete IL-5, IL-10 and TGFβ. These cells seem to be important for the development of oral tolerance, and Th3 cells may intervene to maintain tolerance towards the beneficial commensal micro-organisms that populate our intestinal tract [123].

The role of Tregs in the development of oral tolerance was elucidated in a recent paper by Qamar et al. [124], who studied 58 children with (1) either egg or peanut allergy, (2) recent

acquisition of natural tolerance to egg or peanut, and (3) healthy children without food allergy. In the paper, PBMC were stimulated with relevant antigens for 48 hours, and flow cytometry was performed to characterise both *surface* (CD3, CD4, CD25, CD14, CD19, CD127), and *intracellular, markers* (IL-10, FoxP3 and IL-5). The authors found that there were significantly increased CD3+CD4+CD25+CD127loFoxP3+ cells in unstimulated PBMC from *naturally tolerant* patients, compared to persistently allergic, or control non-allergic, patients. Upon stimulation with the relevant antigens, *naturally tolerant* patients also had significantly increased IL-10-expressing CD25+CD127lo cells (which they identified as *Tr1* cells), FoxP3+ cells, and CD4+ cells. In contrast, no increase was observed in PBMC from persistently allergic or control patients. This up-regulation was antigen specific, and was only seen upon stimulation with the relevant antigen (but not with unrelated antigen), suggesting that *acquisition of tolerance* is an active and antigen-specific process. The authors hypothesized that (1) the increased CD3+CD4+CD125+CD127lo cells at base-line and upon stimulation, and (2) increased induction of IL-10-producing cells of several types, including Tr1 cells from naturally tolerant patients, suggest <u>an important role for regulatory T cell subsets in the acquisition of natural tolerance</u>.

1.3.8 Immunological chain-reaction in a peanut-induced allergic event

Immediately after ingestion and leaving the stomach, peanut allergens will first encounter the gut mucosal immune system. There are a large number of specialised follicle-associated cells called *Microfold (M) cells* that dot the epithelium of the gastrointestinal tract in close proximity to the *Peyer's patches*. Together they form the gut-associated lymphoid tissue (*GALT*), and are responsible for the induction of subsequent immune responses. *Sensitisation* to a food allergen occurs when the exogenous food antigens are taken up by the M cells, and then presented to macrophages or dendritic cells (DC), which serve as the antigen presenting cells [125]. Once internalised by these cells, the antigens are endocytosed, then denatured, and finally degraded into peptides of 12-20 amino acids in length. A minute fraction of these small peptide fragments is then transported intracellularly, and then presented at the cell-surface to *MHC class II* molecules, for specific interaction with CD4+ *T helper* (Th) cells. These (now activated) T-cells then undergo expansion, become *Th2 cells*, and release *Th2 cytokines*. The combination of Th2 cells, IL-4 and IL-5 would promote further differentiation of *B-cells*, and make them *secrete* allergen-specific IgE antibodies [126]. These IgE-producing B-cells then expand in number, and become *plasma cells*, with the sole function of continuously secreting *allergen-specific IgE antibodies*. Subsequent exposure to peanut would result in the binding of peanut

allergens to specific IgE-coated *mast cells* and *basophils*. Cross-linkage of adjacent antigen-receptor-sites provides a potent activation stimulus that results in the *degranulation* of basophils and mast cells. The rapid release of a variety of preformed and newly-formed pro-inflammatory and vasoactive compounds such as prostaglandins, leukotrienes, serine proteases, tryptase, histamine and cytokines into the extracellular fluid ensues to produce inflammatory responses [127], culminating in the clinical features of an acute allergic reaction. Local gastrointestinal symptoms of peanut allergy include abdominal pain, vomiting, cramping and diarrhoea, and are common in occurrence even in mild cases of peanut allergy. This acute yet non-life-threatening reaction causes a transient increase in intestinal permeability, which subsequently allows systemic distribution of macromolecules, such as whole peanut allergens, exacerbating any resulting immune responses [128].

1.4 MEDICAL TREATMENTS OF PEANUT ALLERGY

1.4.1 Currently approved standard of care in managing peanut allergy

The current standard of care for the management of peanut allergy is to advise strict peanut avoidance and provide emergency medications (oral antihistamines, inhaled β2 agonists, oral steroids, and intramuscular adrenaline auto-injectors such as Epipen) whenever necessary [129]. Epipen is generally recommended for patients with a previous history of anaphylaxis after ingestion, or significant reaction to merely a trace amount of peanut, or medical history of significant co-existing asthma. Other factors, such as limited access to emergency medical care, and the patient's age (adolescents are considered to be at higher risk of life-threatening allergic reactions) [129], and very high PPV of diagnostic test readings, may also be taken into account. Educating the families on how to avoid accidental ingestion, or recognise and treat allergic reactions promptly when they have occurred, forms the cornerstone of current management for peanut allergy. In the majority of fatal or near-fatal reactions to peanut, patients were often unaware that the food they had just consumed actually contained peanuts, indicating that attempts at avoidance were often unsuccessful [130]. A comprehensive management plan, including verbal and written advice on nut avoidance, as well as training in recognition and treatment of allergic reactions, with the addition of a written management plan, can be effective in reducing both severity and number of future reactions [131, 132].

1.4.2 Common problems associated with simple peanut avoidance

Avoidance can be difficult because peanuts are widely present in many foods. There is also the risk of contamination with peanut or peanut products during manufacturing process. Labelling can be inadequate or misinterpreted by families and caregivers [133]. As a result, accidental reactions are common. Annual incidence rates for accidental reactions vary between several studies: from 15% [134] to 55% [42], to 75% [135]. It has been estimated that the average peanut-allergic patient will have an allergic reaction from accidental ingestion every 3-5 years [42]. Furthermore, nearly 1/3 of younger nut-allergic children cannot recognise the nut to which they are allergic, and this lack of recognition would put them at increased risk of unintentional ingestion [136]. It is also important to highlight the unpredictability of future accidental reactions, even if the initial reaction on presentation is considered mild. A longitudinal study of a population of children who had developed peanut allergy before the age of 4 years revealed that, of the children with initial non-life-threatening reactions, 44% had at least one potentially life-threatening reaction during follow-up [137].

1.4.3 Which nuts to avoid: just the allergic ones, or should all nuts be banned?

It is well known that a significant proportion of peanut-allergic children (25-50%) are also allergic to one or more tree nuts [138]. Similarly, many children who presented initially with a single tree nut allergy were later found to be allergic to many other nuts, including peanut. In a cross-sectional study of 784 children in the UK, Clark et al. [46] noted that at 2 years of age only 19% of their subjects were multi-sensitised (i.e. tested positive on skin and blood tests but never exposed) and 2% multi-allergic (had a positive history in addition), but by 14 years of age the corresponding figures were raised to 86% and 47% respectively.

In the past, many allergists have simply recommended complete avoidance of all nuts in a child with peanut allergy, because of concern about the possibility of cross-contamination between various nuts, and problems associated with the labelling of food products. In addition, they also argued that children were often unable to recognise the nuts that they were allergic to, and indiscriminate consumption of multiple nuts could put them at increased risk of accidental reactions [136]. A counter-argument to this is that the avoidance of nut consumption in early life could be associated with an increased risk of development of new nut allergies later in life, and it is possible that the early introduction of “non-allergic” nuts may help prevent the development of further nut allergies, and at the same time expand the repertoire of the child’s

diet. More studies are needed in this area in order to make the correct recommendation on nut consumption [129], but opinions are gradually shifting from complete avoidance to the alternative advice of deliberate but cautious consumption of non-allergic nuts.

1.4.4 When should infants start eating peanut? The LEAP Study

The *LEAP Study* is an acronym formed by the first letter of the first four words in "Learning Early About Peanut Allergy". It was a randomized controlled clinical trial, spanning 60 months, designed and conducted by the Immune Tolerant Network led by Gideon Lack of UK. The study's aim was to determine the best strategy to prevent peanut allergy from occurring in young children, based on the optimum time to introduce peanuts to young infants. A total of 640 children between 4 and 11 months of age, who were identified as having high risk for peanut allergy (because of existing egg allergy and/or severe eczema) [70], were enrolled into the study. They were then randomly assigned to either consume or avoid peanuts until 60 months of age. All would have a skin prick test to peanut at the start, resulting in 530 infants being tested negative and 98 being positive (SPT 1-4 mm), while 12 participants were excluded from analysis for a variety of reasons. The primary outcome of the study was the proportion of participants with peanut allergy after 5 years of observation, as confirmed by a peanut OFC at exit. The paper were published in 2015 [139], and has since had a profound effect on the guidelines and recommendations made by professional allergy advice bodies across the world, regarding the best time to introduce peanut to infants.

Among the 530 infants in the intention-to-treat sub-population, who initially had negative results on the skin-prick test, the prevalence of peanut allergy at 60 months of age was 13.7% in the avoidance group and 1.9% in the consumption group ($P<0.001$), representing a 7-fold reduction as a result of early introduction of peanut. Among the 98 participants in the intention-to-treat population who initially had positive skin test results, the prevalence of peanut allergy was 35.3% in the avoidance group and 10.6% in the consumption group ($P = 0.004$), representing a 3-fold reduction. There was no significant between-group difference in the incidence of serious adverse events.

As a consequence of this study, parents are now generally advised to introduce peanut to their infants by 6 months of age [140-142].

1.4.5 Allergen-specific approaches in the treatment of peanut allergy

1.4.5.1 Introduction: desensitisation vs immunologic tolerance

Allergen-specific immunotherapy (sometimes also called allergen desensitisation immunotherapy, or just allergen immunotherapy), refers to the process of modulating the immune response of an allergic person through increasing exposure to the allergen over time, with the aim of making that person able to tolerate an increased amount of the allergen without reaction. The process is commonly abbreviated to just *desensitisation*, but it is important to know that the ability to tolerate an allergen after desensitisation is only short term, and requires ongoing maintenance to prolong its protection. This is because desensitisation is mediated mainly through temporary changes to the effector cells and their products, without a permanent adjustment to the underlying pathogenic immune mechanisms [143]. As a result, a desensitised individual will "remain allergic to the allergen" (as indicated by persistent positive skin prick test and allergen-specific IgE levels), and in the case of food allergy, the ingestion of an allergic food after a long discontinuation period of treatment may lead to a relapse of the original allergic reaction [144]. In contrast, *immunologic tolerance* is a more permanent state of non-reaction, in which large quantities of allergen can be tolerated long after discontinuation of treatment, suggesting a more profound change to the underlying immune system [143]. Such level of tolerance cannot be readily achieved using traditional allergen-specific immunotherapy methods, and the acquisition of immunologic tolerance is now believed to involve combinations of regulatory T cells or other T-cell subsets and/or allergen-specific *anergy*, or *clonal deletion of effector B-cells* [145]. An intermediate state of "reduced allergy" straddling between desensitisation and tolerance is called *sustained unresponsiveness* [146, 147], in which the period of protection from desensitisation is extended by weeks or months, but not quite as permanent as in the case of full tolerance. It is important to make a mental note that here we are talking about the scenario in which a food allergy has already been established, and treatment is therefore aimed at reversing the existing condition (i.e. allergy first, then trying to reverse it next). An alternate scenario will be discussed later.

In food allergy, "*oral tolerance*" is often used instead of "*immunologic tolerance*", because the former implies that the underlying mechanism is through the oral route, and most likely involves gut-associated lymphoid tissues (*GALT*). Obviously, the ultimate goal of immunotherapy is to achieve full oral tolerance, but the exact mechanism of how to achieve this is still not clear, and we are nowhere near a cure for any food allergy, other than through

natural resolution. Immunotherapy appears to be able to alter T-cell responses to allergens by a combination of skewing Th2 response toward Th1 bias, class-switching from IgE to IgG4, less profound changes to dendritic cells, mast cells, basophils, B-cells, and induction of Tregs [148]. As mentioned earlier, two kinds of Tregs could be at play: either natural (nTregs, thymus-derived) or inducible (iTregs, antigen-specific), and both can suppress the immune responses by quite different mechanisms, including secretion of IL-10 and transforming growth factor (TGF)-β [149], and both cytokines have been shown to be important in modulating food allergy [150]. It has been reported that repeated low-dose ingestion of antigen in rodent models can lead to the induction of antigen-specific iTregs that have suppressor activity [151], and depletion of iTreg would lead to the loss of tolerance in these animals [152]. In contrast, naturally occurring thymus derived nTreg do not appear to be required for successful oral tolerance induction [153]. Also, oral tolerance can develop in CD8 knockout mice [154], suggesting that the role of CD8+ T cells (Tc or CTL) may be restricted to maintenance, as opposed to induction, of oral tolerance [123].

Looking from another perspective, food allergy can also be seen as a failure, or loss of, an already established state of oral tolerance (i.e. tolerance comes first, becoming allergic later), due to some unknown impact on the immune system. There is some evidence in support of this scenario, as a small number of peanut-allergic patients could have a clear history of being able to eat peanuts without problem long before they unexpectedly develop an allergic reaction to peanut later in life. Although allergen-specific Th1, Th2 and Treg cells can all be identified in allergic and non-allergic individuals, it is the change of balance between allergen-specific Treg and Th2 cells that provides the critical factor in determining the direction of outcome: i.e. progression to either allergy, or tolerance [155]. Consequently, allergic subjects tend to show a predominance of allergen-specific Th2 reactions with relative deficiency of allergen-specific Treg responses, when compared to non-allergic individuals who tend to go along the opposite direction: with dominant Treg reactions but attenuated Th2 responses, after exposure to the allergen. The loss of tolerance could well be linked to a change of this balance, but this is still just speculation. The functional role of Tregs has been discussed in more detail earlier, in Section 1.3.7.

1.4.5.2 Immunological changes following peanut desensitisation

Despite all the earlier discussions, it is fair to say that a clear picture of mechanisms underlying peanut desensitisation and oral tolerance is still not well elucidated [156].

Interestingly however, whilst the ability of allergen-specific immunotherapy to induce full oral tolerance remains elusive, the former (i.e. desensitisation) has been shown to modulate allergen-specific immune reactions and direct them towards responses typically associated with the development of the latter (i.e. oral tolerance). This suggests that these immune effects may not be the critical determinants of tolerance acquisition, and there could be other factors that are not yet discovered [123], which may convert desensitisation to tolerance. It is well known that oral immunotherapy is most commonly associated with a reduction in allergen-specific IgE and an increase in allergen-specific IgG4 [157]. The immunoglobulin IgG4 is thought to be able to suppress peanut-induced basophil and mast cell activation, by either competing with IgE for binding to peanut allergen, or direct binding to the inhibitory FcγRIIb receptor on the surface of these cells, causing an inhibitory effect [158]. Of the 8 published papers on peanut OIT, an increase in serum levels of psIgG4 had been reported in two papers [159, 160], while reduction of psIgE level had been reported in one [160]. The mechanism mediating the shifting (or class-switching) in allergen-specific antibody isotype from sIgE to sIgG4 during OIT, in the setting of both desensitisation and tolerance, is unclear. There is some evidence that this could involve down-regulation of T-cell apoptotic pathways [160], or clonal deletion of allergen-specific IgE-producing B-cells, together with clonal expansion of allergen-specific IgG4-producing B-cells [157].

Changes in cytokine profile have also been reported following OIT, in which reduced Th2 and increased Treg responses are common findings. It is well known that peanut OIT was associated with reduced allergen-induced Th2 cytokine production [159, 161]. Desensitisation was found to be associated with reduced IL-5 production, while sustained unresponsiveness was associated with a reduction of IL-4 and IL-2 responses.[159]. Jones et al. [160] reported increased IL5, IFNγ. IL-1β, and TNFα production in patients who underwent peanut OIT, while Th1 (INFγ) responses have been noted to increase [160], or remain unchanged [159]. Treg numbers [162] and production of regulatory cytokines such as TGFβ [163] were reported to increase with OIT, although one study noted that the change was only transient [164]. Immunotherapy may have induced expansion of Tregs populations that are capable of suppressing T effector responses to peanut, but some studies have not seen an increase in this cell subset [165]. More detailed characterization of OIT immune effects is required to decipher its mechanism and improve the likelihood of developing a method that can lead to oral tolerance, rather than just temporary desensitisation.

1.4.5.3 Peanut desensitisation using subcutaneous immunotherapy (SCIT)

Although subcutaneous immunotherapy has been used effectively in the treatment of environmental or venom allergies, it has not enjoyed similar success in treating peanut allergy. A few early studies did try to explore its feasibility [166, 167], but at the end the high rate of severe adverse reactions (including one tragic death) was considered to be unacceptable, and this route was completely abandoned a long time ago [167, 168].

1.4.5.4 Peanut oral immunotherapy (OIT)

Peanut oral immunotherapy (OIT) in children is the most-studied peanut desensitisation method, largely because of promising results from similar approaches in the treatment of egg and milk allergies [169-171]. In a typical peanut OIT protocol, increasing doses of the peanut allergen (usually in the form of partially defatted peanut flour) are mixed in a food vehicle and ingested by the subject over an extended period of time. Most OIT studies consist of an initial *escalation* phase, which is usually supervised inside a hospital ward or day unit, followed by a bi-weekly *up-dosing* phase, also supervised at a hospital, and finally a *maintenance* phase, which is usually carried out at home.

Since 2009 (until the writing of this thesis) there have been 8 peanut OIT studies [159-161, 172-175], the first three of which were published almost simultaneously in the same month of August 2009, while the last one was published in March 2015 [176]. Their combined results are summarised below:

- The number of subjects in these studies varied from 4 to 99.
- In the smallest study (4 subjects), no subject withdrew. However, in the other 7 studies, the number of withdrawals varied from 10% to 39%.
- Adverse events during OIT varied from 45% to 93%. Most of them were mild (mouth, tongue or throat itchiness, perioral urticarial, generalised erythema or urticarial, abdominal pains, vomiting, diarrhoea, mild laryngospasm, mild bronchospasm), but a few had required treatment with adrenaline.
- For those who managed to complete OIT, 62-100% can be expected to tolerate a reasonable amount of peanut.

- Maintenance daily doses of peanut varied between 300mg and 4,000mg peanut protein. Post-OIT challenge doses varied between 1,000mg and 6,500mg peanut protein.
- Immunologic studies showed a decrease in skin prick test readings, basophil hypo-responsiveness, drop in psIgE and corresponding rise in psIgG4, decrease in Th2 cytokines (mainly IL-5 and IL-13), and increase in Tregs.

In the most recent (2015) paper, Tang et al. [176] co-administered probiotics during peanut OIT, and called the treatment PPOIT. They reported that 89.7% receiving PPOIT and 7.1% receiving placebo were desensitised, while possible sustained unresponsiveness (assessed 2-5 weeks after discontinuation of PPOIT) was achieved in 23 (82.1%) of 28 PPOIT-treated participants and 1 (3.6%) of 28 placebo-treated participants. However, that study did not have a treatment arm using peanut flour only (to distinguish the individual role between peanut and probiotic), but instead had just an all-placebo arm, so its claim of high rate of sustained unresponsiveness could not be confidently attributed to the process of adding probiotics to peanut during desensitisation. The addition of adjuvant or co-treatment to OIT will be discussed further in two later sections (see section 1.4.5.7 and 1.4.5.9).

It is important to note that patients during OIT, including those in the maintenance phase, can sometimes react unexpectedly to a previously-tolerated dose of the allergen. Common triggers for such reactions include concurrent infections (particularly if febrile), physical activities within 2 hours either side of ingesting a dose, ingesting the allergen on an empty stomach, poorly controlled asthma, pollen season, and menstruation in adolescents and adults [160, 177]. However, no trigger could be identified in at least 28.5% of home reactions in the Barbi et al. series [177].

In conclusion, currently peanut OIT methods are considered to be not ready for clinical use outside a research setting [178].

The outcomes of these eight OIT studies are summarised in the following 2 tables:

Table 1.4.5.4A: Withdrawals and Adverse Events in Peanut OIT Studies

Range of withdrawal rate varied between 0% (only 4 subjects) and 39%; mean = 19%

Adverse events varied between 45% and 93%.

No	Year	Patient No	Summary of reactions
1	2009 Hofmann	28	8/28 (29%) withdrew; 93% reaction at dose escalation (18% with wheeze), 46% at build-up, 3.5% at maintenance, 2 required adrenaline.
2	2009 Jones	39	10/39 (25%) withdrew; 92% experienced reaction during dose escalation.
3	2009 Clark	4	None withdrew, 3 out of 4 (75%) experienced allergic reactions during treatment.
4	2010 Blumchen	23	9/23 (39%) withdrew or dropped out. No data of percentage of patients with adverse events, but total AE for the whole study = 185. AE excluded "subjective" oral pruritus, abdominal pains, nausea and worsening of eczema.
5	2010 Varshney	28 (T19 C9)	3/19 (16%) withdrew; 47% experienced allergic reactions, 2 required adrenaline.
6	2011 Anagnostou	22	1/22 dropped out, 3/22 could not tolerate designated treatment dose and had lower maintenance dose. 12/22 also required transient dose reduction before reaching maintenance. 86% had reactions during treatment.
7	2014 Anagnostou	99 (T49 C50)	15/99 (15%) discontinued, withdrew, or could not reach target dose. 81% had mouth itch, 57% abdominal pain, 33% nausea, 33% vomit, 1% diarrhoea, 13% urticaria, 19% angio-oedema, 21% erythema, 24% rhinitis, 22% wheezing, 1% laryngeal oedema. 19% use of inhaled β2 agonist, 1% adrenaline.

8	2015 Tang	62 (T31, C31)	6/62 (10%) withdrew, 3 from each group. At least 1 severe AE was reported in 45.2% in treatment and 32.3% in Placebo. One child in treatment group has 13 severe AEs. **Severe** AE was defined as any symptom that prevents daily activities and might require therapeutic intervention. 10 **serious** AEs (**SSE**) related to study product had occurred in 7 participants (6 SSE with treatment group and 4 with placebo group). Placebo group ingested "peanut essence" which might explain the adverse reactions.

Definition of SSE = abdominal pains, vomiting, itch throat + difficulty breathing but no objective signs, asthma, wheeze on auscultation, urticarial + hoarse voice, urticarial + wheeze, urticarial + vomiting + wheeze, urticarial + wheeze + cardiovascular symptoms.

Table 1.4.5.4B: Failure/Success Rate of Peanut OIT

Excluding withdrawals and dose-reduced cases, success rate varied between 62% and 100%

No	Year	Patient No	Failure/Success Rate
1	2009 Hofmann	28	8/28 (29%) withdrew. 20/28 completed all three phases of study. No challenge was done at end of study so outcome is unknown.
2	2009 Jones	39	10/39 (25%) withdrew including 4 who could not tolerate OIT. 27/29 can ingest 3.9 g peanut protein (16 peanuts) after treatment "with no more than mild symptoms".
3	2009 Clark	4	None withdrew. 4/4 tolerated 10 peanuts at end of treatment (6 weeks after tolerating 5 peanuts as maintenance).
4	2010 Blumchen	23	9/23 (39%) withdrew because of allergic reactions during treatment. After a median of 7 months 14 patients reached "protective dose" (1 g peanut protein = 4 peanuts).

5	2010 Varshney	28 (T19 C9)	3/19 (16%) withdrew. Remaining 16 could tolerate 5 g peanut protein (20 peanuts) challenge.
6	2011 Anagnostou	22	1 subject (5%) withdrew, 2 went on lower maintenance dose. After 30 weeks maintenance 14/19 (74%) tolerated 6.6 g protein.
7	2014 Anagnostou	99 (T49 C50)	15/99 (15%) discontinued, withdrew or could not reach target dose (800 mg or 5 peanuts). After the first phase, 62% (24 of 39) of active group could tolerate 10 peanuts compared to zero subject for control group.
8	2015 Tang	62 (T31, C31)	6/62 (10%) withdrew, 3 from each group. 89.7% receiving PPOIT and 7.1% receiving placebo were desensitised. Possible sustained unresponsiveness was achieved in 82.1% receiving PPOIT, and 3.6% receiving placebo.

1.4.5.5 Peanut sublingual immunotherapy (SCIT)

Sublingual immunotherapy (SLIT) involves the administration of small drops of allergen extract under the tongue and then either swallowed or spat out after holding there for several minutes. SLIT has been trialled in Europe for a number of years before its first attempted-use in peanut allergy, but mainly for the treatment of allergic rhinitis using pollen extracts [179, 180]. The theoretical advantage of SLIT is that the oral mucosa is rich in tolerogenic antigenic presenting cells, so SLIT may be able to induce tolerance with smaller doses of the allergen, which presumably also means fewer side effects. The typical dose of peanut protein used in peanut SLIT is approximately 1000-times less than that of peanut OIT [181]. In 2011, Kim et al. published the first double-blind study of peanut SLIT in which 18 peanut-allergic children were enrolled and underwent dose escalation up to a maintenance dose of 2 mg of peanut protein [182]. After 12 months of treatment, subjects on active treatment (n=11) could consume up to 1,710 mg peanut protein during oral food challenge (although the amount tolerated by those in the active treatment varied significantly) while placebo subjects (n=7) could only ingest 85 mg before developing an allergic reaction. There were no dropouts from adverse events related to peanut dosing during SLIT, and the side effects were mainly oropharyngeal symptoms,

observed in 11.5% of active and 8.6% of placebo doses. As with OIT, peanut SLIT was associated with decreases in readings of SPT and Basophil Activation Assays. Immunological studies revealed a transient increase in peanut-specific IgE levels over the first 4 months, which then steadily decreased, and a significant increase in peanut-specific IgG4. Th2 cytokines (IL-5 and IL-13) were decreased in those on active treatment with peanut SLIT, and this was not observed in placebo subjects.

In another randomised, double-blind, placebo-controlled multicentre trial of peanut SLIT, with a crossover design involving 40 subjects (age: 12-37 years), Fleischer et al. [183] showed that after receiving 44 weeks of peanut SLIT, 14 out of 20 (70%) subjects were able to consume tenfold more peanut protein than baseline oral food challenge (median successfully consumed dose or SCD increased from 3.5 to 496 mg), compared to 3 out of 20 (15%) subjects receiving placebo. After 68 weeks of SLIT, the median SCD increased to 996 mg compared to 496 mg at the 44-week time point (p=0.05). This study demonstrated that peanut SLIT was well-tolerated, with mainly oropharyngeal symptoms as the notable adverse effects in this study. However, these results, while encouraging, showed that there was only modest level of desensitisation, compared with that of peanut OIT.

Subsequently, in a 3-year, long-term follow-up evaluation of these 40 subjects from the above study, Burks et al. [184] performed a 10-g peanut powder oral food challenge after 2 and 3 years of daily peanut SLIT therapy. At 3 years, SLIT was discontinued for 8 weeks, followed by another 10-g oral food challenge and an open feeding of peanut butter to assess sustained unresponsiveness. Approximately 98% of the 18,165 doses were tolerated without adverse reactions beyond the oropharynx, with no severe symptoms or uses of adrenaline. Unfortunately, a high rate (>50%) of participants had chosen to discontinue therapy. By the study's end, 4 (10.8%) of 37 SLIT-treated participants were fully desensitised to 10-g of peanut powder, and all 4 achieved sustained unresponsiveness. Responders at 2 years showed a significant decrease in peanut-specific basophil activation and SPT titration, compared with non-responders.

1.4.5.6 Direct comparison between OIT and SLIT

In 2013, Chin et al. retrospectively compared oral food challenge outcomes after 12 months of peanut SLIT (maintenance dose 2 mg/day) vs 12 months of OIT (4,000 mg/day), and showed that OIT produced greater immunological changes than SLIT in peanut-allergic children.

Specifically, peanut OIT resulted in greater changes in peanut-specific IgE, IgG4 and IgE/IgG4 ration as well as basophil activation. In addition, eliciting-dose thresholds were lower and more variable during DBPCFC at 12 months in SLIT-treated subjects than in OIT-treated subjects [185].

In a later study, which was a prospective and direct-comparative study of peanut OIT vs SLIT, Narisety et al. [186] randomised 21 subjects aged 7 to 13 years with peanut allergy in a double-blind fashion to receive either active-SLIT/Placebo-OIT or active-OIT/placebo-SLIT. Five discontinued therapy during the blinded phase. Of the remaining 16, all had a greater than 10-fold increase in challenge threshold after 12 months. The increased threshold was significantly greater in the active OIT group (141- vs 22-fold, p=0.01). Significant within-group changes in skin tests and peanut-specific IgE and IgG4 levels were found in both groups, but the overall effects were greater in the OIT group. Adverse reactions were generally mild, but more common with OIT (p<0.001), including moderate reactions requiring medications. Four subjects had sustained unresponsiveness at study completion. The authors concluded that OIT appeared to be "far more effective" than SLIT for the treatment of peanut allergy, but was also associated with significantly more adverse reactions and early study withdrawals. Sustained unresponsiveness after 4 weeks of avoidance was seen in only a small minority of subjects in either group.

1.4.5.7 OIT assisted with Omalizumab

Omalizumab is a recombinant, DNA-derived, humanized monoclonal antibody which binds specifically to free-circulating IgE antibodies in blood and interstitial fluid, and also to IgE-expressing B lymphocytes. It attacks the CHε3 region of the IgE molecule and blocks the attachment of IgE to FcεRI and FcεRII receptors at mast cells and basophils, thereby preventing them from activation and mediator release. When given concomitantly to patients receiving treatment in the up-dosing phase of OIT, it can mitigate adverse events and facilitate successful desensitisation. A pilot study of Omalizumab treatment together with cow's milk OIT showed improved tolerability of OIT [187], and the method was later extended to peanut OIT, again to facilitate rapid oral desensitisation of high risk peanut-allergic patients [188]. In this pilot study, utilising Omalizumab as adjunct therapy to peanut OIT, 13 subjects with confirmed IgE-mediated peanut allergy were pre-treated with Omalizumab for 12 weeks before peanut OIT was initiated. All 13 participants had tolerated the desensitisation doses given over the first day of OIT, and reached the goal dose of 500 mg of peanut flour with minimum or no symptoms.

Twelve of the thirteen subjects (92%) achieved the maximum maintenance therapy dose of 4 gram of peanut protein daily over a median of 8 weeks. At this point, Omalizumab was discontinued and subjects continued on with peanut OIT alone for an additional 6 months, and all 12 subjects were able to pass a 8,000mg peanut flour challenge at the end of this time. Over the course of the study, 2% of total OIT doses (with or without Omalizumab) were associated with reactions, but most were mild. Overall, 6 of the 13 subjects had experienced either no or mild allergic reactions, 5 had moderate reactions, and 2 had severe symptoms, all of which responded rapidly to treatment.

1.4.5.8 Epicutaneous peanut immunotherapy (EPIT)

EPIT for peanut allergy consists of delivering a small quantity of peanut allergen through a special patch that is applied to the skin and then left there, repeated once every 24 hours. Following application, a condensation chamber would develop between the skin and the patch, creating an accumulation of water content that solubilizes the allergen and allows for its entry into the epidermis underneath the patch. The allergen is then captured by the Langerhans cells, which process the allergens and present them to lymphocytes within the draining lymph nodes [189]. The skin patch is called Viaskin Peanut (VP) and made by DBV Technologies, Montrouge, France. There are two different strengths used in clinical trials, 100 μg (VP100) and 250 μg (VP250).

Before its application in peanut allergy, EPIT has been shown to have some success in treating milk allergy based on the results from a small pilot study [190], with common side-effects being mainly pruritus and eczema at the site of application of the patch. The method has also been tried on a murine model [191].

In 2016 Jones et al. evaluated and published the clinical, safety and immunologic effects of Viaskin Peanut EPIT when used for the desensitisation of peanut allergy [192]. It was a multicentre, double-blind, randomised placebo-controlled study involving 74 participants with confirmed peanut allergy (ages 4-25 years). Twenty-five (25) of the participants had treatment with placebo, 24 with VP100, and 25 with VP250. The primary outcome was defined as passing a 5,044mg peanut protein oral food challenge, OR achieving a 10-fold or greater increase in successfully consumed dose (SCD) from baseline to week 52.

After 52 weeks of daily application, it was claimed that "treatment success" was achieved in 3 (12%) placebo-treated participants, 11 (46%) VP100 participants, and 12 (48%) VP250 participants. However, all but one of participants had failed a full OFC (5,044mg peanut protein), while <u>the only person who could consume this amount at OFC was actually in the placebo group</u> (suggesting that the person had probably developed natural tolerance without treatment). Median change in successfully consumed doses (SCD) were: 0, 43 and 130 mg of protein in the placebo, VP100, and VP250 groups, respectively. Overall, only 23% (including a 12% contribution from the placebo group) could tolerate SCD ≥1044mg of peanut protein at OFC, and 14% (including 8% contribution from placebo) could tolerate <u>both</u> SCD ≥1044 mg of peanut protein <u>and</u> 10-fold increase from Baseline at OFC. In short, peanut EPIT can only succeed in achieving a modest treatment response, even less than that of SLIT, while the AE rate was still reportedly high (79.8%), but predominantly at local patch-sites.

1.4.5.9 A comment on the addition of probiotic to peanut OIT (PPOIT)

It was thought that the addition of probiotic as an adjuvant to standard peanut flour might promote tolerogenic mechanisms in the gut, and enhance the ability of OIT to provide longer-term protection – i.e. sustained unresponsiveness - even after the desensitisation process is discontinued [176]. In this probiotic POIT study, Tang et al. completed a double-blind, placebo-controlled trial where 62 children 1-10 years of age were randomized to receive either the probiotic *Lactobacillus rhamnosus* CGMCC 1.3725 (NCC4007), given in combination with peanut OIT (maintenance dose 2 gm peanut protein/day), or placebo for 18 months. Their paper reported that among the subjects receiving OIT plus probiotic, 89.7% were desensitised in comparison to 7.1% of participants in the placebo group. The paper also claimed that possible sustained unresponsiveness was achieved in 82.1% of the OIT/probiotic group (PPOIT) vs 3.6% of children in the placebo group (their definition of sustained unresponsiveness was 2-5 weeks after discontinuation of treatment). Unfortunately, the paper did not include a peanut-OIT-only arm (i.e. no probiotic), so it was impossible to say whether the result of sustained unresponsiveness was actually due to the combined use of probiotic and peanut, or just the POIT itself. Another issue with this study is that the rate of adverse events was still unacceptably high at 45%, and any future study using a similar method must overcome this problem first, before it can be applied to treat large numbers of peanut-allergic children in the community.

1.5 THE PROTEOMIC PROPERTIES OF INDIVIDUAL PEANUT ALLERGENS

1.5.1 An overview of known peanut allergens

Peanut contains 44-56% oil and 22-30% protein [193]. To date, 16 peanut allergens have been identified in the official allergen nomenclature database (http:www.allergen.org/) and in two recent peanut allergen review articles [194, 195]. These allergens have been labelled Ara h 1-17, the letters Ara h denote the botanical name of peanut, *Arachis hypogaea*. Ara h 4 was withdrawn from the list and became an isoallergen of Ara h 3 (it was renamed Ara h 3.02). Ara h 1, Ara h 2 and Ara h 3 are considered to be the three major peanut allergens.

Two published papers have analysed the proportion of peanut allergens present in the peanut protein components, but the figures differed significantly between the two. In the earlier paper [196], Ara h 1 was determined to contribute 12-16%, and Ara h 2 5.9-9.3% of peanut protein content, but the contribution of Ara h 3 was not determined. In the second, later paper [197], Ara h 1, Ara h 2 and Ara h 3 together accounted for 75% of total proteins in peanut, while the rest of peanut allergens accounted for another 10%. This means that about 85% of peanut proteins can potentially become allergens.

The bulk of peanut proteins are formed by storage proteins, dominated by two superfamilies: Cupins (Ara h 1 and Ara h 3) and Prolamins (Ara h 2, Ara h 6 and Ara h 7). Ara h 9 is also a prolamin but not a storage protein, as its role is to be a non-specific lipid transferase protein (the other two non-specific transferase proteins are Ara h 16 and Ara h 17). The remaining 8 allergens belong to a diversified group of specialised protein families: Ara h 8 is a Bet v 1, Ara h 5 is a Profilin, Ara h 10, Ara h 11, Ara h 14 and Ara h 15 are Oleosins, and finally Ara h 12 and Ara h 13 are Defensins.

The classification and relative positions of the 16 peanut allergens are summarised in Figure 1.5.1.

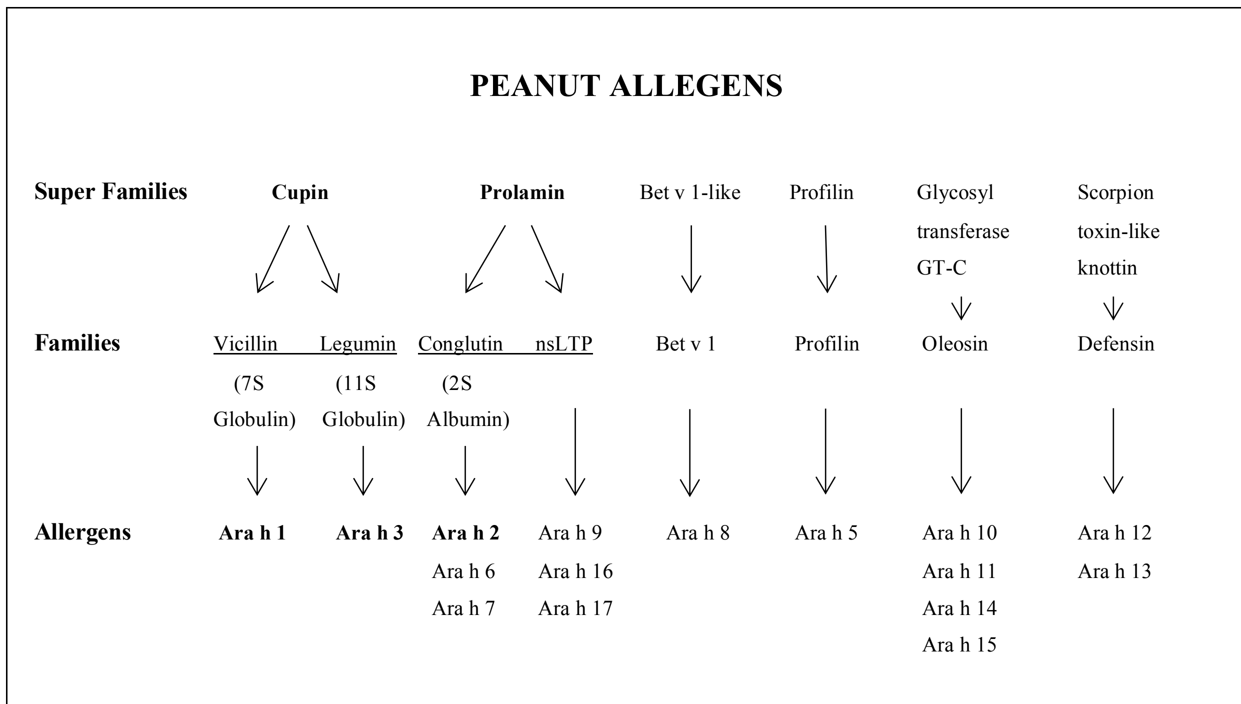


Figure 1.5.1: There are 16 peanut allergens under 6 Super Families and 8 Families

1.5.2 Individual peanut allergens and their cross-reactivity

1.5.2.1 Ara h 1

As mentioned earlier, Ara h 1 is a Vicillin or 7S globulin ("S" refers to the sedimentation coefficient) within the Cupin superfamily [198]. It has a molecular weight of 63.5 kDa and pI of 4.6, and in the native form it is a disc-shaped trimeric protein with a molecular weight of approximately 160 kDa. Its biological function is that of a storage protein, providing nourishment to the growing seedling. Its prevalence of IgE-binding reactivity in peanut allergic patients is determined to be 30-80% [199, 200]. It cross-reacts with other legumes and tree nut vicillins, as well as to Ara h 2 (a conglutin) and Ara h 3 (a legumin) [201, 202]. The paper by Vereda et al. [200] is particularly interesting because it suggests that peanut-allergic patients recognize different allergens and display different clinical symptoms in different areas of the world, possibly due to cross-reactivity from different environmental exposures and also culinary traditions. So far 23 IgE-reactive epitopes have been identified in Ara h1 [203].

1.5.2.2 Ara h 2

Ara h 2 is a Conglutin or 2S albumin within the Prolamin superfamily [204]. Structurally, it consists of 4 alpha helices held together by 4 disulphide bonds [194], which explains

why it is significantly more heat-resistant than most of the other peanut allergens (such as Ara h 1), which have either no or single disulphide bonds. It has two isoforms, designated as Ara h 2.01 and Ara h 2.02, with molecular weights of 16.7 and 18 kDa, and pI of 5.8 and 5.5 respectively [205, 206]. It has ten identified IgE-binding epitopes in its molecular structure, three of which are immune-dominant, and are located in the exposed and structurally flexible regions of the folded protein [207]. These three IgE-binding epitopes have been recently defined as the peptide biomarkers for prediction of symptomatic peanut allergic reactions [208]. They are also found to be responsible for cross-reactivity between Ara h 2 and the other major allergens Ara h 1, Ara h 3, and also Ara h 6 [202], suggesting that Ara h 2 may act as the primary sensitising molecule in peanut-allergic individuals. This may explain why Ara h 2 has such high prevalence in all patients with peanut allergy.

Ara h 2 has 59% amino acid sequence identity to Ara h 6, and 42% to Ara h 7. However, the similarity was low enough for the WHO/IUIS Allergen Nomenclature Subcommittee to recommend that they form separate allergens [194]. Ara h 2 is regarded as the most potent peanut allergen, with a prevalence rate of 42-100% [200, 209] in peanut allergic patients.

1.5.2.3 Ara h 3

Ara h 3 is a Legumin or 11S globulin in the same superfamily of Cupin as Ara h 1. Like Ara h 1, it is also a storage protein [210]. It shares 91% amino acid sequence identity with a peanut allergen previously known Ara h 4, and as a result the latter is now considered to be an isoallergen of Ara h 3, and renamed Ara h 3.02, while Ara h 3 becomes Ara h 3.01 [211]. The polypeptide chain of Ara h 3 has only a single disulphide bond, implying that it is more heat-labile than Ara h 2. In the native form, it is a hexameric protein with a molecular weight of 360 kDa, but after reduction in SDS-PAGE it becomes a monomer, with molecular weight of 60 kDa and pI of 4.6. Its prevalence of IgE binding in peanut allergic patients is reported to be 16-57% [199, 200], and so far four IgE-reactive epitopes have been recognised in Ara h 3 [210].

1.5.2.4 Ara h 5

Ara h 5 is a minor peanut allergen in the Profilin family, which is involved in pollen-associated peanut allergy, as in pollen allergy syndrome. It has a molecular weight of 14 kDa and pI of 4.6, and has a low sensitisation rate among peanut allergic patients, with

only 3.3% in the United States, 9-16% in Northern and Central Europe, and 24% (highest) in Spain [199, 200, 212]. It regulates polymerisation and depolymerisation of actin monomers, and does not cross-react with any other peanut allergen.

1.5.2.5 Ara h 6

As mentioned earlier, Ara h 6 is a 2S albumin and is very similar in structure to Ara h 2, with which it shares 59% of amino acid sequence identity. It has a molecular weight of 15 kDa and pI of 5.5. Unlike Ara h 2, it has a fifth disulphide bond linking the C-terminus to the compact fold of the molecule [213, 214], so it could be even more heat-resistant than Ara h 2. Its prevalence in IgE binding is only second to Ara h 2, in the order of 86-92% [209, 215]. Like Ara h 2 and Ara h 7, it is a source of amino acids for growth in seedlings, but is also involved in their defence against pathogens, a function closer to the more specialised Defensins Ara h 12 and Ara h 13. It has strong cross-reactivity with Ara h 1, Ara h 2 and Ara h 3 in peanut-allergic patients.

1.5.2.6 Ara h 7

Ara h 7 is another 2S albumin protein belonging to the same family of Ara h 2 and Ara h 6 (Conglutin). Like Ara h 2 it has two isoforms, with molecular weights of 16.4 and 17.4 kDa respectively (note: very similar to Ara h 2), but pI of 5.6 and 7.5 respectively (different from Ara h 2). As a result, they are more readily separated using 2-D gel and 2-D Western blot. Its prevalence of IgE-binding reactivity is slightly less than that of Ara h 6, at 43% [215], while its cross-reactivity to other peanut allergens is not known.

1.5.2.7 Ara h 8 and Ara h 9

Both proteins are minor peanut allergens, but can cross-react with allergens from some other plants. Ara h 8 is a Bet v 1-related peanut allergen, and as a result is more prevalent in patients who are also allergic to other Bet v 1 proteins, such as birch pollen. It has a molecular weight of 17 kDa and pI of 5.0. Its prevalence in IgE-binding reactivity is thought to be about 22-66% [199, 200].

Ara h 9 is a non-specific lipid transferase protein (nsLTP). It is of interest because of its digestion-resistant nature. Sensitisation to Ara h 9 is found in southern Europe, mainly in patients who are also suffering from peach allergy, mediated by IgE antibodies to the peach

LTP (*Prunus persica*, Pru p 3) [80, 216], with an IgE-reactivity prevalence of 3-24% in that region [200].

1.5.2.8 Ara h 10-13.

These allergens are collectively described here as a group of minor peanut allergens, with unknown IgE-binding prevalence in population, and unknown cross-reactivity. Ara h 10 has molecular weight of 17.6 kDa, pI 9.6. Ara h 11 has molecular weight of 14.3 kDa and pI 10.1, and Ara h 12 has molecular weight of only 5.2 kDa, the lightest of all peanut allergens, and pI of 7.7. Ara h 13 is only slightly heavier, with molecular weight at 8.4 kDa and pI of 7.5.

1.5.2.9 Ara h 14-17

Ara h 14-17 are four newly-identified peanut allergens that were only recently submitted to IUIS Allergen Nomenclature Subcommittee for provisional acceptance by two groups of investigators, in February and May of 2015 respectively (two allergens from each group). The claims were based on simple studies involving only small number of patients (n=25 and 33), with full details and structures of these allergens still not yet published. Ara h 14 and Ara h 15 (molecular weights 17.5 and 17 kDa respectively) were Oleosins, like Ara h 10 and Ara h 11, while Ara h 16 and Ara h 17 (molecular weights 8.5 and 11 kDa respectively) were non-specific Lipid Transfer Proteins like Ara h 9.

Table 1.5.2: Peanut allergen molecular weights varied between 5.2kDa and 63.5 kDa

Allergen	Molecular weight (kDa)	Isoforms
Ara h 1	63.5	Ara h 1.01
Ara h 2	16.6 18.0	Ara h 2.01 Ara h 2.02
Ara h 3	60 (as monomer) 37 (fragment)	Ara h 3.01 Ara h 3.02
Ara h 5	14.0	Ara h 5.01

Ara h 6	15.0	Ara h 6.01
Ara h 7	16.4 17.4	Ara h 7.01 Ara h 7.02
Ara h 8	17.0	Ara h 8.01 Ara h 8.02
Ara h 9	9.01	Ara h 9.01 Ara h 9.02
Ara h 10	17.6	Ara h 10.01 Ara h 10.02
Ara h 11	14.3	Ara h 11.01
Ara h 12	5.2	Ara h 12.01
Ara h 13	8.4	Ara h 13.01
Ara h 14	17.5	Ara h 14.01
Ara h 15	17.0	Ara h 15.01
Ara h 16	8.5	Ara h 16.01
Ara h 17	11.0	Ara h 17.01

1.6 PREVIOUS STUDIES INVOLVING BOILED PEANUTS

While boiling (wet-heating) reduces the allergenicity (i.e. IgE-binding to peanut allergens) of raw peanuts, roasting (dry-heating) has the opposite effect, and increases the allergenicity when compared to that of raw peanut extracts [217-220]. To date there have been 5 published papers on boiling whole-peanuts, and two on boiling purified peanut allergens Ara h 1 and Ara h 2

respectively. Maximum boiling times in these papers were limited to 60 minutes or less, and one was as short as 5 minutes.

As mentioned earlier, Beyer et al. in 2001 were the first group of people to investigate the effect of boiling on peanut allergenicity [13]. In their paper, two varieties of peanuts commonly grown in the United States (Florunner - a kind of Runner, and Valentia) were roasted (20 minutes), boiled (20 minutes) or fried (5-10 minutes) respectively. Proteins were analysed by using SDS-PAGE. Allergenicity was compared between nuts, by using immunolabelling (Western blot), with sera from 8 peanut-allergic patients. The paper concluded that the method of frying or boiling peanuts (as practised in China) appeared to have reduced the allergenicity of peanuts compared to the method of dry-roasting (as practiced in the United States).

Mondoulet et al. in 2005 published the second paper on boiling peanuts in relation to allergenicity [221]. In their study, raw peanuts of the Virginia variety were boiled for 30 minutes, and afterwards whole peanut protein extracts were prepared from raw, roasted and boiled peanuts respectively. In addition, major peanut allergens Ara h 1 and Ara h 2 were also extracted and purified from these three nut types, using “selective precipitation and multiple chromatographic steps”, and then characterised by electrophoresis and mass spectrometry. Separately, the immuno-reactivity of whole peanut extracts and purified peanut allergens was analysed using ELISA and Inhibition ELISA with sera from 37 peanut-allergic patients. The results of electrophoretic pattern showed that low molecular weight protein bands were less marked in boiled than in raw or roasted peanuts, while the same low-molecular weight proteins were also found in the cooking water. The IgE-binding capacity of whole peanut protein extracts prepared from boiled peanuts was 2-fold less than that of the extracts prepared from both raw and roasted peanuts. No significant difference was observed between extracts prepared from raw and roasted peanuts. The authors noted that the proteins in the cooking water were also recognisable by the IgE-containing sera from peanut-allergic patients, and postulated that the decrease in peanut allergenicity was mainly due to a transfer of the low molecular weight allergens into water during cooking. The IgE-immunoreactivity of purified Ara h 1 and Ara h 2 prepared from roasted peanuts was higher than their counterparts prepared from raw or boiled peanuts, suggesting that roasting has actually increased peanut allergenicity.

The third paper was only partly related to boiling peanuts, and was published by Maleki et al. in 2010 [222]. In it the authors compared the values of skin prick tests on 19 peanut-allergic

patients and 4 controls (non-peanut-allergic), using three different commercial peanut extracts (all prepared from raw peanuts) and three of their own-laboratory-made, specially prepared extracts from raw, roasted or boiled peanuts respectively. The heat-processed peanuts were either boiled or roasted for 5 minutes only. The SPT results showed that the boiled peanut extract had the highest specificity (67%) compared to the other extracts (42%-63%).

In 2011 Blanc et al. boiled purified Ara h 1 peanut allergen, which was extracted from either raw (native) or roasted peanuts, for 15 minutes at 100°C [223], and observed partial loss of Ara h 1 secondary structure, and formation of rod-like branched aggregates with reduced IgE-binding capacity and impaired ability to induce mediator release from activated T-cells. They concluded that Ara h 1 aggregates formed by boiling were morphologically distinct from those formed by roasting, and had lower allergenic activity.

In the same year Vissers et al. (who were the same group of investigators in the above paper but with the order of authorship slightly changed) also looked into the boiling of purified Ara h 2 allergen for 15 minutes, but in a "heated solution" at a higher boiling temperature of 110°C in the presence of glucose (which caused additional glycation during boiling) [224]. They concluded that boiling and glycation at 110°C of Ara h 2 isolated from raw peanut had resulted in extensive denaturation, hydrolysis and aggregation of the protein, while Ara h 2 and Ara h 6 isolated from roasted peanut managed to retain their native conformation. It was not clear whether this higher temperature reading was due to the presence of glucose in solution (thus raising the boiling point), as this was not specifically discussed in the paper.

Also in 2011, Cabanillas et al. [225] boiled raw peanuts at 100°C for 60 minutes and compared changes to allergenicity with roasted peanuts that have been autoclaved at 121°C (low, 1.18 atmospheric pressure) for 15 and 30 minutes, and also at 138°C (high, 2.56 atmospheric pressure) for 15 and 30 minutes. They demonstrated significant reduction of IgE immunoreactivity (based on Western blot, ELISA and skin prick tests in peanut-allergic patients) at extreme conditions of autoclaving (2.56 atmospheric pressure 30 minutes), when compared to both roasted and simply boiled peanuts.

In 2013 Kim et al. [226] evaluated IgE-mediated reactivity to major peanut allergens Ara h 1, Ara h 2 and Ara h 3 in 42 Korean children with peanut specific IgE levels of 15 kU/L or higher, using protein extracts prepared from boiled (10 minutes), roasted (10 minutes), fried (10

minutes) and pickled (boiled peanuts left in white vinegar overnight). SDS-PAGE and Western blot were used for analysis of IgE-reactivity. They found that Ara h 2 was less prevalent in Korean children compared to Western children, and that vinegar treatment of boiled peanuts seemed to be the most effective way of reducing IgE-reactivity of all three peanut allergens, when compared with the other methods of heat processing.

1.7 TRANSLATION FROM EXTENDED BOILING OF PEANUTS TO SEQUENTIAL ORAL IMMUNOTHAPY

I first came across Dr Beyer's paper in 2001, which has left an indelible mark in my memory. In that paper, Dr Beyer pointed out that the prevalence of peanut allergy in China was low compared to Western world, and hypothesized that this was because Chinese children ate boiled peanuts rather than peanut butter or roasted peanuts. She went on to show that peanuts that had been boiled for 20 minutes were less allergenic than peanuts roasted for a similar period. I was impressed with her hypothesis and thought that she was probably correct, but dissented in that her choice of boiling time (only 20 minutes) seemed surprisingly short. I knew in China peanuts were often cooked and eaten with other foods, but the cooking time was more like 2 hours. I wondered why Dr Beyer did not bother to boil peanuts a little bit longer and make them even less allergenic.

Then from the beginning of 2009 a series of papers on peanut oral immunotherapy started to appear. A common message from these studies was that the rate of adverse events (AEs) from treatment was unacceptably high, anywhere between 45% and 93%, and as a result all up-dosing procedures had to be closely monitored at hospitals. Then, about 6 years ago, I suddenly made a translational connection between the findings of Dr Beyer's paper and these studies, and pondered whether desensitisation using hypoallergenic boiled peanuts might mitigate some of the AEs.

Two immediate questions sprang to mind. One was how long should peanuts be boiled? I suspected that Dr Beyer's original 20 minutes of boiling time was way off the mark, and intuitively felt that it should be at least 2 hours, the same time that Chinese people cooked

peanuts with their foods. Then I realized that to find out the correct answer in a proper way I would need to learn more about proteomics and study for a PhD, as there was insufficient information on extended boiling of peanut in the literature.

The second question was that (if extended boiling could indeed make peanuts hypoallergenic), would its exclusive use in desensitisation lead to full protection? Then I quickly came up with a viable solution: why not use a two-step approach, ingesting hypoallergenic boiled peanuts first and then roasted peanuts second in a sequential manner, which would then guarantee full desensitisation?

In early 2012 I decided to enrol myself to do a part-time PhD over 4 to 5 years (part-time because I will still need to see patients and recruit them for the clinical trial). I spent the next two years learning proteomic techniques (which formed the first half of my thesis) and then another two years to complete a proof-of-concept pilot study (thus concluding my PhD). I made three hypotheses as I started my study:

1. Extended boiling progressively reduces peanut allergenicity, but for practical use in oral immunotherapy the minimal boiling time is 2 hours.

2. Desensitisation with hypoallergenic peanut can mitigate adverse events, and as a result up-dosing can be done at home, avoiding hospital supervision.

3. Using hypoallergenic boiled peanut alone may not be able to provide full protection, but the subsequent use of roasted peanut in a sequential manner can compensate for the deficiency and achieve full desensitisation.

This thesis was written primarily to validate these three hypotheses, but its real goal is to transform the landscape of peanut oral immunotherapy in the future, making it readily available to all peanut-allergic children and adults who want to be desensitised, and do that in more friendly settings outside hospitals.

Chapter 2

Materials and Methods

SECTION 2A: MATERIALS

2.1 Materials used for *in vitro* evaluation of peanut allergenicity

2.1.1 Peanut and other materials for making extracts

- "Jumbo" raw peanuts with no shells but intact kernel skins, weighing approximately 1 gram each, were purchased from Charlesworth Nuts Adelaide, Australia. Kernel skins were manually removed immediately before boiling. Similarly treated skinless kernels were also used as source of raw peanuts.
- Protease inhibitors for stabilizing proteins during solubilisation or storage were usually prepared fresh immediately before use, by dissolving Roche cOmplete EDTA-free tablets in PBS according to manufacturer's instruction. The resultant "cocktail" comprised 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.3 M phenylmethanesulfonyl fluoride.
- Protein measurement kits for measuring protein concentration**:** either EZQ® Protein Quantitation Kit (Invitrogen) or Bio-Rad DC Protein Assay Kit (Bio-Rad Hercules, California USA) could be used.

2.1.2 Peptides for vaccination of animals to raise polyclonal IgG antibodies

- 20-mer Ara h 1 and Ara h 2 peptides were designed in-house and then manufactured by order from Mimotopes, Melbourne, Australia. Sequences of these peptides were described in detail under Methods.

2.1.3 Primary antibodies

- Human peanut-specific IgE antibodies were provided by sera from 10 peanut-allergic patients with their serum concentrations pre-determined by ImmunoCAP assay (Phadia, Uppsala, Sweden). The sera were stored at -80˚C immediately after collection.
- Rabbit or sheep polyclonal IgG antibodies to Ara h 1 and Ara h 2 were raised in-house from vaccinated animals. Antibodies were lyophilized and stored at -20˚C as dry powder in aliquots.

2.1.4 Secondary antibodies

- Biotinylated goat anti-Human IgE antibody was purchased from Invitrogen/Life Technologies, Carlsbad, CA, USA.
- Biotinylated donkey anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch, West Grove, PA, USA.
- Biotinylated rabbit anti-sheep IgG antibody was purchased from Jackson ImmunoResearch, West Grove, PA, USA.

2.1.5 Common materials for 1-D Electrophoresis (also called 1-DE, 1-D gel or 1-D SDS-PAGE), and 2-D Electrophoresis (2-DE)

- Precast AnykD 18-well Criterion Stain-Free TGX polyacrylamide gels for 1-DE (Bio-Rad Hercules, California USA) and Western blot.
- Precast 4-20 kD IPG+1 well Criterion Stain-Free TGX polyacrylamide gels for 2-DE (Bio-Rad Hercules, California USA).
- Molecular weight markers: Precision Plus Protein Dual Colour Standards and Precision Plus Protein Unstained Standards, (both from Bio-Rad Hercules, California USA).
- DTT (Astral Scientific, Gymea, NSW, Australia) to reduce proteins before electrophoresis.
- Bromophenol blue (Sigma-Aldrich, Castle Hill, NSW, Australia) to indicate electrophoresis front with a blue colour.

2.1.6 Materials for Western blot

- Immobilon PVDF Transfer membrane, low-florescence 0.2 µm (Millipore Billerica, MA, USA).
- Extra thick Protean XL blotting paper (Bio-Rad Hercules, California USA).
- HRP-conjugated Streptavidin (Pierce, Waltham, MA, USA) as enzyme. HRP is short for Horse Radish Peroxidase.
- SuperSignal West Pico chemiluminescent substrate (ThermoFisher Scientific, Rockford, IL, USA).
- Blocking-agents: Bovine Serum Albumin, BSA high grade (Gibco, ThermoFisher Scientific, Rockford, IL, USA) or Diploma skim milk powder (Fonterra brand, Mount Waverly, Vic, Australia).

2.1.7 Materials for 2-D gel electrophoresis

- ReadyStrip 2-D gel strip for isoelectric focusing (Bio-Rad Hercules, California USA).
- Agarose for 2-D gel (Sigma-Aldrich, Castle Hill, NSW, Australia).
- 4% (w/v) CHAPS (an abbreviation for 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate).
- TUC buffer: 2M thiourea, 7M urea.
- Strip holder cleaning solution (GE Healthcare, Little Chalfont, UK)
- IPG buffer: Bio Lyte 3-10 buffer from Bio-Rad Hercules, California USA as 100x 20% bottles.
- Equilibration buffer: 100 mM tris-HCl, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, pH = 8.0.
- Equilibration solutions 1 and 2: (1) 1% (w/v) DTT in equilibration buffer; (2) 4% (w/v) iodoacetamide in equilibration buffer, 0.003% (w/v) bromophenol blue.
- Whatman No.1 filter paper

2.1.8 Materials for Inhibition ELISA

- 96-well high protein-binding Microlon plate (Greiner Bio-One, Kremsmünster, Austria).
- TMB Peroxidase EIA Inhibition ELISA substrate (Bio-Rad Hercules, California USA).
- Blocking-agents: Bovine Serum Albumin, BSA (high grade) or Diploma skim milk powder
- ELISA “stopper” to terminate enzyme activity on substrate: 1M sulphuric acid.

2.1.9 Materials for mass spectrometry

- *Vivaspin* 500 3-kDa centrifugal concentrators.
- Acetonitrile, LiChrosolv® hypergrade for LC-MS grade, 99.9% purity (Fluka Analytical, division of Sigma).
- Formic acid, Puriss, P.A. for mass spectrometry grade, 98% purity (Sigma).
- Propanol, Chromasolv® Plus for HPLC grade, 99.9% purity (Sigma).

2.1.10 Materials for T-cell proliferation test

- Cell Proliferation dye eFluor® 450 from eBioscience, San Diego, USA
- Cell culture medium RPMI-1640 from Sigma Life-sciences, MO, USA

- Staphylococcal enterotoxin B (SEB) from Sigma-Aldrich, MO, USA
- Tetanus toxoid (TT) from CSL Pharmaceuticals, Melbourne, Australia
- Monoclonal anti-CD antibodies for staining T-cells: anti-CD3 (clone SK7), anti-CD4 (clone SK3), anti-CD25 (clone 2A3) and anti-CD134 (clone ACT35) from BD Biosciences, San Jose, USA
- Viability dye eFluor® 660 from eBioscience, San Diego, USA (to discriminate live from dead T-cells)
- Raw, 2-hour and 12-hour boiled peanut extracts made at Flinders Proteomics Facility

2.1.11 General purpose buffers

- 4x 1-D Sample buffer: 250 mM tris-HCl, 40% (v/v) glycerol, 8% (w/v) SDS, 400 mM DTT, 0.04% (w/v) bromophenol blue, pH 6.8
- Transfer buffer: 25 mM tris, 192 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS
- Blocking buffer: 0.5% (w/v) skim milk powder in TBST, filtered.
- 2.2.22 1x Running buffer: 25 mM tris, 192 mM glycine, 0.06% (w/v) SDS, pH 8.3
- Inhibition ELISA coating buffer: 50 mM sodium bicarbonate buffer containing 0.01% thimerosal, pH 9.6
- ELISA washing buffer: 20 mM tris, 150 mM NaCl, 0.1% (w/v) sodium azide, 1 mM EDTA, 0.3 mM PMSF, pH 7.4
- 10x PBS (phosphate buffered saline): NaCl, KCl, Sodium hypophosphate, Potassium hypophosphate.
- TBS (Tris buffered saline): 20 mM tris, 150 mM NaCl, pH 7.4
- TBST (Tris buffered saline with Tween-20): 0.1% (v/v) Tween-20 in TBS

2.2 Materials Used for Skin Prick Tests (SPT)

2.2.1 Lancets for making skin pricks

Unilet GP superlite single-use general-purpose lancets (Owen Mumford, UK).

2.2.2 Commercially available raw peanut extract for SPT

Raw peanut extract in 50% glycerol from Hollister Stier, Washington, USA

2.2.3 Raw and boiled peanut extracts specially made at Flinders Proteomics Facility

The following peanut extracts were produced in-house under sterile conditions at Flinders Proteomics Facility, for exclusive use in SPT. Extracts were normalised to a concentration of 2 µg/µl of peanut protein, 50% v/v autoclaved glycerol and 0.9% benzyl alcohol.

- Raw peanut extract
- 1-hour boiled peanut extract
- 2-hour boiled peanut extract
- 4-hour boiled peanut extract
- 1-hour boiled leachate
- 2-hour boiled leachate
- 4-hour boiled leachate

2.3 Materials Used for the Pilot Study

2.3.1 Printed documents, either given to participants and their parents or kept in patient files for record (printed on A4 papers, some more important documents are listed in Appendix section). They include:

- Letter of Invitation
- Participant Information and Informed Consent Form (PICF)
- Patient demographic and medical history data
- Referral letter to Flinders Medical Centre Day Unit for oral peanut challenges
- Biphasic peanut OIT protocol given to every participant with full explanation
- Detailed addition advice to avoid adverse events during OIT treatment
- Patient treatment and compliance diary
- Record of adverse events

2.3.2 Two-hour boiled peanuts for the first phase of oral immunotherapy

- Jumbo-sized raw peanuts in 2 Kg batches were boiled (simmered) for 2 hours and then dehydrated for 3 days in two Sunbeam DT5600 food dehydrators at the main factory of Charlesworth Nuts Adelaide. After delivery, dehydrated boiled peanuts were weighed and individually packed at Flinders Proteomics Facility for distribution to participants.

2.3.3 Roasted peanuts for the second phase of oral immunotherapy

- Jumbo-sized raw peanuts were roasted at Charlesworth Nuts Adelaide and sold as "roasted peanuts" at their retail outlets. After delivery, the nuts were weighed and individually packed at Flinders Proteomics Facility for distribution to participants.

SECTION 2B: METHODS

2.4 Methods Used for *in vitro* Evaluation of Peanut Allergenicity

2.4.1 Collection of sera from patients with high serum peanut-specific IgE

1. Blood samples were collected from 10 peanut-allergic children (age 8-14, M6:F4,) with high peanut-specific IgE-reactivity ranging from 91.8 kU/L to >100 kU/L as determined by ImmunoCAP assay (Phadia, Uppsala, Sweden).
2. Sera were separated from bloods after centrifuging at 4,000 r.p.m. or 2465 g for 10 minutes, divided into aliquots, and stored at -80ºC.
3. Human Ethics approval for use of patient sera in *in vitro* experiments was obtained from Southern Adelaide Clinical Human Research Ethics Committee (SACHREC), approval number 126.13.

2.4.2 In-house production of peanut-specific animal-origin IgG antibodies

2.4.2.1 Acknowledgement

All animal IgG antibodies were produced by the *Flinders University Polyclonal Antibody Production Facility*. Animals were nursed and vaccinated at the *Animal House Facility* in Flinders Medical Centre. My involvement was limited to assisting the design of 20-mer peptides for vaccination, performing literature search and using software tools available at Flinders Proteomics Facility to select the best candidates (see below). Animals either sheep or rabbit were used to raise anti-Ara h 1 or anti-Ara h 2 polyclonal IgG antibodies, depending on availability.

2.4.2.2 Design and synthesis of 20-mer peanut peptides

A list of 20-mer peanut T-cell epitopes from either Ara h 1 or Ara h 2 which could be suitable for vaccination were selected from literature [227, 228]. Potential candidates were screened using *MacVector v12* software to identify regions of high antigenicity by engaging the following *modules*: Parker antigenicity, Profusion index antigenicity, Welling antigenicity and Antigenic index. Window settings for the software were 11 for each module except for Antigenic index, which had a setting of 7. Regions of high antigenicity were then blast-searched using *NCBI BLASTg* algorithm to determine if other peanut allergens shared significant regions of homology. At the end of screening, one Ara h 1 and two Ara h 2 20-mer peptides were identified to be suitable (Table 2.4). The sequences of selected epitopes were then sent to Mimotopes Pty Ltd of Clayton, Victoria, Australia for synthesis.

Table 2.4.2: Details of Peanut Allergens Used for Production of Animal Antibodies

One Ara h 1 and three Ara h 2 antibodies were produced from 3 rabbits and 1 sheep

Peanut Antigen	Animal involved	Catalogue Number	20-mer peptide sequence
Ara h1	Rabbit	Rb1-180913-WS Rb1-180913-AP	FQNLQNHRIVQIEAKPNTLV
Ara h2a	Rabbit	Rb3-080812-WS Rb3-080812-AP	ELNEFENNQRCMCEALQQIM
Ara h2b	Rabbit	Rb2-180913-WS Rb2-180913-AP	DSYGRDPYSPSQDPYSPSQD
Ara h2b	Sheep	Sh2-100914-WS Sh2-100914-AP	

2.4.2.3 Vaccination, antibody production and purification

Vaccination was completed over a 16 weeks period using the following protocol:

1. Synthesized peptides were conjugated to *Keyhole Limpet Hemocyanin.*
2. 400 µg of the conjugated product in 1 ml 0.9% sodium chloride was mixed with 1 ml of Complete Freunds Adjuvant, and injected into the animal (sheep or rabbit) once a week for the first 2 injections, and then bi-weekly for the following 14 weeks.

3. Antibodies raised were polyclonal anti-Ara h 1 or anti-Ara h 2 IgG specific to the amino acid sequence of synthesized 20-mer T-cell epitopes. Antibody production was monitored bi-weekly with ELISA, using the same original synthetic peptide as antigen.
4. Animal was euthanized when antibody production reached a plateau, as indicated by the ELISA results. Blood was taken and allowed to clot. It was later centrifuged at 4,000 r.p.m. or 2465g for 10 minutes to yield serum. Specimens were aliquoted and stored at -80°C before affinity purification at a later date.
5. Affinity-purification was performed using 1-ml Hi-Trap NHS-Activated Affinity Column (GE Healthcare, Little Chalfont, UK) with the original peptides incorporated into the beads.
6. Purified and lyophilized antibodies were stored as dry powder in aliquots at -20˚C.

2.4.2.4 Animal Ethics. Ethics approval was obtained from Flinders University Animal Welfare Committee, approval number 843/12.

2.4.3 Boiling of peanuts and solubilisation of proteins to produce extracts

2.4.3.1 Boiling of peanuts

Peanut skins were manually removed from kernels in 6 batches of raw peanuts of 30g each, with the first 5 batches destined for "boiling". More accurately, these de-skinned kernels were not physically "boiled" at atmospheric pressure (i.e., at 100ºC), but were actually heated in 300ml of deionized water at a constant temperature of around 98ºC, inside a closed Schott bottle, which was submerged in a continuously boiling water-bath. Although the water temperature <u>outside</u> the Schott bottle was 100ºC as a result of constant boiling, the water temperature <u>inside</u> could only reach 98ºC (determined in a separate experiment) because the thickness of glass had produced an impedance to heat conduction. The lids of the bottles were screwed tight during water-bath to prevent leakage. The periods of boiling were 30 minutes, 1, 2, 4, or 12 hours respectively. Pre-measured volume of water in each Schott bottle (10x v/w) was preheated and equilibrated with surrounding boiling water-bath before the peanuts were added, to minimise the water heat-up time at start of count-down. The cooking water (leachate) in each bottle was retained for further use at each time-point. After the designated time of cooking, the bottle was removed from water-bath and each batch of boiled peanuts was dehydrated for 24 hours in a Sunbeam DT5600 food dehydrator prior to defatting. Dehydration was not required for raw peanuts because they had not been exposed to water.

2.4.3.2 Solubilisation of peanut proteins to produce extracts

Proteins were extracted from both raw and boiled peanuts using a combination of methods described by Beyer et al. [13] and Maleki et al. [222]. Briefly, each batch of raw or boiled peanuts was ground to a smooth paste using initially a small hand-held coffee grinder to produce a rough grind, and then a mortar and pestle to produce the paste. Each batch was then defatted in 20 volumes of acetone inside Falcon tubes (4 Falcon tubes per batch, containing 2 grams of peanut paste in 40 ml acetone for each tube). The Falcon tubes containing peanut and acetone were agitated for 4 hours at maximum speed of a Patek Instruments Multi-mixer, followed by centrifuging at 2,500*g* for 20 minutes at 4ºC. Afterwards, the acetone was discarded while the pellets were washed again with 5 ml of new acetone, with the mixture vortexed during washing. The newly washed mixture was centrifuged again, and after discarding the small amount of supernatant acetone (which removed the last trace of fat), the remaining pellets were allowed to air-dry in the Falcon tubes (with lids removed but loosely covered with perforated alfoil), for 24 hours inside a ducted fume hood with exhaust fan turned on throughout drying.

The dried pellets were then re-suspended in 5 volumes of PBS (pH 7.4) to solubilise the protein contents, in the presence of protease inhibitors. To achieve solubilisation, mixtures went through 5 cycles of (1) vortexing for 1 minute and (2) pulse-sonicating for 1 minute using a micro-tip (Misonix S-4000 Ultrasonic Processor) at 80% power. After completion of the 5 cycles, extracts were centrifuged at 12,000*g* for 15 minutes at 4°C.

The proteins in the leachates were already solubilised, so they were only centrifuged at 12,000*g* for 15 minutes at 4°C, without preceding sonication. Both the peanut extracts and leachates were then filter-sterilized using 0.2 μm filters. Filtrates were stored in aliquots at -80ºC.

2.4.4 Determination of protein concentration in extracts

Two methods were available for quantifying protein concentration. The first method was used earlier in the investigations, while the second method was learned later.

2.4.4.1 EZQ protein quantitation assay

The EZQ (Invitrogen) assay is a fluorescence-based procedure using a fluorescent dye that stains proteins. After staining, fluorescence was produced under ultraviolet light and the intensity can be quantified using an UV imager.

Before assay, protein standards were prepared by reconstituting 2 mg ovalbumin (Invitrogen) with 1 ml of deionised water (dH_2O) to give a 2.0 mg/ml stock solution. Serial dilutions were made to yield standards of 1.0, 0.5, 0.2, 0.1, 0.05 and 0.02 mg/ml.

The assay paper was fitted into a microplate-based housing which was then covered with a perforated stainless steel template called the "backing plate". 1 µl drops of either standard or the sample solutions to be assayed were spotted onto the assay paper in triplicates, guided by the template perforations. The spots on assay paper were allowed to dry at room temperature, and afterwards the paper was removed from the housing, trimmed, and treated in 40 ml of methanol in a Perspex tray for 5 minutes, on an orbital shaker (~50 RPM) at room temperature. After treatment, the paper was dried on "low heat" inside an Easy Breeze Gel Dryer. The dried paper was then submerged in 35 ml of EZQ Protein Quantification Reagent in a new tray. After returning to the Orbital Shaker for 30 minutes at room temperature, the paper was washed with EZQ de-staining solution (10% methanol and 7% acetic acid in de-ionized water) 3 times for 3 minutes each on the Orbital Shaker.

The assay paper was scanned using a Gel Doc EZ Imager (Bio-Rad Hercules, California USA) connected to a MacBook Air lap-top computer. The Carestream Molecular Imaging Software Version 5.0.6.20 (Carestream Health Inc., Rochester, NY) was used for quantification.

The EZQ method can only provide an estimate of protein concentration by comparing fluorescence readings between test-protein samples and the ovalbumin standards, but unfortunately the composition of the two proteins are not identical. It is therefore not a true quantitative method of protein assay.

2.4.4.2 DC protein assay

This assay (*DC* means detergent compatible) is a colorimetric assay to detect protein concentration after detergent solubilisation. In the process, a special reagent will react with

the amino acids *tyrosine* and *tryptophan* (and to a lesser extent *cysteine* and *histidine*), causing a colour change from clear to blue, with maximum absorbance at 750 nm and minimum absorbance at 405 nm of light wavelength. Because the assay quantifies proteins in solution rather than on a porous absorbent paper as used in EZQ, it is more accurate in measuring smaller-sized proteins and peptides, because in EZQ a significant amount can be lost through lack of binding or being removed during the washing steps.

The DC package came with two reagents, A and B. *Reagent A* contains an alkaline solution of copper tartrate while *Reagent B* contains a dilute Folin reagent. Mixing the two reagents with the solubilised protein will lead to a blue-colour change, which can then be measured using a plate reader capable of measuring absorbance at 620 nm.

Protein standards were prepared the same way as in EZQ, using serial dilutions of ovalbumin with a range from 0.2 mg/ml to 1.5 mg/ml, using PBS as blank. After setting aside the prepared standards, peanut extracts were diluted to 1:1, 1:5, 1:10 and 1:20 serially.

Using a Socorex brand "non-movable" pipette (which ensured consistency of pipette volume because a large numbers of wells had to be filled serially), 5 µl each of standards, blanks, and sample dilutions were added to the 96 wells of a microplate in triplicates. Afterwards, 20 µl of Reagent A and 200 µl of Reagent B were added sequentially to each well, using a multi-channel pipette to save time. The plates were agitated for 15 minutes at room temperature, and then read at 620 nm wavelength using a DTX 880 plate reader (Beckman Coulter) with a Bio-Rad Hercules, California USA DC photometric reader.

Like EZQ, it has the same flaw in being able to provide only an estimate of protein content of the test-protein, comparing specifically with ovalbumin as the standard. It is therefore also not a true quantitative method.

2.4.5 One-dimensional gel electrophoresis (also called 1-D gel, 1-DE or 1-D SDS-PAGE)

Precast *AnyKd* TGX stain-free gels in 18-well format were selected for performing 1-D SDS-PAGE and Western blot experiments because most peanut allergens fell into the 10-70 Kd molecular weight range. With 2-D gel performed on 2-hour boiled peanuts (and raw peanuts for comparison), the precast *4-20Kd* stain-free gels were deemed more suitable because protein

fragments formed by boiling were smaller than the original molecules and would therefore be visualized better in the lower density-gradient gel.

All precast gels were stored inside an individually-sealed pack at 4ºC, with the gel protected by, and securely sandwiched between, two clear plastic sheets with a built-in upper tank arrangement. In preparation for electrophoresis, the gel with its plastic sheets were rinsed with deionized water and lowered into a Criterion Electrophoresis Tank filled with Running-buffer up to the recommended line, which became the "lower tank". The upper tank of the precast gel was then filled with the same Running-buffer. Extract samples were later instilled into the wells at the top of the gel, with the tips dipping through the buffer solution in the upper tank before releasing the pipettes. Up to 17 samples could be instilled for each gel. A dual colour molecular weight marker (MWM) was instilled into the first lane to become the reference column.

Usually about 20 µl of an extract containing 10-15 µg of protein was instilled into a well. After calculations, sufficient quantities (at least double the volume required for each well) for each sample were prepared and kept in separate 1.7 ml Eppendorf tubes, buried in crushed ice inside an Esky.

Proteins were *reduced* before SDS-PAGE by adding a small quantity of DTT (made up by adding 8 mg of DTT to 200 µL of 4x sample buffer + the dye *bromophenol blue* to give it a blue colour). The DTT was added to all samples prior to heat treatment. Heat treatment was performed on all Eppendorf tubes using a heating block at 95ºC for 2 minutes. Afterwards, all tubes were centrifuged briefly to leave any solid as a pellet at the bottom of the Eppendorf tube. The calculated amount of sample solutions was then pipetted into each well of the gel.

After instillation, the gel was electrophoresed at 300V constant voltage until the *bromophenol blue* dye front reached approximately 0.5 cm above the bottom edge of the gel (this usually took 20-25 minutes and closer observation was recommended towards the end of the run). The blue front must not be allowed to pass below the bottom line of the gel or else the proteins would leak into the buffer solution in the tank, invalidating all results.

After electrophoresis, the gel was removed from the tank and the protective plastic sheets carefully separated, exposing the soft gel. The gel must be handled with care, and after rinsing with deionized water on a reading plate the gel was imaged under UV light inside a Bio-Rad

Gel Doc Imager. Images could be optimised by altering exposure time and reproduced in a variety of colours, with blue the most popular to mimic Coomassie Blue staining.

2.4.6 One-Dimensional Western blot

1-D Western blot was completed in the following steps:

1. *Starting with 1-D gel:* It is necessary to make a standard gel from raw and boiled peanuts before the proteins can be transferred from the gel to a membrane. The procedure of how to make 1-D gel was described above in Section 2.4.5.
2. *First imaging:* The 1-D gel was imaged in a Gel-Doc EZ (Bio Rad), using initial 5 minutes activation time and subsequent exposure to achieve the best visualisation. This step was required to make sure that the gel quality was good for transferral.
3. *Transferring:* Proteins must be transferred onto a membrane first before they can be probed with primary antibodies. A small sheet of PVDF membrane and two pieces of extra-thick filter paper (Bio-Rad Hercules, California USA) were cut to the same size as the gel. The membrane was pre-soaked in 100% methanol for 30 seconds, and then the gel, the membrane and filter papers were separately equilibrated in 1x-transfer buffer (using different trays) on an Oxyos Shaker, with speed set at 50 r.p.m., for 15 minutes. The gel and membrane were then put together close to each other and sandwiched between the two filter papers. The gel-and-membrane combination was carefully laid out in a Bio-Rad *TransBlot Turbo* system in the correct orientation so that proteins were transferred from gel (negative pole) to membrane (positive pole) and not vice versa. The TransBlot Turbo was set at 25V for 30 minutes.
4. *Second imaging:* A needle was used to pierce holes in the membrane, guided by the visible bands of the coloured molecular weight marker. This is necessary because the colours of the marker, while still visible immediately after the transfer, will be permanently lost from the membrane after subsequent washings and incubation with primary and secondary antibodies, leaving the membrane with no reference marks except the holes. The membrane was then imaged with Gel-Doc EZ to check its quality.
5. *Blocking:* After imaging, the membrane was blocked with 5% BSA in PBS-T for 1 hour, to fill up all un-occupied binding sites on the membrane.
6. *Incubation with primary antibody:* After blocking, the membrane was washed 3x of 5 minutes each, in PBS-T. A *primary antibody* was then added to the membrane to probe the antigens trapped in it. Primary antibodies could be either pooled peanut-allergic patient sera (IgE, diluted 1:20 in PBS-T), or rabbit / sheep polyclonal anti-Ara h 1 / anti-

Ara h 2 antibodies (IgG, 1 µg/ml in PBS-T), which were raised locally as described in Section 2.4.2. The membrane and the primary antibody were incubated overnight at 4ºC on a Stuart mini gyrocker, with speed set at 50 r.p.m. This was done inside a large glass-door refrigerator with temperature set at 4ºC.

7. *Incubation with secondary antibody:* After washing with PBS-T 3 times for 5 minutes each, the membrane was now incubated with a *secondary antibody* for 10 minutes. Commercially available anti-human IgE antibody, anti-rabbit IgG antibody, or anti-sheep IgG antibody would be chosen depending on the nature of the primary antibody. Secondary antibodies were diluted 1:15,000 (2 µl in 30 ml of PBS-T) before incubation.
8. *Incubation with Streptavidin-HRP:* Commercial secondary antibodies were already biotinylated and would therefore conjugate strongly to the enzyme Streptavidin-HRP. The membrane with the secondary antibody already bound to the primary antibody was washed with PBS-T 3 times for 5 minutes and then incubated with diluted HRP-conjugated Streptavidin (Thermo Scientific-Pierce) at 1:10,000 (1.2 µl in 12 ml PBS). Incubation time was 1 hour in darkness (usually done by covering the tray with alfoil), at room temperature.
9. *Incubation with substrate:* The membrane, now with the enzyme firmly conjugated to the secondary antibody, was double-washed with PBS-T 3 times for 5 minutes and then PBS 1 time for 5 minutes. The substrate (Clarity Western ECL Bio-Rad Hercules, California USA) was made fresh, by mixing its 2 components at 1:1 ratio (6 ml of each solution). The mixture was poured on top of the membrane and incubated for 5 minutes.
10. *Third and last imaging:* Detectable *chemiluminescence* from the interaction between the enzyme and the substrate was imaged under ultraviolet light using a Fujifilm SAS-4000 CCD imager. Exposure time was set at "automatic" first, and then fine-tuned to produce the best result.
11. *Quantification:* Individual lane reactivity could be quantified by drawing a "transect" through each lane, subtracting the background, and then integrating the total reactivity of image in each lane.

2.4.7 Two-dimensional (2-D) gel electrophoresis

Each 2-D gel took 3 days to complete, following the protocol below:

1. *Day 1: Protein extract cleanup and soaking of gel strip. Cleanup* improves spot detection and produces sharper image for 2-D electrophoresis. The procedure worked by quantitatively precipitating and concentrating proteins in a sample while leaving

behind and washing away substances such as ionic detergents, salts, nucleic acids and lipids, all of which were known to interfere with isoelectric focusing. Sample extracts were cleaned up using ReadyPrep 2-D Clean-up kit (Bio-Rad), following manufacturer's instructions. Briefly, 100 µl of extract was cleaned up each time, using Precipitating Agent 1 and 2 to precipitate the proteins, and Washing agent 1 and 2 to wash the precipitants. After repeated centrifuging at 6000g for 5 minutes between reagents and subsequent removal of supernatants, the protein pellets were re-solubilized using rehydration/sample buffer (containing TUC) back to the original volume of 100 µl. Cleaned extracts were stored at -20°C in aliquots either from a previous preparation or for future use. EZQ assays on cleaned extracts were performed which showed that raw peanut extract had a protein concentration of 20 µg/µl while the 2-hour boiled peanut extract had a protein concentration of 2.5 µg/µl. Consequently, to prepare fresh solutions of either raw or 2-hour boiled peanut for gel strip soaking (the two experiments must be performed separately as each 2D gel procedure took 3 days to complete), 1 µl of cleaned raw peanut extract or 8 µl of 2-hour boiled peanut extract had to be diluted respectively with 275 of TUC buffer containing 0.4% DTT, 0.5% ampholyte solution, and 0.5 µl of bromophenol blue solution.

For *soaking*, approximately 275 µl of the diluted extract was added to the bottom of a ceramic gel electrophoresis container, also known as the "coffin". A dry gel strip was removed from its package with forceps, and placed into the extract, with the writing side up and gel side down. The gel strip must be fully immersed in the extract with the end marked "positive" in close proximity with the positive pole of the coffin. About 1.5 ml of mineral oil was added evenly on top of the gel strip to ensure full coverage before the lid was closed. The loaded coffin was placed in the middle of an IPGphor unit, with the positive end of the coffin in the positive section of the base. The plastic cover of the IPGphor was then pressed down onto the coffin. The unit was set at Rehydration Mode: O/N rehydration, 20°C, 50V. The strip was allowed to rehydrate (soak) in the coffin overnight.

2. *Day 2: Isoelectric Focusing to make the first dimension*. The IPGphor unit was stopped in the morning after returning from overnight. The coffin was removed from the unit and placed on a clean disposable towel. The gel strip was lifted from the coffin and cleaned carefully with IPG detergent and deionized water. The inside of the coffin was also cleaned, and small squares (2 by 2 mm) of wet filter paper (using de-ionized water) called "wicks" were placed over the electrodes of the coffin at both ends. The wet gel

strip was then maneuvered to fit snugly between the wet wicks, making connection to the electrodes for isoelectric focusing (IEF). The gel strip must be placed with the gel side down and positive end hard-up against the positive pole to ensure correct orientation. The coffin was then put back onto the IPGphor unit and set at "IEF protocol". A potential gradient was generated up to 10,000 Volt, until 55,000 total Volt-hours was reached, and then the voltage was held at 1,000 volts. The program was terminated on the next day (Day 3), but before the power was switched off the recording of volt hours must be checked to ensure that the IEF was done properly.

3. *Day 3: Making of second dimension with SDS-PAGE.* A special "TGX Stainfree IPG+1 well" precast gel, from Bio-Rad Hercules, California USA, was used for this purpose. It was called "IPG+1" because it only had a single well at the top of the gel, skewed to one side, while the remaining part of the gel-top next to the single well was all flat, designed to precisely accommodate the 13 cm gel strip after IEF. The gel strip was joined to the main gel using boiling hot agarose to fill-up the gap, aided by the two transparent plastic sheets on each side of the precast gel, the purpose of which was to protect the gel and provide a built-in upper tank. The single well was used for 1-D SDS-PAGE of the original extract, providing a reference for the 2-D image.

   The standard method of SDS-PAGE was then applied to the Precast gel, but with some modifications, both before and during this last step of 2-D electrophoresis. Firstly, the gel strip had to be "equilibrated" (EQ) before joining with the precast gel as described above, using special EQ solutions 1 and 2, the first one containing DTT and the second one containing Iodoacetamide and Bromophenol Blue. The process was carried out inside an equilibration tube, and the equilibration was performed in two steps, using Solution 1 first and then Solution 2 next. Once equilibration was ready, the Criterion Gel Tank was prepared as described in Section 2.4.5. Separately, low melting point agarose (1% agarose in 1x running buffer) was prepared using a microwave oven until the agarose had melted and was bubbling (it would take approximately 1 minute). The gel strip was then taken out from the equilibration tube and carefully lowered onto the top of the precast gel, next to the single well, protected by the two clear plastic sheets. Any gaps between the gel strip and the precast gel, or next to the single well, were all sealed with boiled agarose, carefully avoiding (and removing) bubbles formed between the strip and the gel as they could interfere with subsequent smooth electrophoresis. The usual procedure of SDS-PAGE was then followed, and the final product was imaged

using a Bio-Rad Hercules, California USA Gel-Doc EZ Imager (activating time approximately 5 minutes).

2.4.8 Inhibition ELISA

Inhibition ELISA is basically an Indirect ELISA, but the primary antibody is *pre-incubated* with a *related antigen* (e.g. boiled peanut in relation to raw peanut) in a separate container (usually an Eppendorf tube) prior to the mixture being added to the Microlon plate, which has already been coated with the original antigen (e.g. raw peanut). This *pre-incubation* produces an "inhibition" effect on the primary antibody in the Eppendorf tube, or a "competition" effect on the immobilized antigen on the plate. Therefore the terms *Inhibition ELISA* and *Competition ELISA* are often used synonymously. After inhibition, the subsequent steps of detection are exactly the same as conventional Indirect ELISA. However, because of the pre-incubation process, the relative antigenicity of related antigens can be conveniently compared with each other.

Inhibition ELISA was performed using the following protocol:

1. *Protein concentrations* in raw and boiled peanut extracts were pre-determined using EZQ. For *coating*, raw peanut extract was diluted with coating buffer to produce a protein concentration of 10 ng/µl. For *inhibition*, raw and boiled peanut extracts were all normalized to produce a uniform protein concentration of 1,000 ng/µl.
2. *Coating*. A volume of 100 µl of the diluted raw peanut extract (at 10 ng/µl) was added to each well of the Microlon plate and incubated overnight at 4°C. The plate was washed 5 times in the following morning with PBS-T.
3. *Blocking*. A volume of 200 µl of 0.5% BSA in PBS-T was added to each well and incubated for 30 minutes at room temperature. After incubation, the plate was again washed 3 times with fresh PBS-T.
4. *Dilution of patient serum prior to inhibition*. Sera from peanut allergic patients were used as the source of primary antibody. The sera were pooled from 9 peanut allergic subjects who all had psIgE ≥ 100 IU/L, and then diluted with PBS-T. After initial testing, it was found that the original serum was far too strong for the performance of Inhibition ELISA without dilution. In a subsequent experiment, 4 different dilutions of pooled sera at 1:4, 1:20, 1:40 and 1:100 were tried in an Inhibition ELISA setting against raw and 2-hour boiled peanuts, and it was determined that 1:40 dilution of the pooled

sera provided the best results. 100 µl of the diluted serum would be used for each inhibition process after mixing with known quantities of raw or boiled peanuts.

5. *Preparation of known quantities of raw and boiled peanuts for use in inhibition*. The original normalized raw and boiled peanut extracts for inhibition, with protein concentration = 1,000 ng/µl (see step 1 above), were further diluted to produce 3 additional concentrations of 100 ng/µl, 10 ng/µl and 1 ng/µl respectively. From these 4 samples, a total of 10 different quantities of raw or boiled peanuts were derived and then added individually to each volume of 100 µl of diluted sera in Eppendorf tubes to produce a series of 10 inhibition data. The resultant concentrations of the 10 peanut proteins in each 100 µl volume of diluted sera were (*in ng/µl*): 0.01, 0.05, 0.1, 0.2, 0.5, 1, 1.5, 5, 10 and 15 respectively. The corresponding quantities of peanut proteins were (*in ng*): 1, 5, 10, 20, 50, 100, 200, 500, 1,000 and 1,500 ng respectively. Because Inhibition ELISA was done in triplicates, the actual amount of sera and proteins in each Eppendorf tube had to be multiplied by a factor of > 3 (resulting in total volume inside each Eppendorf tube being about ≥320 µl).
6. *Inhibition*. Inhibition was then carried out with incubation time set at 1 hour and temperature at 37ºC.
7. *Adding post-inhibition mixture to plate*. 100 µl of each post-inhibition mixture was added in triplicates to the antigen coated wells and incubated for 1 hour at 37ºC. The original uninhibited sera (at 1:40 dilution) and 0.5% BSA in PBS-T respectively were used as positive and negative controls, also done in triplicates.
8. *Adding secondary antibody to plate*. After washing 3 times with PBS-T, 100 µl of a 1:10,000 dilution (in PBS-T containing 0.5% BSA) of biotin-conjugated goat anti-Human IgE antibody was added to each well and incubated for 1 hour at 37ºC. The plate was then washed 3 times with PBS-T.
9. *Adding enzyme to plate.* After above washing, 100 µl of a 1:10,000 dilution (in PBS-T) of HRP-conjugated streptavidin was added to each well and incubated for 20 minutes at room temperature, allowing the enzyme to be firmly conjugated to the biotin molecule at the secondary antibody, which in turn was firmly bound to the primary antibody.
10. *Adding substrate and development of plate.* The plate was washed 3 more times with PBST. Afterwards, 100 µl of TMB Peroxidase EIA substrate (Bio-Rad Hercules, California USA) was added to each well, incubated for 30 minutes at room temperature, and then stopped with 100 µl/well of 1M sulphuric acid. The absorbance of each well

was read at 450 nm with a DTX880 Multi-Mode microplate reader (Beckman Coulter, Pasadena, CA, USA).

11. *The percentage of inhibition* was calculated using the following equation:

$$\text{Inhibition of IgE binding (\%)} = \frac{(\text{absorbance of positive control - absorbance of inhibited sample})}{(\text{absorbance of positive control})}$$

The reading at 50% Inhibition provided the simplest means to compare the relative antigenicity between raw and boiled peanuts at different boiling times.

2.4.9 Mass spectrometry

2.5.9.1 Acknowledgement

This part of investigation was performed mainly by my supervisor Dr Tim Chataway and Dr Alex Collela. My role was limited to the preparation of raw and boiled peanut extracts before HPLC.

2.4.9.2 Performance of mass spectrometry

The mass spectrometer at Flinders Proteomics Facility is an *AB Sciex TripleTOF* 5600+ mass spectrometer. It comprises three separate components: a High Performance Liquid Chromatography (*HPLC*) unit with two C18 columns upfront (hence this kind of setup is also called *LCMS*), an electrospray ionization (*ESI*) unit in the middle (which is called a "*nanospray*" if the ionised particle output is nano-sized), and the main body at the end, which does all the analysis. The main purpose of HPLC is to separate and purify the peptides, while that of the ESI is to convert peptide solutions into gaseous state and ionise the droplets (particles) at the same time. The main body contains a vacuum space, and charged ions produced by the ESI are accelerated and deflected in a magnetized space created by a quadrupole, until they hit the detector. The time travelled by these charged ions is called Time of Flight (*TOF*). The speed of movement of these ionised particles depends on two factors: their individual *masses* and the number of electron *charges* on the surface of each particle. The relation is expressed as a ratio, known as the *m/z or mass-to-charge* value. When the accelerated particles reach the detector, TOF data is calculated by a computer, producing three data sets: *time, m/z, and ion intensity (counts)*, upon which the sequence of amino acids can be deduced. The data is then checked with a known protein database, such as *Uniprot*, using a search engine such as *ProteinPilot*.

Mass spectrometry of individual raw and boiled peanut extracts was performed separately according to the following protocol:

1. *Preparation of raw and boiled peanut extracts.* Proteins from raw and boiled peanuts were solubilized in 5xPBS as described in Section 2.4.3. Solutions were ultra-centrifuged at 125,000*g* for 30 minutes and then filtered using a 3 kDa cutoff Vivaspin 2 centrifugal concentrator (GE Healthcare) to remove larger particles (>3kDa) from the analyte, leaving the smaller peptides in the filtrate to be analysed.
2. *HPLC and ESI.* 3µl of each sample was analyzed using the AB Sciex TripleTOF 5600+ mass spectrometer equipped with a nanospray source. The peptide solution was initially applied to a Polar 3 µm precolumn (0.3 x 10 mm, SGE Analytical Science) and eluted onto a combined spray tip / C18 column (5 µm, 75 mm x 150 mm with a bead pore size of 100 Å) (Nikkyo Technos), using an Eksigent Ekspert 415 nanoLC. Initial elution was performed using a 35 minute gradient from 5% to 25% acetonitrile containing 0.1% formic acid at a flow rate of 300 nl/minute over 35 minutes. A second elution followed with a larger C18 column, with gradient to 40% acetonitrile over 7 minutes and a further step to 95% acetonitrile for 11 minutes.
3. *Mass spectrometry analysis.* The mass spectrometer was operated in positive-ion mode with one MS scan of mass/charge (m/z) 350–1,500, followed by collision-induced dissociation fragmentation of +2 to +5 charge state ions that were greater than 10 counts per second for a maximum of 100 candidate ions. Exclusion of former target ions was performed for 30 seconds after 1 occurrence with a mass tolerance of 50 mDa. Rolling collision energy and dynamic accumulation were also applied. Product ion scans were from 100 to 1,500 Da in high sensitivity mode, and accumulation times were >0.05 second for MS1, and >0.01 second for MS2.
4. *Matching MS data with known database.* MS data was searched using *ProteinPilot* version 4.5 beta against the *Uniprot peanut protein database* (2014_09). *Proteinpilot search settings* were: Identification mode, Cys Alkylation = Iodoacetamide, Protease = none, Instrument = TripleTOF 5600, biological modifications mode and thorough ID were selected, detected protein threshold [Unused ProtScore (Conf)] was >0.05 and false discovery rate analysis set at 1%.

2.4.10 *Ex-vivo* T-cell stimulation test using flow cytometry

2.5.10.1 Acknowledgement

This part of investigation was performed by Dr Preethi Eldi, a post-doctoral cellular immunologist at the Experimental Therapeutic Laboratory located at Sansom Institute, University of South Australia. My role was limited to the identification of suitable patients for donating PBMC, and the supply of raw and boiled peanut extracts to Dr Eldi.

2.4.10.2 Collection of blood, preparation of PBMC, and Ethics approval

Blood samples from 3 peanut-allergic patients and 3 non-allergic volunteer controls were collected at SA Pathology on Frome Road, Adelaide. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA anti-coagulated whole blood using Lymphoprep density gradient centrifugation (Stemcell Technologies, Vancouver, Canada). PBMCs were labelled with Cell Proliferation dye eFluor® 450 (eBioscience) as per manufacturers recommendation, using 5 µM dye concentration.

Ethics approval to perform these investigations was obtained from the University of South Australia Human Ethics Committee, approval number 32966.

2.4.10.3 Culture and stimulation with mitogen controls and peanut allergens.

1. 1x 10^6 PBMCs/well were cultured for 7 days at 37ºC, 5% CO_2 in RPMI-1640 (Sigma Life-sciences, MO, USA) supplemented with 10% human AB serum, 2Mm L-glutamine, antibiotics and antigens at specific concentrations.
2. Staphylococcal enterotoxin B (SEB) and Tetanus toxoid (TT) were used at a final concentration of 1 µg/ml and 2 LfU/ml respectively as mitogen controls.
3. Raw, 2-hour and 12-hour boiled peanut extracts were used at a final concentration of 10 µg/ml.

2.4.10.4 Flow cytometry analysis

1. At the end of the culture period, cells were stained with anti-CD3 (clone SK7), anti-CD4 (clone SK3), anti-CD25 (clone 2A3) and anti-CD134 (clone ACT35) in PBS containing 2% serum for 30 minutes at 4ºC.
2. Fixable viability dye eFluor® 660 (eBioscience) was used for live-dead cell discrimination.

3. Data from the stained samples were acquired on BD FACSARIA™ Fusion using the FACSDiva software and analysed using FlowJo software (v10, TreeStar Inc.).

2.5 Methods Using Skin Prick Test to Evaluate Peanut Allergenicity

2.5.1 Invitation to participate and getting Informed Consent

Parents of 20 consecutive paediatric patients (age 2-16, M14:F6) attending Allergy SA (my private allergy practice) with known peanut allergy were invited to participate. Parents and children were asked if they would be willing to receive additional skin prick tests on top of their standard panels of peanut and tree nut commercial extracts, which were planned originally. Informed Consent was obtained before skin prick tests were performed. The additional tests were labelled as: "Flinders raw peanut" (as distinguished from the commercial raw peanut extract), 1-hour boiled, 2-hour boiled and 4-hour boiled peanuts, as well as 1-hour boiled, 2-hour boiled and 4-hour boiled leachates.

2.5.2. Preparation and normalisation of skin prick test extracts

Production of local extracts was described earlier under Section 2.4.3. Commercial raw peanut extract (Hollister-Stier) and the additional peanut extracts were normalized after their protein concentrations were individually determined using EZQ assay. Leachates were concentrated using Vivaspin 2 Sample Concentrators (GE Healthcare Life Sciences) while the peanut extracts were diluted with sterile deionized water so that each sample contained a final concentration of 2 µg/µl of peanut protein, 50% v/v autoclaved glycerol, and 0.9% benzyl alcohol in 1 ml.

2.5.3. Performance of skin prick tests

Skin pricks were performed using Unilet GP superlite single-use general-purpose lancets (Owen Mumford, UK). Skin reactivity was recorded at 15 minutes as the mean of two perpendicular diameters of the wheal, with the first diameter chosen visually as the longest length of the wheal.

2.5.4. Ethics approval

Human Ethics approval for performing additional SPT on these subjects was obtained from Southern Adelaide Clinical Human Research Ethics Committee (SACHREC), approval number 435.13.

2.6 Methods of Peanut Oral Immunotherapy in the Pilot Study

Note: Chapter 4 provides a full account of the pilot study. A summary of the methods is described here.

2.6.1 Study design and aim

The study was an open, non-randomised and non-controlled proof of concept intervention trial. The aim was to show that a novel sequential style oral immunotherapy protocol using hypoallergenic boiled peanuts first before roasted peanut will mitigate AE during treatment and allow up-dosing to be conducted safely at home instead of at a hospital. Due to the home-based up-dosing nature of the protocol, a cautious approach was adopted, which excluded severely allergic children. *Inclusion criteria* were a recent history of peanut allergy (mild to moderate reactions), skin prick test (SPT) 7-14 mm of mean wheal diameter, and a positive OFC. *Exclusion criteria* were a history of severe anaphylaxis and/or SPT>14 mm.

2.6.2 Invitation and enrolment

A consecutive sample of children matching the inclusion criteria attending my private practice at Allergy SA were invited to participate in the study. Participants were required to undergo an oral peanut challenge (OFC) to confirm the diagnosis. Sample size was determined by the number of OFC that could be arranged at the Paediatric Day Unit of Flinders Medical Centre over a 1-year study period, to a maximum of 15 subjects. At the end, 7 male and 8 female children aged between 8 and 15 had accepted the invitation to participate. Informed consents were obtained from each family before the oral peanut challenge.

2.6.3 Oral peanut challenge

Open labelled oral peanut challenges (OFCs) were performed on all participants to confirm their peanut allergy status. All OFCs were carried out at the Day Unit of Flinders Medical Centre, South Australia, which involved administration of increasing doses of peanut butter, starting at 17.5 mg of peanut protein, given every 20 minutes until a definite and objective

reaction was observed. The OFCs were supervised independently by Dr Christine Ziegler and Dr Henning Johannsen, who were staff paediatric allergists at Flinders Medical Centre.

2.6.4 Treatment Protocol

The 12-month protocol comprised a sequential 7-month initial phase of 2-hour boiled raw peanuts and a 5-month second phase of roasted peanuts. This pilot study used peanuts (1 g per peanut) boiled for 2-hours, as it was considered to be an appropriate balance between sufficient hypoallergenicity while still retaining allergens to commence desensitization. Boiled peanuts were prepared by boiling for 2 hours and then dehydrating for 24 hours as previously described. Boiled and roasted peanut phases followed the same schedule for the first 18 weeks. Dosing began with 250 mg peanut daily (peanuts contain approximately 25% peanut protein), and frequency was increased weekly over the first three weeks from once a day to three times a day. From 4th to 6th week the dose was increased each week to 500 mg peanut three times a day. From 7th to 9th week it was increased to 1g peanut three times a day. From 10th to 18th week it was increased to 10 g peanuts per day in 3 divided doses, which became the maintenance dose.

Table 2.6.4 Conversion table from peanuts to peanut protein

Peanut in portion or number	¼ peanut	½ peanut	1 peanut	2 peanuts	3 peanuts	4 peanuts	10 peanuts
Average weight of Peanut	250 mg	500 mg	1 gram	2 grams	3 grams	4 grams	10 grams
Weight of Peanut protein	62.5 mg	125 mg	250 mg	500 mg	750 mg	1000 mg	2500 mg

2.6.5 Precautionary measures during OIT

Precautionary measures included (1) not to ingest peanuts with empty stomach, (2) avoid exercise 2-hours before and after peanut ingestion, (3) temporary pause of OIT during sickness (up to 1 week). On resuming OIT, the “restarting” dose was set at half the previously tolerated amount and gradually raised back to original dose over 1-2 weeks.

2.6.6 Monitoring progress

1. Skin prick test wheal sizes and serum antibody titres were recorded at entry (baseline) and at completion of each OIT phase.
2. Serum peanut-specific IgE and IgG_4 levels were measured by ImmunoCAP. Results were recorded at entry and at completion of each OIT phase.
3. Ara h 2-specific IgE levels were only measured at entry.

2.6.7 Statistical analysis

Skin prick test readings, peanut-specific IgE, and peanut-specific IgG4 at baseline, end of boiled peanut OIT and end of roasted peanut OIT were used for statistical analysis. Differences at each time-point were evaluated using Wilcoxon signed ranks test. Statistical comparisons were undertaken using Stata 14 (Stata Corp, College Station, Texas). All tests were 2-sided with an alpha level of 0.05.

2.6.8 Ethics approval

Ethics approval was obtained from Southern Adelaide Clinical Human Research Ethics Committee (approval number 473.13).

2.6.9 Clinical Trial Registry

The trial was registered with **Australian New Zealand Clinical Registry** (Trial ID ACTRN12614000919617).

Chapter 3

The Effect of Extended Boiling on Peanut Allergenicity

Chapter 3 describes a series of experiments which combine to show that extended boiling progressively reduces peanut allergenicity while still retaining T-cell reactivity.

3.1 Protein Concentrations in Raw/Boiled Peanut Extracts and Leachates

The EZQ assay (see Section 2.4.4.1) was used for the determination of protein concentrations in most of my earlier experiments. At first glance the assay seemed to have performed according to expectation, at least in the first 4 experiments. However, a subsequent Experiment 3.1.5 showed that there was a significant flaw with EZQ, which will be fully discussed later.

1. **Experiment 3.1.1**: Comparing protein concentrations in commercial and locally produced raw peanut extracts.
   Table 3.1.1 showed that the protein concentrations of both raw extracts were similar.

Table 3.1.1 Commercial peanut extracts (due to the presence of 50% glycerol) had half as much protein content as locally produced peanut extract, which had no glycerol

Extract	Protein concentration mg/ml
Commercial raw peanut extract (containing 50% glycerol)	12.26
Locally produced raw peanut extract (containing no glycerol)	26.45

2. **Experiment 3.1.2**: Comparing protein concentrations of raw and boiled peanut extracts.
   Table 3.1.2 showed that, as boiling time increased, there was a progressive reduction of protein concentration in peanut extracts, presumably from (1) increasing fragmentation of the original proteins, and (2) leaching of proteins and fragments into the cooking water. Short boiling time of less than 1 hour may denature some proteins but might not be able to break them down into smaller molecules due to lack of sustained heat energy. Extended boiling, in contrast, could do both because of its increased energy source, causing fragmentation through hydrolysis of peptide bonds as well as denaturation.

Table 3.1.2 Protein concentrations of peanuts fell progressively as they were boiled for up to 2 hours

Extract	Protein concentration mg/ml (EZQ assay)
Raw peanut	29.8
Half-hour boiled peanut	7.24
1-hour boiled peanut	4.84
2-hour boiled peanut	3.98

3. **Experiment 3.1.3**: Comparing protein concentrations of 2-hour boiled peanut extract with ½-hour, 1-hour and 2-hour boiled leachates.

Table 3.1.3 Protein Concentrations of 2-hour Boiled Peanut vs Three Leachates, showing protein concentrations in leachates increased with extended boiling time

Extract	Protein concentration mg/ml (EZQ assay)
2-hour boiled peanut	3.80
Half-hour boiled leachate	0.40
1-hour boiled leachate	0.46
2-hour boiled leachates	1.53

The results in Table 3.1.3 showed that there was incremental leaching of proteins and their fragments into cooking water, as the boiling time was increased.

4. **Experiment 3.1.4**: More comparison of protein concentrations in boiled peanut extracts with corresponding leachates.

Results of Table 3.1.4 suggested that the protein contents in boiled peanuts became "stabilized" after 4 hour boiling, while that in cooking water continued to rise with additional boiling time. It could be because more components of the "insoluble structural proteins" in peanut became fragmented or solubilized, presumably from heat-induced hydrolysis, and then moved into the cooking water.

Table 3.1.4 Protein Concentration in 12-hour leachate was higher than either boiled peanuts or 4-hour boiled leachate

Extract	Protein concentration mg/ml
4-hour boiled peanut	6.61
12-hour boiled peanut	6.01
4-hour leachate	2.63
12-hour leachate	7.03

5. **Experiment 3.1.5**: Reassessment of protein concentrations in boiled peanuts using Bio-Rad DC Protein Assay.

   The decision to re-assess protein concentrations in boiled peanut extracts and leachates was prompted by the late realization (which only occurred to me in the second half of my PhD study when I started to think more deeply about various proteomic methods) that the EZQ method could be fundamentally inaccurate in estimating small molecular weight proteins and peptides, which were produced as a result of extended boiling. The main concern was related to the fact that EZQ used a porous absorbent paper material for "spotting" the extracts (at 1 μl per spot), and as a result small molecules of protein and peptides initially adherent to the paper were likely not to bind as tightly as full-length proteins, causing loss during the staining and washing processes, and subsequent under-estimation of smaller molecular weight proteins present in the paper. In contrast, the *Bio-Rad DC Protein Assay* (see Section 2.4.4.2) would perform assay in solution and therefore quantify all reactive amino acids present in it, making it a superior method for the detection of smaller proteins and fragments than EZQ. Ironically, previous

investigations at the proteomic lab did not involve comparison between larger intact and smaller fragmented proteins, so the EZQ method had worked well for those purposes but not necessarily for my boiled peanut experiments.

However, the DC assay is not without problems. It is more susceptible to the presence of interfering compounds than EZQ, and the results of protein assays need to be interpreted with caution. As well, it still uses egg ovalbumin as the standard, just like EZQ. Before boiling, peanut proteins were fully sized and their molecular weight profile might well-match that of the ovalbumin. After boiling, many resultant proteins became smaller and their profiles might not match that of the ovalbumin anymore.

Five peanut extracts (1 raw and 4 boiled) and one 2-hour leachate were reassessed using the new method, in triplicates to calculate the mean and standard deviation.

Table 3.1.5: Bio-Rad DC Protein Assay showed that protein concentrations in raw and boiled peanuts were actually similar (EP = Extractable Protein; TOP = Total Extractable Protein in the original 30 gram peanut)

	EP (mg/ml)	**TOP (mg)**
Raw peanut	7.3+/- 0.9	545.2 ± 54.5
1-hour boiled	7.3+/- 0.8	547.7 ± 59.1
2-hour boiled	6.8+/- 1.8	512.8 ± 140.3
4-hour boiled	6.5+/- 0.9	487.5 ± 65.9
12-hour boiled	8.5+/- 0.9	637.8 ± 66.4
2-hour leachate	0.9+/-0.3	294

As can be seen, results from Table 3.1.5 gave a completely different picture from that obtained using the EZQ method. The earlier EZQ data suggested that extended boiling progressively reduced soluble proteins in boiled peanuts, but the new DC Assay data

indicated that the protein contents in boiled peanuts actually remained constant despite extended boiling. One possible explanation could be that although the original proteins were broken down into smaller components, the majority of the fragments were still trapped inside the structure of the boiled peanut, as these peanuts had maintained their shape during boiling. It would be an interesting exercise to find out if boiling ground-up peanut flour instead of intact peanuts would produce similar data as in Table 3.1.5, especially in comparing the protein concentration of centrifuged peanut pellet with intact boiled peanut. However, at the end this was not done because only intact boiled peanuts were given to participants in the pilot study, not pellets from boiled peanut flour.

An interesting observation from Table 3.1.5 was that, after 2 hours of boiling, the leachate contained a significant amount of "new" proteins without corresponding reduction of protein contents in the 2-hour boiled peanuts. They could have come from initially "non-extractable" structural proteins in the peanuts, which were later converted to more soluble forms after extended boiling and then moved into the cooking water.

3.2 Determination of Water-loss in Raw Peanuts after Dehydration and Oil-loss in Raw and Boiled Peanuts after Defatting

Raw peanut contains approximately 50% oil, 25% protein, and 4% intrinsic water. In making high-quality boiled peanut extracts, the peanuts must be initially boiled for ½ to 12 hours, dehydrated for 24 hours, and then defatted over 2 days. It is therefore important to determine the effect of dehydration (using a dehydrator) on the original water-content of raw peanut. Similarly, it is also important to find out whether extended boiling can actually cause significant oil-loss from peanuts to the cooking water.

1. **Experiment 3.2.1:** Loss of water in raw peanuts after 24 hour dehydration.

Table 3.2.1: Raw peanut lost 2% intrinsic water after dehydration.

Weight of raw peanut before dehydration	Weight of raw peanut after dehydration	Difference (= water loss)	Percentage of difference
30.02 gm	29.94 gm	0.60 gm	2 %

Table 3.2.1 showed that raw peanut lost 2% of its intrinsic water after 24-hour dehydration with a dehydrator.

2. **Experiment 3.2.2:** Loss of oil after defatting of raw and boiled peanuts.

Table 3.2.2: Raw and boiled peanuts all lost about 50% of weight after defatting

	Original de-hydrated weight (grams)	Weight after defatting (grams)	Oil lost to defatting (grams)	Percentage of oil lost to original weight
Raw peanut	8.20	4.26	3.94	48.0
½ hour boiled	8.04	4.07	3.97	49.4
1 hour boiled	8.08	4,16	3.92	48.5
2 hour boiled	8.07	4.13	3.94	48.8
4 hour boiled	8.07	4.06	4.01	49.7
12 hour boiled	8.08	3.97	4.11	50.9

Table 3.2.2 indicated that, even after 12 hours of boiling, no significant amount of oil had left peanuts and leached into the cooking water. There could be two possible explanations: (1) the boiling point of oil is higher than that of water, so the oil inside peanut was likely to stay within its structure, despite being surrounded by hot water; (2) the oil inside peanut might not be free oil, but firmly bound to its structure, to the effect that as long as the overall shape of peanut during boiling was kept intact, the oil was unlikely to leave the peanut and move into the surrounding water. This restriction might help prevent newly formed peptides (after extended boiling) from moving freely into cooking water, forming a counter-balancing force to the hydrophobicity of peptides. Peptides are important players in immunotherapy, so having as many peptides as possible retained in boiled peanuts can be a decidedly favourable factor in their use in oral immunotherapy.

3. **Experiment 3.2.3:** Visual inspection of cooking water after ultracentrifugation.
   This is a very simple experiment. After extended boiling for up to 12 hours, leachates were ultracentrifuged at 55,000 rpm (~130,000g) for 1 hour at 4°C and then visually inspected. The layer of oil on top of the cooking water was barely visible, and would have formed less than 1% of the water content. This basically supported the results of Experiment 3.2.2.

3.3 1-Dimensional SDS-PAGE of Raw/Boiled Peanuts and Leachates

The 1-D SDS-PAGE of raw and boiled peanuts (including leachates) delivered a powerful visual image of progressive proteomic changes to peanut proteins after extended boiling. The proteins were separated by electrophoretic forces to line up as horizontal bands in a vertical column in the gel according to their molecular weights.

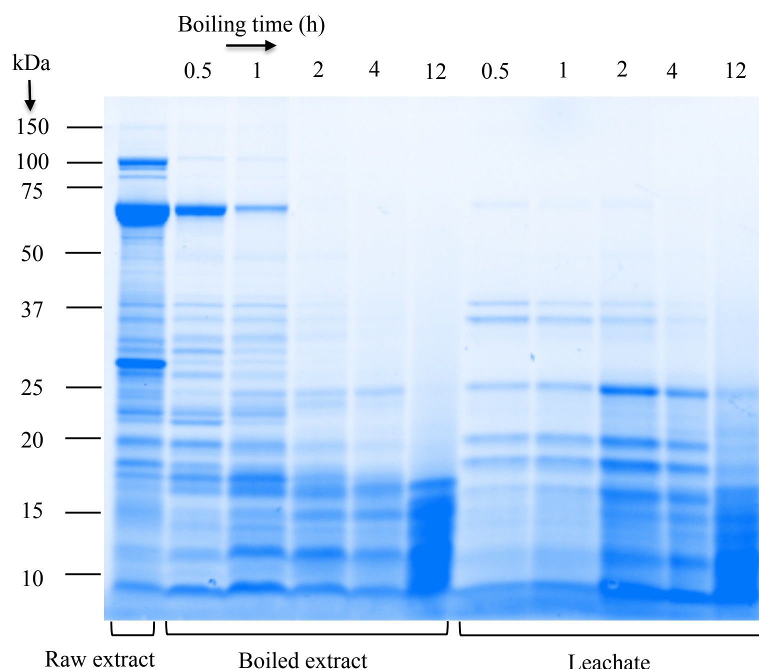


Figure 3.3.1: Boiling induced fragmentation of peanut proteins. SDS-PAGE of 20 µg of peanut extract or concentrated leachate revealed an increase in lower molecular weight proteins in boiled extracts and leachates.

Figure 3.3.1 was created using an 18-well AnyKd precast gel containing 10 µg of protein per well before electrophoresis (see Section 2.4.5, under Methods). It can be seen that, as cooking

time was increased from ½ hour to 12 hours, the protein pattern progressively changed in accordance, in both boiled peanuts and leachates. However, the more visually-dramatic changes actually occurred at 2 hours and 12 hours. It is interesting to remember that in Dr Beyer's original paper in 2001 she had only boiled peanuts for 20 minutes, even less than the minimum half-hour cooking time shown here.

The results indicated that extended boiling had caused increasing fragmentation of the peanut proteins into lower molecular weight polypeptides, but additionally there was also some transferring (leaching) of proteins into the cooking water (leachate). By 2 hours, most peanut proteins were fragmented to less than 25 kDa, and by 12 hours extensive hydrolysis of peanut proteins was evident by the smeared appearance of the gel-track and absence of protein bands greater than 18 kDa. The proteins transferred to the leachate showed a similar profile to the boiled nuts. Intact proteins were still visible at 4 hour, but were mostly fragmented by 12 hour, including those in the leachates.

Among the peanut allergens, Ara h 2 (seen as a pair of double bands at 17 and 19 kDa) and Ara h 6 close-by seemed to be significantly heat-resistant, and would only break down in boiled peanut and leachate at the 12-hour boiling-time. This was because Ara h 2 contained 4 disulphide bonds in its molecular structure and Ara h 6 contained 5, which significantly increased their resistance to hydrolysis by boiling.

3.4 Human IgE Western Blot on Raw/Boiled Peanuts and Leachates

3.4.1 The Western blot image. Western blot, also known as Immunoblot, uses an antigen-specific primary antibody (in this case the peanut-specific IgE antibody from highly peanut-allergic children) to probe and display the target proteins (in this case the raw and boiled peanuts, and also in leachates). The proteins are first separated using SDS-PAGE and then transferred to a membrane, after which the membrane is probed with the primary antibody.

Figure 3.4.1 demonstrated the progressive changes to IgE reactivity as the boiling time was increased. The blot was performed under denaturing conditions (heating extracts to 95° in the presence of denaturing detergent SDS) and used pooled sera from 9 peanut allergic subjects as primary antibody. Raw peanut showed strong IgE binding to Ara h 1 (63 kDa), Ara h 2 (17 and 19 kDa) and Ara h 6 (15 kDa), and weaker reactions to other peanut allergens.

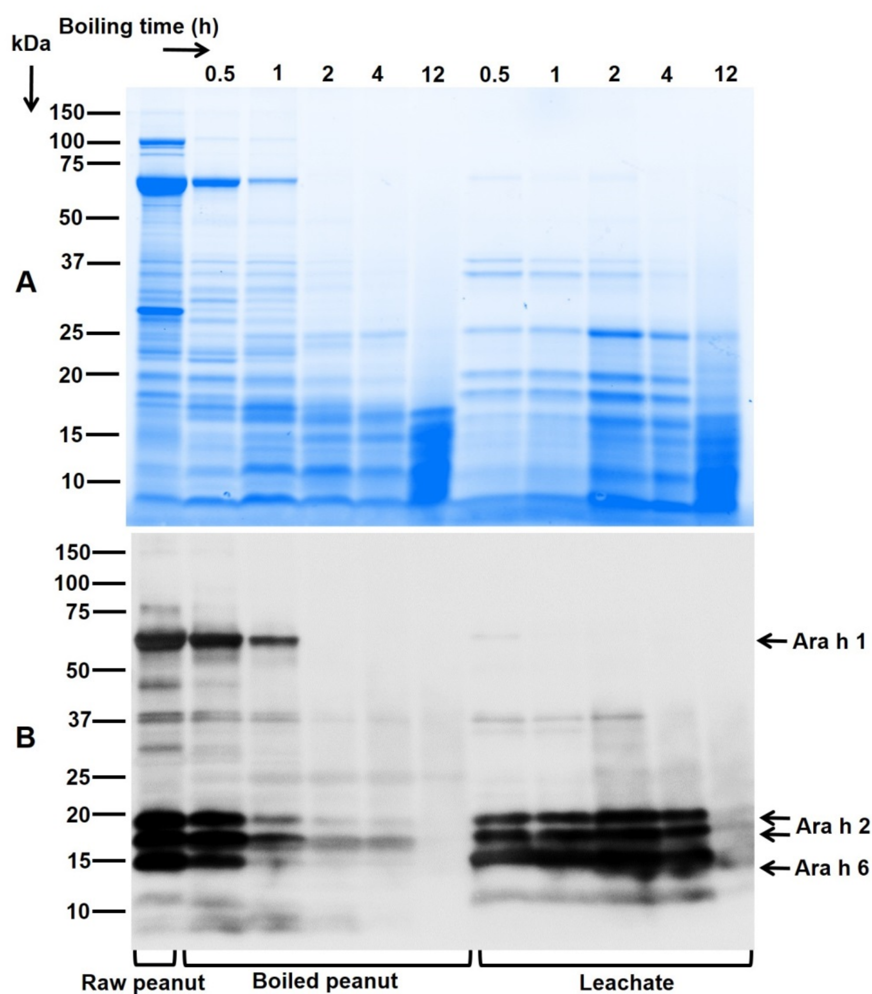
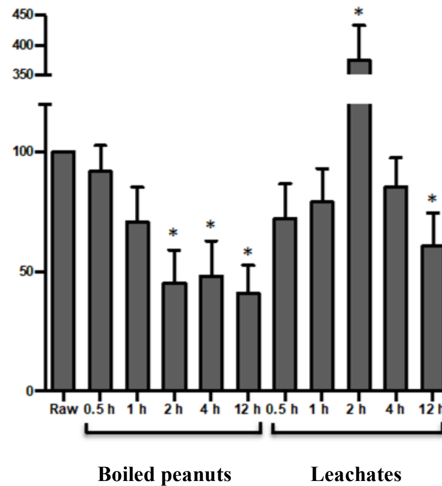


Figure 3.4.1: Boiling progressively reduces human IgE reactivity to peanut proteins as shown by Western blot (B) directly under original SDS-PAGE (A)

Identities of the allergens were confirmed separately by mass spectrometry, using gel cuts. At 2-hour boiling, IgE-binding to intact Ara h 1 became undetectable for both boiled peanut and leachate, indicating that it was comprehensively fragmented into smaller molecules or peptides. This observation was supported by the loss of the band corresponding to intact Ara h 1 on the corresponding SDS-PAGE gel. In contrast to Ara h 1, reactivity of Ara h 2 in boiled peanuts decreased only slowly over the 12-hour period, with an associated increase in leachate, indicating transference of intact Ara h 2 during cooking. However, by 12 hours of boiling, even the 17-19 kDa bands were significantly depleted in both boiled peanut and leachate.

3.4.2 Densitometry analysis. Separate Western blots of raw and boiled peanuts were produced using individual sera from 9 peanut-allergic children. The data were then quantitatively analyzed using densitometry (see Section 2.4.6).



*** p<0.05 compared to raw peanut IgE reactivity**

Y-axis = optical density

Figure 3.4.2: Densitometry of Western blot from 9 individual allergic subjects indicated a significant decline in IgE reaction after boiling for two hours or more, and transferral of IgE reactive protein to leachates. ($P < 0.05$ compared to raw extract. Error bar indicate standard error of the mean (SEM)

The results of Figure 3.4.2 showed significant decreases in total IgE reactivity to 45% of raw extract after 2-hour boiling, and to 40% after 12-hour boiling. IgE binding to concentrated 2-hour leachate was significantly higher than all boiled nuts at any point, even the raw peanut. However, because EZQ was used for estimation of protein in the original SDS-PAGE, the real protein concentrations in the leachates could be inaccurate (as explained earlier in Experiment 3.1.5), resulting in over-representation of protein in leachates in the Western blot. These experiments were not repeated using DC Protein Assay because it was decided to be unnecessary as leachates were not used in the peanut oral immunotherapy pilot study.

3.5 Animal IgG Western Blot to Ara h 1 and Ara h 2 in Raw and Boiled Peanuts and 2-hour Leachate

Western blot was also performed using animal polyclonal IgG antibodies to probe Ara h 1 and Ara h 2 respectively, in raw and boiled peanuts, and 2-hour boiled leachate. These animal

antibodies were raised in-house using synthetic 20-mer peptide-epitopes as previously described in Chapter 2, Section 2.4.2.

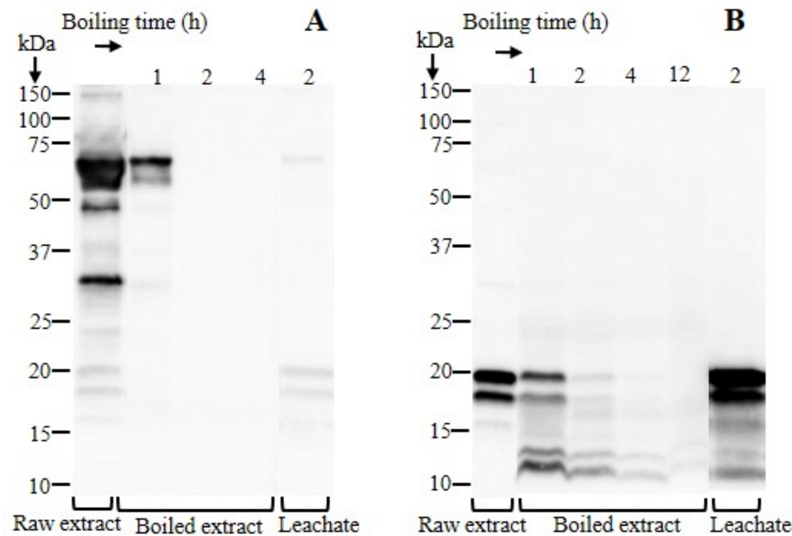


Figure 3.5.1: Animal IgG Western Blot of Ara h 1 (A) and Ara h 2 (B) showed that Ara h 1 became undetectable after 2-hour boiling, while Ara h 2 was more heat-resistant until 12-hour boiling.

In Figure 3.5.1, the Ara h 1 antibody (Part A) detected a major band at 63 kDa, corresponding to intact Ara h 1. The Ara h 2 antibody (Part B) similarly detected a pair of double bands at 17 and 19 kDa. The results were consistent with human IgE Western blot (see Figure 3.4.1), and the identity of these allergens had been previously confirmed by Mass Spectrometry using SDS-PAGE gel cuts (as described under Section 3.4). Part A showed that intact Ara h 1 was no longer detectable after 2 hours of boiling, with only faint bands observable in 2-hour leachate, suggesting that the majority of Ara h 1 was either broken down before transferal, or afterwards. Part B showed that Ara h 2 was more resistant to boiling, with intact Ara h 2 still visible at 4-hour boiling in both boiled peanuts and leachate. However, by 12 hours only Ara h 2 remnants of less than 15 kDa were present in the boiled peanuts. Ara h 2 contained four disulphide bonds, which stabilized its protein conformation and made it more resistant to heat treatment.

3.6 2-Dimensional SDS-PAGE Comparing Raw and 2-hour Boiled Peanuts

My first 2-D gel was performed using peanut extracts that had not been properly cleaned up before isoelectric focusing, and the resulting product was an inferior image. I have deliberately

included this first image here (see Figure 3.6.1, below) in order to highlight the benefit of cleaning the extracts properly before performing the 2-D Gel (Figure 3.6.2). The Bio-Rad 2-D gel clean-up kit was used for the cleaning in the repeat experiment.

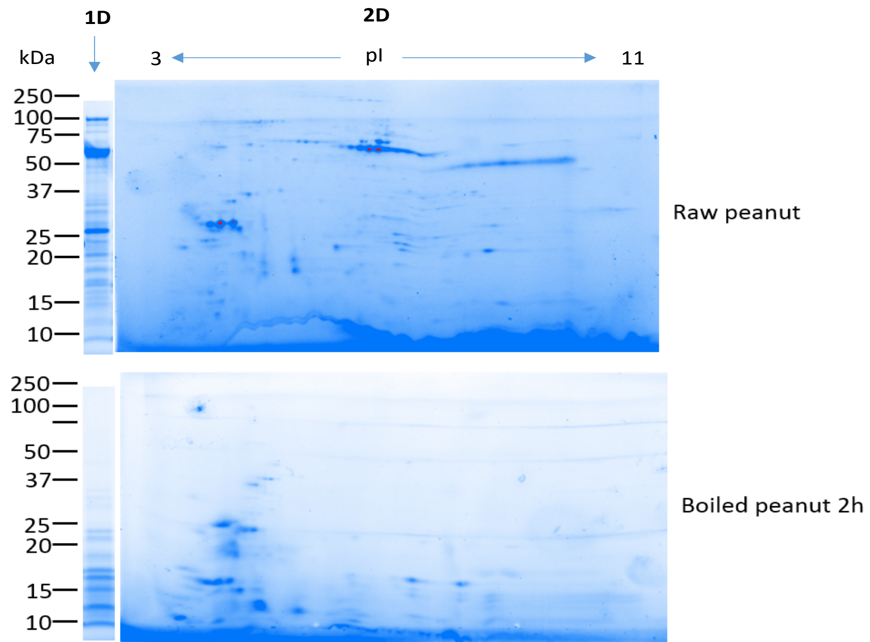


Figure 3.6.1: Poor quality of 2-D SDS-PAGE without proper cleaning of extracts before IEF

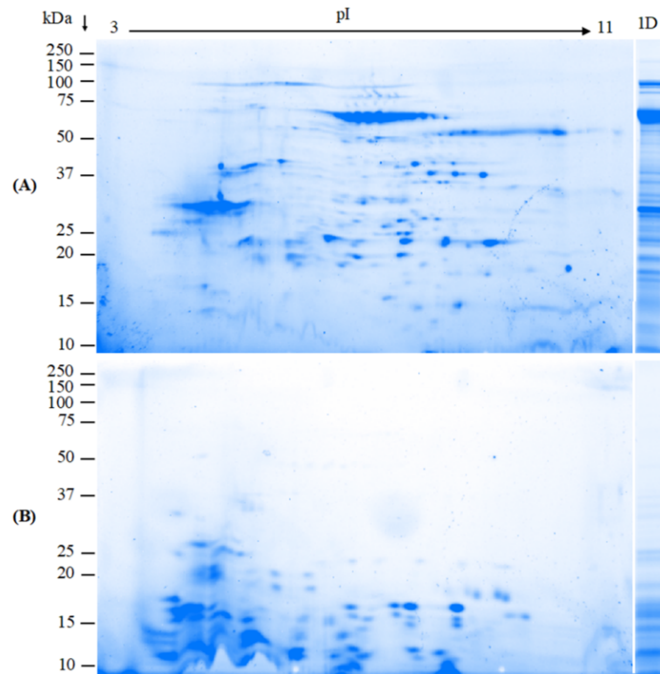


Figure 3.6.2: Significant improvement of quality with proper cleaning before IEF

The contrast between the first and second image (after proper cleaning) was quite obvious as shown in Figure 3.6.1 and 3.6.2. In Figure 3.6.2 A (raw peanut), approximately 170 peanut protein spots were detectable, with molecular weights ranging from 12 kDa to 100 kDa and pI from 3.5 to 11. In Figure 3.6.2 B (after 2-hour boiling), there were only 80 protein spots with molecular weights ranging from <10 kDa to 25 kDa and pI from 3.5 to 9.5. It can be concluded that there was significant reduction of the complexity of peanut proteome after 2-hour boiling.

3.7 Inhibition ELISA of Raw and Extensively Boiled Peanuts

While the visual impact of a Western blot (Figure 3.4.1) can be stunning in being able to demonstrate the progressive fall of peanut IgE reactivity as a result of increasing boiling time, it does have a significant intrinsic weakness when quantitative data is required. This is because in the making of a Western blot (see Section 2.4.6) it is necessary to use the reducing agent DTT (dithiothreitol) and the anionic detergent SDS (sodium dodecyl sulphate) to facilitate electrophoresis. Unfortunately, the downside of using these agents is that most secondary and tertiary protein structures are destroyed in the process. Peanut allergens contain both linear (continuous) and conformational (discontinuous) epitopes, but in a Western blot only linear epitopes are retained in the native form. Consequently, densitometry analysis of a Western blot can only provide partial data, while in real life conformational epitopes are actually the major players in IgE-mediated reactivity at mast-cell level.

ELISA does not have such problem, because there is no denaturing condition and both linear and conformational epitopes are equally preserved. An Inhibition ELISA is therefore the method of choice for investigating real changes in peanut allergenicity during extensive boiling.

The following experiments were performed systematically to document changes after extended boiling. The boiling time was progressively increased from half hour to 12 hours towards the end of the study.

1. **Experiment 3.7.1.** Finding the optimum dilution of pooled sera**.**
   Inhibition ELISA relies on the interaction between an antigen and a primary antibody when they are incubated in a closed environment, and as a result the concentrations of both antigens and antibodies are very important in producing good data. For the purpose of this Inhibition ELISA study, pooled sera of 10 highly peanut-allergic children with

serum peanut-specific IgE levels of 100 kU/L or greater were used as the source of primary antibody. Such a potent source could be too strong for direct interaction without further dilution. Therefore, this first experiment was designed to find out the best dilution for pooled sera before it could be used for future operations. Sera without added peanut extract (no inhibition) was used as the positive control and PBS-T was used as the blank/negative control (100% inhibition).

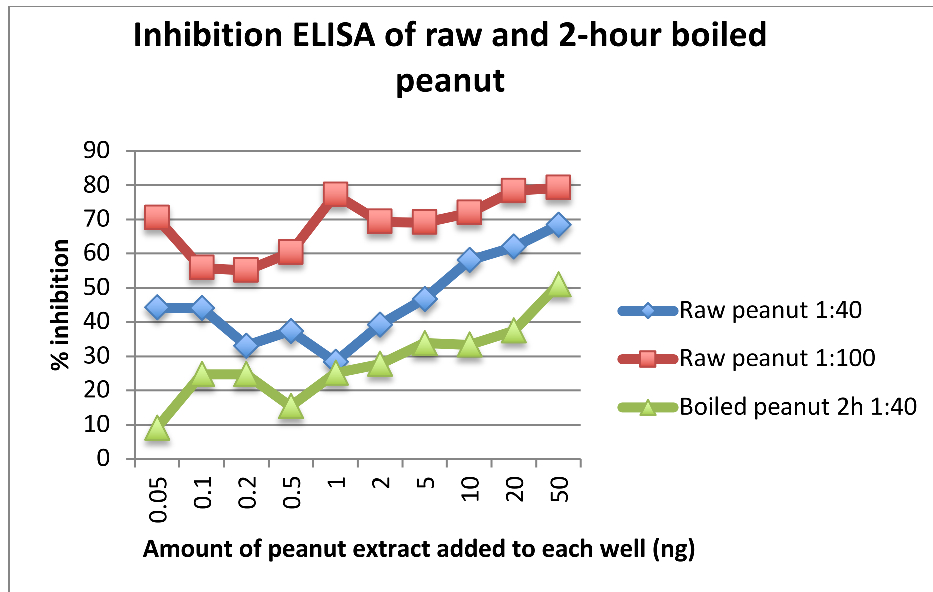


Figure 3.7.1. Inhibition ELISA showed that 1:40 dilution of sera was more suitable than 1:100 dilution

The result in Figure 3.7.1 indicated that 1:40 dilution was the most appropriate choice.

2 **Experiment 3.7.2.** Inhibition ELISA comparing raw peanut and peanuts that have been boiled for 2 and 4 hours.

This second experiment can be seen as a practice run to develop a good hand on Inhibition ELISA technique. Only raw peanut, 2-hour boiled and 4-hour boiled peanuts were used here for incubation with 1:40 diluted sera to create separate inhibitions. Afterwards, the post-inhibition mixtures were added to the immobilized raw peanut antigen in microplate wells for further detection using a combination of secondary antibody, enzyme, and substrate (for details of the method see Section 2.4.8).

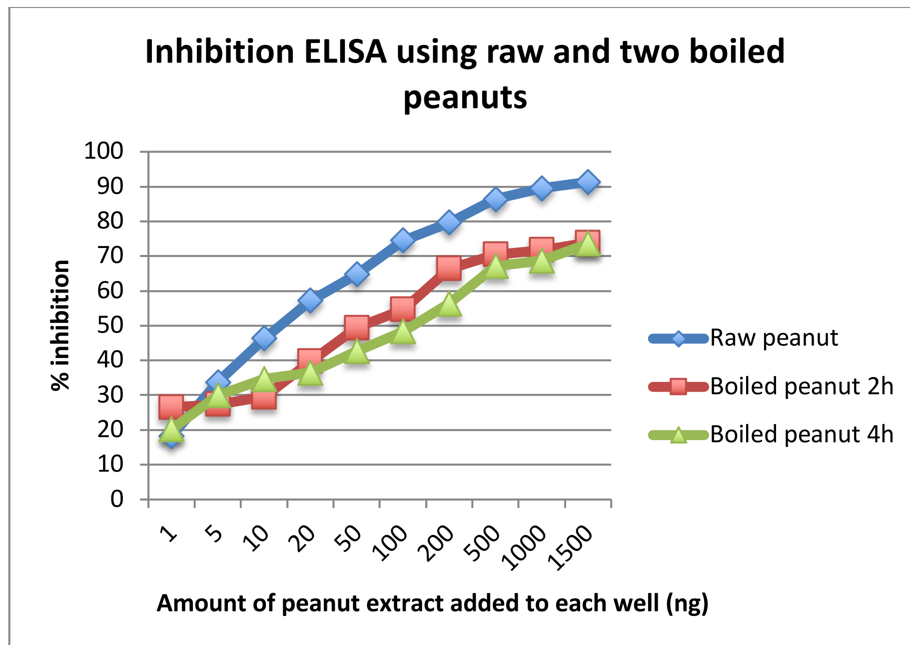


Figure 3.7.2. Inhibition ELISA showed that raw peanut had stronger inhibition power than either 2-hour or 4-hour boiled peanut

The result of Figure 3.7.2 showed smooth and steady rise of inhibition as the concentration of peanut proteins was increased. It also showed that the inhibition power of raw peanut was clearly stronger than either 2-hour boiled or 4-hour boiled, which was as expected. However, what was slightly unexpected was that there was not much difference between the inhibition powers of 2-hour boiled and 4-hour boiled peanuts, suggesting that the doubling of boiling time from 2 hours to 4 hours was not very worthwhile.

3 **Experiment 3.7.3.** Three repeats of the above experiment using new and freshly prepared raw and boiled peanut extracts.

It was decided that the above experiments should be repeated three times using separately prepared extracts of raw peanut, 2-hour boiled and 4-hour boiled, so that calculations of mean and standard error could be performed. The x-axis was also slightly modified to show the peanut protein increments as log-multiples of the first smallest quantity, which was set at log 0. The results were shown in Figure 3.7.3.

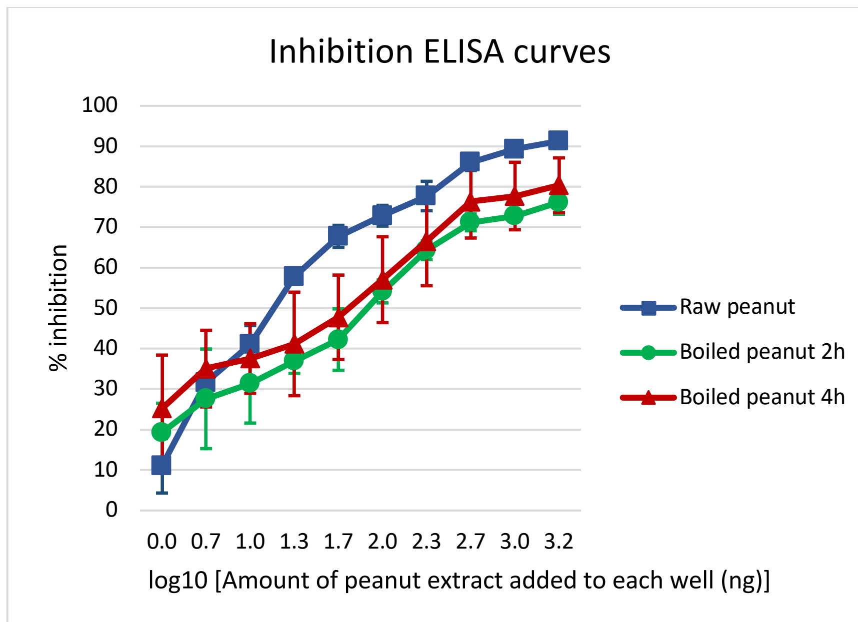


Figure 3.7.3: Results of Experiment 3.7.2 were repeated independently 3 times, showing error bars (standard error of the mean)

Figure 3.7.3 showed that the inhibition powers of 2-hour boiled and 4-hour boiled peanuts were indeed very close and, interestingly, that of the 4-hour boiled peanut was actually higher than that of the 2-hour boiled. The explanation was not immediately clear when these experiments were performed, but several months later, after head-to-head comparison of protein assay data using different methods of EZQ and DC Assay (see Experiment 3.1.5), one possible explanation for this discrepancy could be that the protein concentrations in 2-hour boiled and 4-hour boiled peanuts were both slightly inaccurate due to the use of EZQ as the assay method, which was affected by the fact that many of the original proteins in boiled peanuts had broken down into smaller fragments and escaped detection. More discussion will be provided in the next experiment.

4 **Experiment 3.7.4.** Inhibition ELISA comparing raw peanut with peanuts that have been boiled throughout the time interval of half-hour to 12 hours.

The next experiment was to perform Inhibition ELISA on 6 different kinds of peanut extracts all at once, and compare their relative inhibition powers: raw peanut, half-hour boiled, 1-hour boiled, 2-hour boiled, 4-hour boiled and 12-hour boiled. The results were shown in Figure 3.7.4.

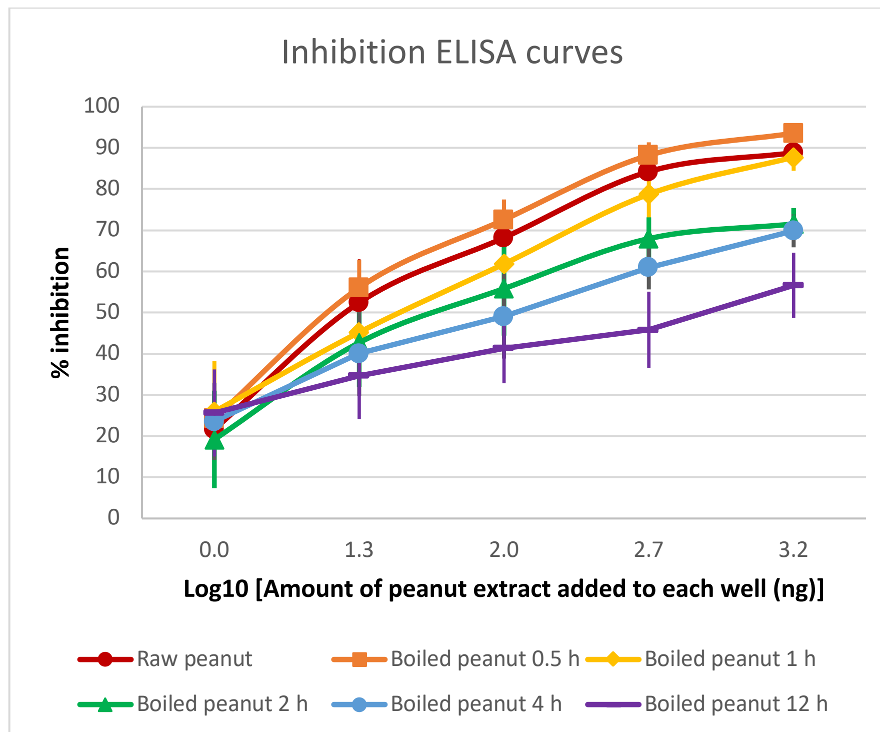


Figure 3.7.4. Inhibition ELISA Comparing 6 Different Peanut Extracts showed progressive loss of inhibition power with increasing boiling time.

Again there was smooth and progressive rise of inhibition as the protein concentrations of raw and boiled peanuts were increased, with each peanut inhibition curve neatly separated from each other. The x-axis was also slightly modified to show the peanut protein increments as log-multiples of the first smallest quantity, which was set at log-0. Importantly, Figure 3.7.4 also indicated that boiled peanuts had restricted ability (2-h ~70%, 12-h ~50%) to block the binding of patient IgE to raw peanut, even when the protein content was increased by 3 logs of 10, suggesting that boiled peanuts possess an incomplete repertoire of epitopes.

A surprise finding in this experiment was that the inhibition power (measured as IgE-binding) of half-hour boiled peanut was actually higher than that of raw peanut, as can be seen in Figure 3.7.4, top line. There could be three possible explanations for this.

The first possibility was that an error was made in under-estimating the protein content of 1⁄2-hour boiled peanut extract at the start, and as a result all subsequent dilutions for

Inhibition ELISA activities actually contained a higher quantity of 1⁄2-hour boiled peanut protein in all preparations, causing an erroneous indication of increased inhibition as shown in the figure. Also, I have used stain-free methodology to perform Western blot, which normalises each protein load on the membrane, meaning that if there was a small error in protein measurement for the 30-minute boiled peanut extract then this would have been corrected by the protein normalisation applied to each membrane. This did not happen using Inhibition ELISA, so any error in miscalculation would persist throughout the serial dilutions.

The second possibility was that the raw peanut extract was coated onto the plate in a high pH carbonate buffer to give the protein a negative charge, allowing it to bind to the positively charged plate. It is possible that this high pH could dissociate protein-protein interactions and cause partial unfolding of the bound protein. This could explain why even raw peanut extract could not completely inhibit the binding of antibody to the plate, because the partially denatured and plate-bound raw peanut had now acquired newly-exposed epitopes that were not present in the competing raw peanut extract during inhibition in a separate environment from the plate. This is the least likely explanation, however.

The third possibility is that the half-hour boiled extract might have acquired greater inhibitory properties than that of raw peanut extract because the short heat-treatment time had exposed previously hidden epitopes in peanut, which then became attached to the primary antibodies that would otherwise bind to the exposed epitopes on the plate. In other words, when peanuts were boiled for only ½ hour, the tertiary structure of peanut protein might become slightly unfolded, causing a temporary net-increase of exposed conformational epitopes, and as a result more antibodies were bound to the ½-hour boiled peanut protein during the inhibition phase, compared to raw peanut. Such partial denaturation and unfolding of peanut proteins could also apply to roasting giving them higher IgE-binding than raw peanut. Indeed, this has been previously reported [217-220]. Western blot of ½-hour boiled peanut extract (Fig 2.4.1 and Fig 3.4.2) demonstrated a decrease in IgE-binding in contrast to the Inhibition ELISA data. Western blot denatures proteins and removes the majority of conformational epitopes so that antibodies recognise primarily linear epitopes on the membrane. However,

Inhibition ELISA in this instance is the more accurate measurement as it is monitoring the change in IgE binding to native protein conformation.

In summary, ½ hour boiling resulting in the exposure of novel epitopes is likely to be the cause of a small increase in IgE binding, similar to that seen for roasting of peanuts. These are basically speculations and a logical step would be to repeat the experiment but using the DC method instead of EZQ to assay protein contents. Unfortunately the Inhibition ELISA had consumed so much human peanut-specific IgE stock after a large number of experiments (including the one below which did not include half-hour boiled peanut) that there was not enough serum left to repeat this one.

5 **Experiment 3.7.5.** Direct comparison of 50% inhibition of IgE reactivity between raw and boiled peanuts and 2-hour leachate.

The most effective and concise way to demonstrate the difference of allergenicity between raw peanuts and boiled peanuts is to compare their 50% inhibition point of IgE reactivity. In this experiment, Inhibition ELISA was repeated three times on raw peanut, 1-hour boiled, 2-hour boiled, 4-hour boiled and 12-hour boiled, and also 2-hour leachate. Data were entered into a Microsoft Excel file and logistic regression analysis was used to calculate the 50% inhibition mark of IgE binding. The results were shown as a histogram in Figure 3.6.5. Each peanut extract was tested three times independently in order to calculate the mean, standard error and statistical significance.

Compared to raw peanut, an 8-fold increase of 2-hour boiled, 11-fold increase of 4-hour boiled, and 19-fold increase of 12-hour boiled peanut were required to achieve the same 50% inhibition of IgE binding. Two-hour leachate also required approximately 8-fold more extract to achieve 50% inhibition when compared to raw peanut extract.

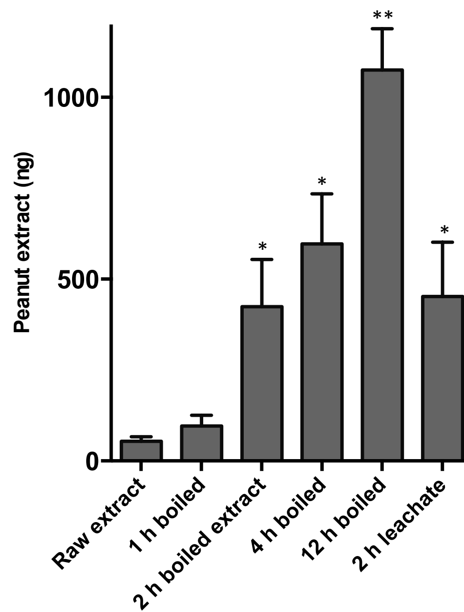


Figure 3.7.5. Boiling reduces IgE binding capacity of peanut proteins under non-denaturing conditions. Inhibition ELISA demonstrated that significantly more boiled peanut extracts than raw extract was required to cause 50% inhibition binding of peanut-allergic sera to raw extract. Logistic regression analysis was used to calculate 50% inhibition of IgE binding. N=10 patient sera. * $p < 0.05$ ** $p < 0.001$ compared to raw extract. Error bars indicate SEM.

The previous densitometry analysis of Western blot studies (performed in Section 3.4.2) had shown that the reductions in IgE reactivity of 2-hour boiled and 12-hour boiled peanuts were about 45% and 40% respectively. The magnitude of reduction achieved in Inhibition ELISA was considerably higher, down to 12.8% (about 8-fold reduction) for 2-hour boiled peanut, and 5.3% (about 19-fold reduction) for 12-hour boiled compared to raw peanut. As explained earlier, Western blot could only quantify linear epitopes because of its use of denaturing agents such as DTT and SDS, which destroyed conformation epitopes. Consequently, the very high reduction results from Inhibition ELISA suggested that there must be substantially more conformational epitopes in raw peanut, and that these were lost with extended boiling. Conformational epitopes are reportedly more important than linear epitopes for binding IgE and for activating basophils and mast cells, so the 19-fold reduction in IgE binding at 12-hour boiling pointed to dramatically reduced allergenicity.

3.8 Mass Spectrometry (MS) of Raw and Boiled Peanuts, and 2-hour leachate

3.8.1. Acknowledgement.

The Mass Spectrometry data of this section were generated by Dr Tim Chataway and Dr Alex Collela. I was only involved in the supply of extracts, analysis and searching of data and the creation of peptide alignment maps.

3.8.2. Overall MS results.

Previous experiments using 1-D and 2-D SDS-PAGE have indicated that proteins in raw peanuts were broken down into fragments, with also leaching into the cooking water. It was not sure if leaching would have significantly reduced the quantity of peptides in boiled peanuts, or even lost them completely. Mass spectrometry was therefore performed to determine if there were any meaningful quantities of allergen peptides retained by boiled peanuts. Small molecular proteins and peptides from 2 independently boiled peanut preparations were separated from larger proteins by filtration using a 3 kDa cut-off filter and then analyzed by nanospray qTOF MS, using an **AB Sciex TripleTOF** 5600+ mass spectrometer.

The results were shown in Figure 3.8.2. To everyone's relief, the all-important peptides, so critical for desensitization, were actually found in abundance in boiled peanuts and not lost to the cooking water. In fact, the content of peptides within boiled peanuts actually increased with longer boiling time, suggesting that new peptides were indeed created by extended boiling (as a result of protein fragmentation), but were then largely retained by the internal structure of boiled peanuts, rather than being lost to the cooking water. Before boiling, raw peanut extracts contained approximately 15 individual (unique) peptide sequences derived from known peanut allergens, but boiling increased the number of unique allergen peptides present in the boiled peanuts by more than 5-fold at 2 hours, and by 42-fold at 12 hours. The majority of the peptides were identified as originating from Ara h 3. Peptides were also sequenced from allergens Ara h 1, 2, 6, 7, 10, 11, 14, 15.

The retention of peanut peptides in boiled peanuts is crucial to their functional use in oral immunotherapy of peanut-allergic patients, without which the desensitisation process may not be able to proceed satisfactorily. Peptides are generally hydrophilic and will stay with water. However, peanut contains 50% oil so the environment in structurally intact peanut is

actually highly hydrophobic. If there are any free peptides present, the quantity would be small and they tend to stay with the internal structure of the peanut. During boiling, heated water intrudes into the internal structure of the peanut and makes the peanut swell. It is unlikely that extended boiling will cause pockets of water inside peanuts, but will rather mix with the oil and form micelle-like micro-environments inside peanuts, so that the peptides are actually retained within the structure of the boiled peanuts. It is possible that if peanuts are ground into a paste first and then boiled for extended hours, much more peptides could be lost to the cooking water, making boiled peanut paste a poor choice for oral immunotherapy. I have not investigated this possibility because my pilot study used boiled and then dehydrated intact peanuts, not boiled peanut paste.

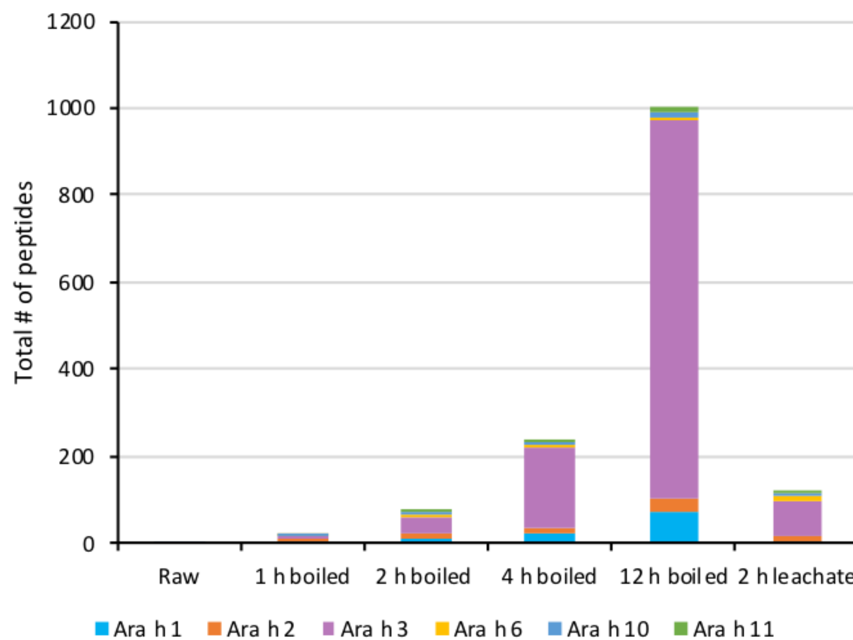
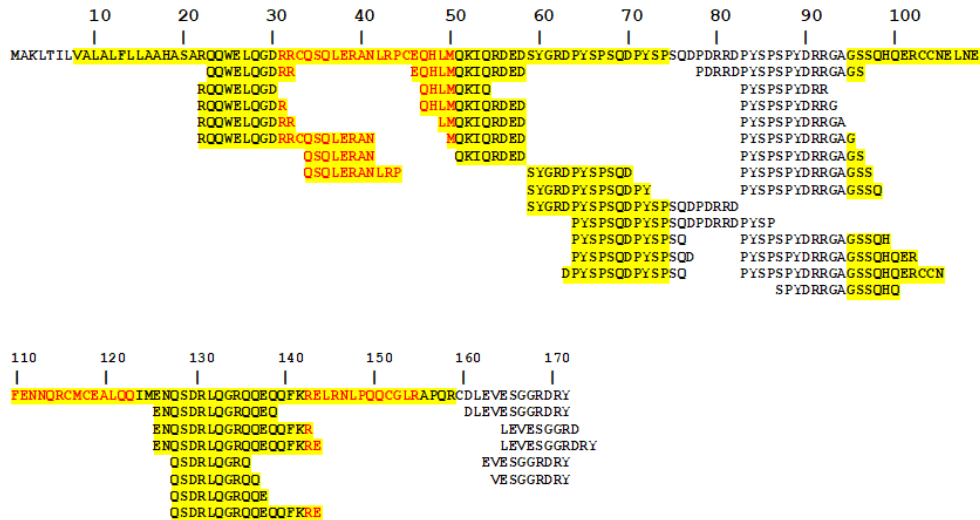


Figure 3.8.2. Allergen peptides are retained in boiled peanuts. Peptides of <3 kDa were extracted from 2 independent raw or boiled peanut preparations and sequenced by mass spectrometry with the mean number of unique allergen peptides determined.

3.8.3. Ara h 2 Peptide Alignment Map.

As shown above, the 12-hour boiled peanut contained the most significant amount of peptides after boiling, so it was used to create a Peptide Alignment Map of Ara h 2, the most important peanut allergen. The map was created by matching and aligning the peptides found by MS in 12-boiled peanut, along the full sequence of Ara h 2, which contain 172

amino acid radicals. Known T-cell epitopes, identified by two published papers, were either coloured red [229] or shown in yellow background [228]. Sequence coverage was found to be 65.7% in 12-hour boiled peanut. Size of peptide length for all allergens was between 7 and 28 amino acids and the mean length of peptides did not change significantly with boiling time.



Published Ara h 2 T cell epitope regions, Pascal et al, Clin Exp Allergy (2012)

Published Ara h 2 T cell epitopes, Prickett et al JACI (2010)

Figure 3.8.3. Sequence coverage of Ara h 2 peptides. Peptides from 12 hour boiled peanut extracts were isolated using a 3 kDa Mw cut-off filter, sequenced by qTOF mass spectrometry and aligned with an Ara h 2 reference sequence. Sequence coverage of Ara h 2 was 65.7%. Published T cell reactive sequences were indicated.

3.8.4. Ara h 1 Peptide Alignment Map.

Similar exercise was performed in producing a Peptide Alignment Map of Ara h 1, using peptides found by MS from 12-hour boiled peanuts. The figure was not shown here because Ara h 1 has 619 peptides (about 3.5 times longer than Ara h 2's 172 peptides), and the map would be too large for a simple figure.

Sequence coverage was found to be 20.1% in 12-hour boiled peanut. Size of peptide length for all allergens was between 7 and 28 amino acids and the mean length of peptides did not change significantly with increased boiling time.

3.8.5. Ara h 3 Peptide Alignment Map.

Peptide Alignment Map of Ara h 3, using peptides found by MS from 12-hour boiled peanuts, was also performed but not shown here because Ara h 3 has 512 peptides, and like Ara h 1 the map cannot be easily fit into a simple figure. Also, there has been no published study on its T cell epitopes, unlike Ara h 1 (1 study) and Ara h 2 (2 studies).

Sequence coverage was found to be 61.9% in 12-hour boiled peanut. Size of peptide length for all allergens was between 7 and 28 amino acids, and the mean length of peptides did not change significantly with increased boiling time.

3.9 Ex-vivo T cell Stimulation Using PBMC from Peanut-allergic Children Incubated with Raw and Boiled Peanut Extracts

3.9.1. Acknowledgement:

The data of this experiment were generated by Dr Preethi Eldi, post-doctoral cellular immunologist at the Experimental Therapeutic Laboratory located at Sansom Institute, University of South Australia, using the equipment there. My roles were limited to the identification of suitable patients for donating PBMC, and the supply of raw and boiled peanut extracts to Dr Eldi.

3.9.2. T cell stimulation study.

To investigate if boiled peanuts (2 and 12 h boil) retained T cell stimulatory activity, antigen-specific T cells in peanut allergic patient blood samples were first identified by flow cytometry. Previous studies have detected human antigen-specific CD4 T cells in a clinical setting of chronic infection using dual expression of CD25 and CD134 in cultures with cognate antigen [119]. To test if these markers could be used to identify peanut antigen-specific CD4 T cells, PBMCs from peanut allergic patient blood samples were stimulated with Streptococcal enterotoxin B (SEB, as polyclonal mitogen control), Tetanus toxoid (TT, as antigen-specific control) or raw peanut extract for 7 days.

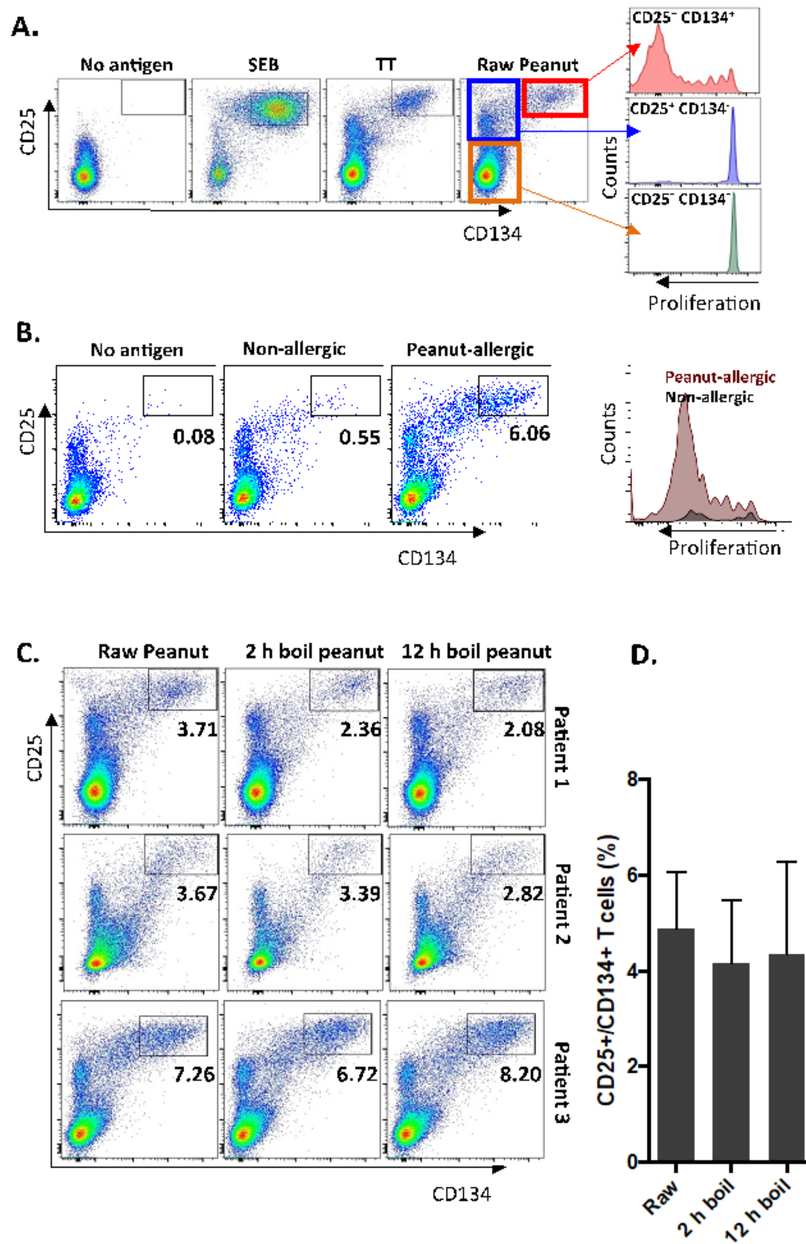


Figure 3.9.2. Boiled peanuts retain ability to stimulate T cell proliferation. (A) PBMC from peanut-allergic individuals were left unstimulated (no antigen) or stimulated with SEB, TT or raw peanut for 7 days and CD4 T cells were analysed by flow cytometry. The gated population showed the antigen-specific CD4 T cells that are CD25 and CD134 double positive. (B) PBMC from non-allergic and peanut-allergic individuals were left unstimulated or stimulated with raw peanut for 7 days and the antigen-specific CD4 T cells population analysed by flow cytometry. Representative plot is shown from 3 different subjects. (C) CD4+,CD25+, CD134+ antigen-specific T cells in response to raw, 2-hour and 12-hour boiled peanut. The

number within the gate indicates the percentage of CD4 T cells that are antigen-specific (CD25+, CD134+) and are summarised in (D).

It was observed that the CD4 T cell proliferative response to the antigen was restricted to the CD25 and CD134 double positive cell population (Figure 3.9.2 A). In addition, there was a 6-fold greater antigen-specific T cell population in ex-vivo peanut stimulated cultures of allergic patients compared to non-allergic controls (Fig 3.9.2 B). These results confirmed the specificity of the flow cytometric assay in detecting antigen-specific CD4 T cells in a clinical setting of allergy.

PBMCs from three peanut allergic patients were then cultured for 7 days with equal concentrations of raw, 2-hour boiled and 12-hour boiled peanut extracts. Flow cytometric pseudo-colour plots of peanut antigen-specific T cells in response to the three stimulatory conditions were depicted in Figure 3.9.2 C, and summarized in Fig 3.9.2 D. Using t-test, no statistically significant differences could be detected in the $CD4^+$, $CD25^+$, $CD134^+$ viable T cell population between raw, 2-hour boiled and 12-hour boiled peanut-stimulated cultures.

3.10 Skin Prick Tests Comparing Raw, 1-4 Hour Boiled Peanuts, and Corresponding Leachates.

To evaluate the allergenic properties of boiled peanuts versus raw peanuts in vivo, skin prick tests (SPT) were carried out on 20 peanut allergic children (Figure 3.10.1). In comparison to the unboiled raw peanut extract, skin prick testing showed a significant reduction in wheal size to 55% and 36% for the 2-hour and 4-hour boiled peanut extracts respectively. No significant difference was seen between a commercial raw peanut extract and our own laboratory-produced raw peanut extract. The SPT was performed in conjunction with a range of other nuts on volunteer patients who agreed to participate in this study as they attended an allergy follow-up clinic, and due to ethical consideration of limiting additional skin prick tests imposed on these patients unrelated to their original nut allergy, SPTs using 12-hour boiled peanut and leachate were not performed. The 2-hour leachate generated a similar wheal size to the 2-hour boiled extract, while the 4-hour boiled extract elicited a significantly lower wheal size when compared to the 2-hour boiled extract.

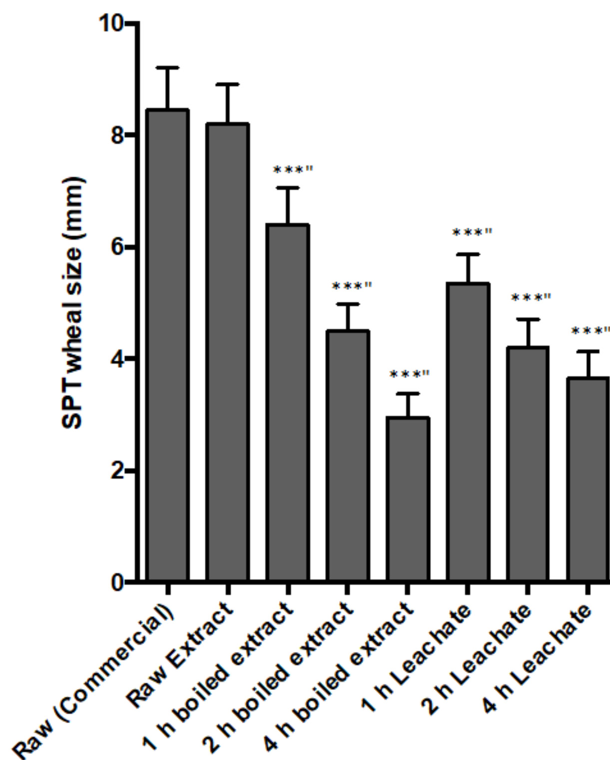


Figure 3.10.1. Skin Prick Test demonstrated that boiling led to significantly reduced wheal size in peanut-allergic subjects (N=20). *** $p < 0.05$ compared to raw peanut. Error bars indicated SEM.

3.11 Discussion: The effect of Extended Boiling on Peanut Allergenicity

Inside an intact peanut the protein allergens were confined within an extremely hydrophobic environment surrounded by the intricate peanut structure that included 50% oil. The boiling method used here allowed peanuts to maintain their shape and structure, even after boiling, and as the boiling time was increased the peanuts could be seen to swell from the absorption of hot water. This “wet heating” process (in contrast to dry roasting) could induce 3 principal changes. Firstly, it could cause leaching of peanut allergens into the cooking water, as shown by the appearance of new protein bands in leachates in SDS-PAGE image (Figure 3.3.1). Secondly, the heated water could cause fragmentation of proteins through hydrolysis, with appearance of new smaller protein molecules in the same SDS-PAGE image. As a result, by 12 hours most of the original peanut allergens had broken down into fragments less than 17 kDa in molecular weight (Figures 3.4.1). Thirdly, boiling could presumably denature the three-dimensional structure of peanut allergens, resulting in reduced binding of conformation-dependent IgE

antibodies. This was illustrated by the reduction of IgE-reactivity in Inhibition ELISA (Figure 3.7.5).

Extended boiling was likely to cause reduction in IgE reactivity to both linear (continuous) and conformational (discontinuous) epitopes. The use of SDS as a denaturing agent prior to Western blotting resulted in the loss of most of the secondary and tertiary protein structures, and as a result Western blot antibody-binding would primarily represent recognition of linear epitopes, although reformation of some discontinuous epitopes could occur during or after transfer of proteins to the membrane. In contrast, Inhibition ELISA did not use denaturing agents and was able to recognise both linear and conformational epitopes. As such, the 45% and 40% reduction in IgE reactivity in Western blot (compared to raw extract) for 2 hour and 12 hour boiled extracts suggested a reduction in mainly linear epitopes. The 8- and 19-fold reduction in IgE binding capacity in the 2-hour and 12-hour boiled extracts seen with inhibition ELISA suggested that there were substantially more conformational epitopes in raw peanut before boiling, and that these were lost afterwards. Conformational epitopes were reportedly more important than linear epitopes for binding IgE and for activation of basophils and mast cells, so the 19-fold reduction in IgE binding epitopes at 12-hour boiling is likely to dramatically reduce the allergenicity of these peanuts.

Ara h 2 contained four disulphide bonds, which stabilized the protein conformation and was likely to be responsible for the requirement of extended boiling to reduce its allergic properties. Ara h 2 was the most prevalent and potent peanut allergen, and the result here had demonstrated that at least 2-hour boiling time was required to reduce the allergenicity of this major allergen, probably even longer. This is significant, as previous investigations have focused on boiling times of 1 hour or less [221, 222, 225, 226]. Rabbit polyclonal IgG antibody WB under denaturing reducing conditions (which quantifies the relative amount of total Ara h 2 protein including fragments) revealed decreases to only 22% and 13% for 2-hour and 12-hour boiled peanut extracts respectively. By comparison, boiling for 2 hours or longer almost completely abolished detection of intact or fragmented Ara h 1, indicating that Ara h 1 is relatively heat labile.

The reduction in IgE reactivity of boiled peanut extracts was demonstrated *in vivo* using skin prick tests on 20 peanut-allergic subjects, with a reduction of mean SPT diameter to 55% (2-hour boiled) and 36% (4-hour boiled) when compared to raw extract. Skin prick test wheal sizes

are known to be a non-linear measurement of clinical allergy. Tripodi et al. demonstrated that a 256-fold dilution of allergen only elicited a 50% reduction in wheal size. MS was employed to determine whether allergen peptides were retained in the boiled nuts. The number of unique allergen peptides sequenced by MS in boiled peanut extracts increased with boiling time, culminating in a 42-fold increase in unique allergen peptides at 12-hour boiling. This provides not only direct evidence of the progressive fragmentation of peanut allergens with increasing boiling time, but also that peptides are retained within the boiled peanuts. T cell reactive peptides have previously been reported for Ara h 1 and Ara h 2. Peptides sequenced from peanuts that have been boiled for 12 hours covered 49% of the Ara h 1 T cell reactive peptides [227], and 62% / 45% [228, 229] of the Ara h 2 T cell reactive peptides respectively, indicating that, despite the substantial reduction in IgE binding epitopes that occurred with 12 hours of boiling, a large number of the Ara h 1 and Ara h 2 T cell reactive peptides were still present.

Results in this chapter had demonstrated for the first time that peanut antigen-specific CD4 T cell activation could be detected by up-regulation of CD134 and CD25 and that this population exclusively contained proliferating cells. The proportion of activated proliferating T cells was higher in peanut-allergic patients than non-allergic controls. While extended boiling reduces IgE-reactivity, the boiled peanut extracts did not lose the capacity to stimulate antigen-specific T cells, as shown by activation and proliferation.

In summary, the Western blot, inhibition ELISA and SPT data have collectively indicated that boiling for at least 2 hours has generated hypoallergenic peanuts that have significantly reduced IgE reactivity. In addition, boiling has caused fragmentation of peanut allergens, but fragmented peptides are retained in boiled peanuts. Significantly, boiling does not quantitatively alter the ability of boiled peanuts to stimulate antigen-specific T cells. Oral administration of T cell epitope peptides has been shown to reduce responses to allergen challenge in mouse models of egg and cow's milk allergy. Therefore hypoallergenic boiled peanuts are attractive candidates for oral immunotherapy, as they have diminished IgE reactivity while still retaining fragmented peptides to stimulate T cell activity, an important indicator to the development of allergy tolerance. Boiling of peanuts for up to 60 minutes as previously described is likely to be insufficient to reduce allergenicity and avoid treatment-related allergic reactions, whereas two, and especially twelve hour boiled peanuts appear to be preferable candidates for oral immunotherapy.

Chapter 4

Sequential Boiled and Roasted Peanut Oral Immunotherapy

4.1 Background

Peanut allergy affects 1-3 % of population in Western communities, and the majority of them will not be able to outgrow their allergy. Oral immunotherapy (OIT) using roasted peanut flour has been shown to be able to desensitize peanut-allergic children but is considered not ready for clinical practice due to the high rates (≥45%) of adverse events (AEs), especially during the up-dosing phase of treatment. This mandates medically supervised up-dosing in hospitals (usually requiring visits once every two weeks), which puts a severe limit on the number of patients that can be treated with this approach. A break-through is therefore in waiting for an oral immunotherapy method that can be safely performed at home without hospital involvement.

In 2001 Beyer et al. proposed that the prevalence of peanut allergy in China was lower than that of the Western world because peanuts consumed in China were boiled, not roasted. They showed that boiling peanuts for 20 minutes was able to reduce IgE binding *in vitro* when compared to roasted peanuts. Chapter 3 of this thesis basically confirmed their findings, but had also revealed that extended boiling was necessary to make the reduction of IgE reactivity relevant for practical clinical application. Using 50% Inhibition ELISA as an index for comparison, it was shown that extended boiling would progressively reduce peanut IgE binding to 12.5% at 2 hours, and to 5.3% at 12 hours, from that of raw peanut. Importantly, T cell reactivity, which was required to induce tolerance, was not attenuated but actually retained by the boiled peanuts. This makes hypoallergenic boiled peanut a suitable candidate for oral immunotherapy, with the potential of having significantly fewer adverse events during treatment.

However, there could also be a serious deficiency if only boiled peanuts were used exclusively for desensitisation. The Inhibition ELISA data in Chapter 3 had shown that the ability of boiled peanuts to block patient IgE binding to raw peanuts seemed to plateau out at just below 80%, (2-hours of boiling ~70%, 12-hours of boiling ~50%), suggesting that extended boiling probably had also significantly impaired the original repertoire of peanut epitopes. To remedy this situation, a supplementary step would be required to ensure full desensitisation.

The novel idea of using sequential boiled and then roasted peanut in oral immunotherapy was devised to achieve this goal. In this model, oral immunotherapy began with hypoallergenic boiled peanuts to reduce adverse events. After a period of partial desensitisation, roasted

peanuts were then introduced, following a similar protocol. Because the patients would have been partially protected from the first phase of boiled peanut desensitisation, the subsequent ingestion of roasted peanuts would also be accompanied by fewer adverse events. The pilot study in this Chapter is therefore a proof-of-concept clinical trial based on this hypothesis. Due to the nature of home-based up-dosing procedure a cautious approach was adopted, which excluded the treatment of severe peanut allergy.

4.2 Study Design and Subject Enrolment

The study was an open, non-randomised and non-controlled intervention trial. Fifteen children who attended my private practice at Allergy SA and satisfied the inclusion criteria were invited to participate. The sample size was determined by the number of oral food challenges (OFC) that could be arranged at Flinders Medical Centre over a 1 year study period. The OFC was used to confirm the allergic status of these children.

The 12-month protocol comprised a sequential 7-month initial phase of ingesting 2-hour boiled peanuts and a 5-month second phase of ingesting roasted peanuts. This pilot study used large peanuts (approximately 1 gram per peanut) for oral immunotherapy, starting with 2-hour boiled samples because it was considered to be an appropriate balance between sufficient hypoallergenicity while still retaining enough allergens for desensitization. Peanuts were sourced from a local nut supplier (Charlesworth Nuts of South Australia), who slow-boiled the peanuts for 2 hours and then dehydrated them for at least 24 hours at their factory, in compliance with instructions given to them and as described in Chapter 2. Commercial roasted "jumbo" peanuts were also sourced from the same supplier for the second stage of oral immunotherapy.

It is worthy to point out here that the protocol used in this pilot study was different from all other previously published regimes in many ways, one of which was to split the daily ingestion of peanuts into three separate doses (morning, afternoon and night). The purpose of having multiple daily doses was to minimise adverse reactions during dose-escalation. The other noted differences were the 2-step desensitisation protocol and the home-based up-dosing which was not supervised at hospitals.

After OFC, one participant decided to withdraw from study after receiving adrenaline at challenge. As a result, only 14 subjects were enrolled to commence oral immunotherapy.

4.3 Ethics Approval and Clinical Trial Registration

Ethics approval was obtained from Southern Adelaide Clinical Human Research Ethics Committee (approval number 473.13). The trial was registered with Australian New Zealand Clinical Registry (Trial ID ACTRN12614000919617). Informed consents were obtained from parents before OFC.

4.4 Inclusion Criteria

1. A recent history of peanut allergy (mild to moderate reactions). The following is how the severity of clinical reaction is graded [134] [132]: Grade 1 = cutaneous urticaria / angioedema; grade 2 = gastrointestinal symptoms (e.g. vomiting, abdominal pains), grade 3 = rhino-conjunctivitis; grade 4 = mild throat swelling and asthma symptoms such as cough, wheeze, stridor. Overall, grade 1-3 = mild, 4 = moderate [230].
2. Skin prick test (SPT) reading of 7-14 mm mean wheal diameter.
3. A positive oral food challenge test.

4.5 Exclusion Criteria

1. A history of severe anaphylaxis.
2. SPT >14 mm.
3. Serious medical co-morbidities.
4. Parents unable to supervise properly or unwilling to participate in tests.
5. In addition, children whose challenge test results are too severe (i.e. graded > 4: presence of cyanosis, hypotension, confusion, collapse, loss of consciousness, incontinence) will be excluded from this current study.

4.6 Clinical Reactions at Oral Food Challenge

All oral food challenges were performed at the Paediatric Day Unit at Flinders Medical Centre according to hospital protocol, and independently supervised by two staff allergists at the hospital. At challenge, increasing doses of peanut paste, starting at 50 mg, were given every 20 minutes until a definite and objective reaction was observed, to a maximum of 5,000 mg peanut paste (1,250mg peanut protein).

A positive challenge outcome was defined as the presence of any of the following: three or more welts >3mm persisting for more than five minutes away from the mouth, angioedema of the lips or eyes, rhino-conjunctivitis, generalised skin erythema, vomiting, wheeze, cough,

stridor or hypotension. The challenge procedure was terminated at the first sign of a positive reaction, and appropriate treatment provided by the staff at the Day Unit. Intramuscular epinephrine was administered at the discretion of supervising allergists, based on severity of symptoms.

The outcomes of individual OFC were shown in Table 4.6.1. As mentioned earlier, a total of 15 children underwent OFC but one child withdrew after adrenaline intervention at OFC. Maximum eliciting doses at OFC ranged from 200 mg to 2500 mg peanut butter (48 to 600 mg peanut protein). All 15 participants had experienced a positive challenge, with 9 of 15 requiring IM epinephrine at termination. Symptoms at termination of OFC were described in detail in Table 4.6.1.

Table 4.6.1: All 14 participants demonstrated positive reactions at challenge, with 8 requiring adrenaline intervention.

Patient ID	Gender	Age	Symptoms at termination of OFC	Adrenaline intervention
1	F	9	itchy palate and throat, urticaria, lip, eyelid or face swelling; persistent abdominal pain with or without vomiting	yes
2	M	13	itchy palate and throat, urticaria, lip, eyelid or face swelling; not responding to oral antihistamine and steroid	yes
3	M	11	itchy palate and throat, urticaria, lip, eyelid or face swelling; angioedema of tongue, chest or throat tightness; tachycardia, signs of dyspnoea	
4	M	15	itchy palate and throat, urticaria, lip, eyelid or face swelling; angioedema of tongue, chest or throat tightness	
5	M	15	itchy palate and throat, urticaria, lip, eyelid or face swelling; persistent abdominal pain with or without vomiting; angioedema of tongue, chest or throat tightness; looking pale or unwell, becoming drowsy or complaining of dizziness, sweating	yes

6	F	11	itchy palate and throat, urticaria, lip, eyelid or face swelling; persistent abdominal pain with or without vomiting	
7	F	11	itchy palate and throat, urticaria, lip, eyelid or face swelling	
8	F	13	itchy palate and throat, urticaria, lip, eyelid or face swelling; persistent abdominal pain with or without vomiting; tachycardia, signs of dyspnea; looking pale or unwell, becoming drowsy or complaining of dizziness, sweating	yes
9	F	15	persistent abdominal pain with or without vomiting; looking pale or unwell, becoming drowsy or complaining of dizziness, sweating	yes
10	M	15	persistent abdominal pain with or without vomiting; angioedema of tongue, chest or throat tightness; persistent coughing	yes
11	M	15	itchy palate and throat, urticaria, lip, eyelid or face swelling; persistent abdominal pain with or without vomiting; angioedema of tongue, chest or throat tightness; looking pale or unwell, becoming drowsy or complaining of dizziness, sweating	yes
12	F	7	itchy palate and throat, urticaria, lip, eyelid or face swelling; persistent abdominal pain with or without vomiting; angioedema of tongue, chest or throat tightness	
13	M	9	itchy palate and throat, urticaria, lip, eyelid or face swelling; persistent abdominal pain with or without vomiting; persistent coughing	
14	F	14	itchy palate and throat, urticaria, lip, eyelid or face swelling; persistent abdominal pain with or without vomiting; looking pale or unwell, becoming drowsy or complaining of dizziness, sweating	yes

4.7 The Sequential OIT Protocol

The protocol was designed in such a way that both boiled and roasted peanut phases could follow the same schedule in the first 18 weeks of each. Dosing began with 250 mg (= ¼ peanut) daily, and peanuts contained approximately 25% peanut protein. The dose frequency was increased weekly over the first three weeks from once a day to three times a day. From 4th to 6th week the dose was increased each week to 500 mg (= ½ peanut) three times a day. From 7th to 9th week it was increased to 1 gram (= 1 peanut) three times a day. From 10th to 18th week it was increased to 10 gram (= 10 peanuts) per day in 3 divided doses, which became the maintenance dose, but the schedule was then gradually consolidated to once a day (e.g. 5:5, then 6:4, 7:3, 8:2, 9:1, 10). A detailed schedule of this biphasic protocol was shown in Appendix.

Precautionary measures included (1) not to ingest peanuts with empty stomach, (2) avoid exercise 2-hours before and after peanut ingestion, (3) temporary pause of OIT during sickness (up to 1 week). On resuming OIT, the "restarting" dose was set at half the previously tolerated amount and gradually raised back to original dose over 1-2 weeks.

4.8 Final Outcome at Completion of OIT

At the beginning there were 14 subjects who commenced OIT after OFC, but two of them withdrew during the first boiled-peanut phase. One child withdrew because of refusal to ingest more than ¼ boiled peanut per dose (despite absence of allergic symptoms). A second child withdrew because of social issues impacting on treatment adherence. While their SPT reading, peanut specific IgE and Ara h 2 reactivity were near two ends of the reactivity spectrum, neither of them had experienced any AE during treatment and their withdrawals were unlikely to be related to their allergy status. The subjects encountered psychosocial issues during their treatment as described earlier, which were more likely to be the cause of their withdrawal. The remaining 12 children all completed ingestion of boiled peanuts and advanced to roasted peanuts. At the end of full OIT, 11 reached the target dose of 10 roasted peanuts daily and continued on this dose until final OFC. One child stopped while eating 8 roasted peanuts per day, as a result of Dengue Fever complicated by chronic fatigue syndrome. At the end of the pilot study, all 11 children were witnessed to ingest 10 roasted peanuts at my office without reaction, which were carried out within 4 weeks of completion of OIT.

4.9 Incidence of Adverse Events

All AEs are described in detail in Table 4.9.1. During the boiled peanut phase, three out of 12 children experienced mild AEs but proceeded to complete full treatment including roasted peanuts. One child reported 3 episodes of mild upper lip angioedema after eating ¼ boiled-peanut in the first 3 days with no medication required. A second child experienced two isolated episodes of urticaria, which responded to oral antihistamine. A third child experienced recurrent itchy mouth, mild abdominal discomfort and lip swelling while ingesting 250 mg boiled-peanut, but all symptoms resolved when the dose was reduced to 60 mg of boiled-peanut and then gradually increased back to 250 mg. No medication was required.

During the roasted-peanut phase two AEs were reported. One child experienced mild abdominal pain with a brief emesis while travelling home after ingestion of first dose of 250 mg roasted-peanut, and was treated with a single dose of oral antihistamine. A second child reported a brief sensation of oral swelling that was not evident visually on day 3 after taking 250 mg roasted-peanut, with no treatment required.

Table 4.9.1. Three participants recorded mild adverse events with boiled peanuts and two participants with roasted peanuts.

Patient ID	AE at which phase?	Description
1		
2	**Roasted** peanut	Mild abdominal pain with a brief emesis while travelling home after ingestion of first dose of roasted OIT
3	**Boiled** peanut	3 episodes of mild upper lip angioedema in the first 3 days of the first week of boiled OIT
4		
5	**Boiled** peanut	2 episodes of urticarial responding to oral antihistamine

6		
7	**Boiled** peanut	Itchy mouth, mild abdominal pain after first dose of first week OIT
8		
9		
10	**Roasted** peanut	Brief sensation of oral swelling that was not evident visually on Day 3 of first week of roasted peanut OIT
11		
12		
13		
14		

Blank cell means no adverse events

4.10 Statistical Analysis

Skin prick tests were performed using commercial raw peanut extract (Hollister-Stier, Spokane, Washington, USA) as described in Chapter 2, Section 2.5.3. Serum peanut-specific IgE and IgG_4 were measured by ImmunoCAP (Phadia AB, Uppsala, Sweden). Skin prick test wheal sizes and serum antibody titres were recorded at baseline and at completion of each OIT phase. Differences at each time-point were evaluated using Wilcoxon signed ranks test. Statistical comparisons were undertaken using Stata 14 (Stata Corp, College Station, Texas). All tests were 2-sided with an alpha level of 0.05. Results were shown in Table 4.10.1 and Figure 4.10.1.

Table 4.10.1. Boiled-to-roasted peanut OIT reduces SPT wheal size and increases psIgG$_4$ with unchanged psIgE

Study I.D. and (age)	Ara h 2 (kU/L)	Skin Prick Test (mm)			Peanut-Specific IgE (kU/L)			Peanut-Specific IgG$_4$ (mg$_A$/l)		
	BL	**BL**	**BP**	**RP**	**BL**	**BP**	**RP**	**BL**	**BP**	**RP**
1	5.67	14	6	2	8.2	9.1	3.42	0.45	13.2	43.9
2	0.56	14	15	12	0.41	0.69	0.59	0.01	0.01	0.04
3	1.05	11	5	4	4.2	4.1	2.2	0.16	2.34	3.53
4	3.61	13	9	5	19	19	12	0.16	0.18	0.49
5	>100	11	7	4	>100	>100	>100	1.98	11.4	21.7
6	0.56	7	6	NA	0.37	0.35	NA	0.05	0.08	NA
7	60.4	14	8	5	>100	>100	>100	1.24	11.4	9.24
8	11.1	12	7	5	11	12	5.32	0.2	1.52	0.84
9	0.53	14	7	4	0.65	NA	0.46	0.13	NA	0.45
10	4.01	8	5	6	11	8.8	10	0.3	0.38	1.4
11	9.81	12	6	4	12	27	13	0.15	3.41	23.7
12	0.75	10	8	5	2.4	4.8	5.1	0.16	1.18	9.3
13[W]	0.68	9	NA	NA	2.6	NA	NA	0.16	NA	NA
14[w]	64.7	14	NA	NA	94	NA	NA	0.7	NA	NA
Median	3.81	12	7	5	9.6	9.1	5.32	0.16	1.52	3.53

[W] = Withdrawn while in Phase 1 of boiled peanuts

Table 4.10.1 showed that the boiled treatment phase was associated with a 41% reduction in SPT wheal diameter and an increase of 9.5 fold in IgG$_4$ levels. The roasted peanut phase further reduced the SPT wheal diameter by 28%. These changes were statistically significant (Figure 4.10.1). While IgG$_4$ increased following the roasted peanut phase, this was not statistically significant. No significant change was observed in peanut-specific IgE following either phase.

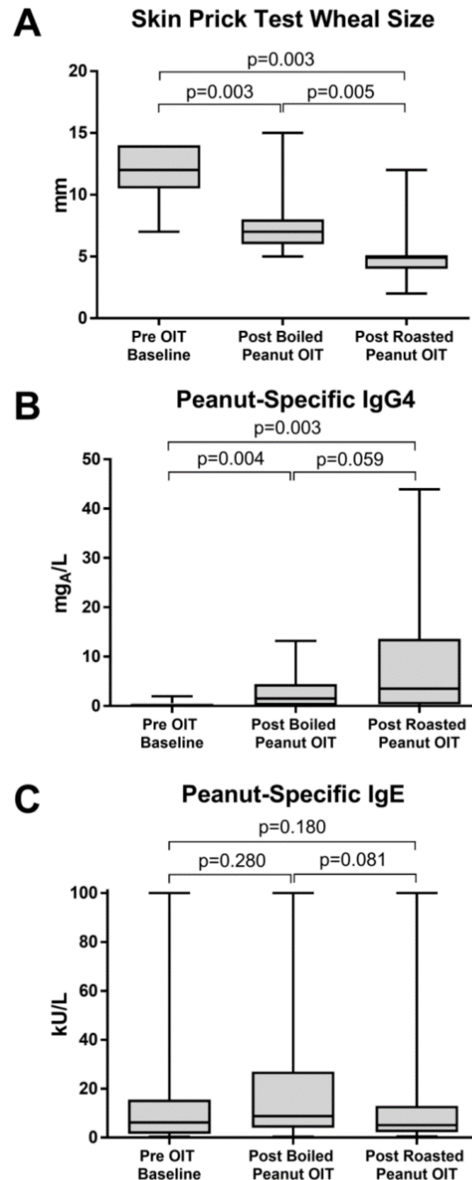


Figure 4.10.1. Boiled-to-roasted peanut OIT leads to a statistically significant decrease in SPT wheal size in both phases (A), statistically significant increase in IgG_4 in Phase 1 (B) but no significant change in psIgE (C)

4.11 Discussion

This study is the first to utilize boiled peanuts within a structured desensitization regimen prior to oral desensitization using roasted peanuts. Turner et al. have previously reported in a research letter [231] a case series of 4 paediatric patients with either confirmed or presumed peanut allergy where 3 were treated with boiled peanuts and achieved variable tolerance. Boiling preparation varied from 2-16 hours at initiation. One patient described was treated with boiled peanuts and "over the course of 2 years she was transitioned to daily raw peanut". Two further

patients were reported to have only received boiled peanut, with a fourth untreated. Raw peanut OFCs to confirm allergy were performed on only 2 patients, with a further patient tolerating a boiled peanut OFC. Post treatment raw peanut OFC was only performed on 1 patient. The lack of a consistent treatment regimen and description of adverse events prevents the assessment of the efficacy and safety of boiled nut treatment in that study. This pilot study differs in that all patients had OFCs to confirm allergy and a standardized regimen of boiled peanut desensitization was utilized, followed by a standardized sequential roasted peanut desensitization phase. In addition, Turner et al. made conclusions regarding post treatment-related changes to peanut specific IgE-reactivity by comparison to an unrelated control, rather than comparing pre and post treatment as we have done in this study.

The data from this pilot study indicate that children with peanut allergy can be desensitized to roasted peanut with few adverse events by using hypoallergenic peanut prior to roasted peanut OIT. The apparent protective effect of boiled peanut in reducing adverse reactions to subsequent roasted peanut OIT has potential clinical significance. The finding that boiled peanut OIT reduces SPT wheal size to raw peanut extract and increases production of peanut-specific IgG_4 is also novel. This indicates that the boiled peanuts are immunologically active and provides a biological basis for the clinical observations. Desensitization to the ingestion of 8-10 roasted peanuts occurred in 12/14 children who commenced the biphasic OIT. This is considerably more successful than current data pertaining to roasted peanut OIT alone. The biphasic regimen used in our study avoided any hospital-based supervision. This contrasts with current practice for roasted peanut OIT which mandates hospital supervision. This observation suggests, within study limitations, the potential for greater cost effectiveness compared to stand-alone roasted peanut OIT.

There are limitations however, and the pilot study findings should be interpreted with caution. The initial OFC was not double-blinded, and children with more severely reactive SPTs or anaphylaxis history were excluded. Furthermore, the study was not randomized with a control group. Hence selection bias cannot be excluded and no comment can be made regarding efficacy in more severely allergic children. Until these limitations are addressed in future clinical trials, e.g. see https://clinicaltrials.gov/ct2/show/NCT02149719 and https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=373026&isReview=true, the role of boiled peanut OIT in clinical practice requires clarification. It seems that adequate

characterization and quality control of the range and nature of allergens induced by boiling deserve careful consideration in future clinical trials using a biphasic approach.

In summary, the combination of graded-dose hypoallergenic boiled peanuts followed by a similar regimen using roasted peanuts has the potential to optimize the safety and efficacy of peanut OIT.

Chapter 5

Future Directions

Oral immunotherapy (OIT) is one of several desensitisation methods that can be used to treat peanut allergy but only approved for clinical trial in a research setting. It is more effective than the other methods but is also hampered by the high rate of treatment-related adverse events (AEs, 45-93%). As a result all up-dosing steps must be done at hospitals and require visits to a Day Unit every 2 weeks for up to 8 months before maintenance can be phased in. Such reliance on hospital is inconvenient, expensive, and restrictive in the number of patients that can be treated. Sublingual immunotherapy (SLIT) and Epicutaneous immunotherapy (EPIT) are better tolerated but are much less effective in achieving desensitisation targets. A method that can be both safe and effective is destined to become a game changer.

This thesis describes a novel OIT strategy that involves the use of boiled peanuts as the starting point for desensitisation in peanut allergic children. The original idea that inspired the thesis came from a paper published more than 16 years ago, which suggested that peanuts could be made hypoallergenic by boiling. The idea was later translated into a clinical trial, the aim of which was to show that desensitisation with hypoallergenic boiled peanuts could indeed reduce AEs. However, using boiled peanuts to start desensitisation is only half of the equation: the other half is contributed by the sequential oral immunotherapy strategy using boiled peanuts first and then roasted peanuts later. This is necessary because boiled peanuts do not possess the full repertoire of conformational epitopes, and the exclusive use of them in desensitisation may end in failure unless there is compensation from fully allergenic roasted peanuts in a subsequent step. This combination strategy is unique in design and stands head and shoulders above all other methods.

The clinical trial mentioned above was a proof-of-concept pilot study that was published in November 2017. The study confirmed that sequential oral immunotherapy using extensively boiled and then roasted peanut was both safe and effective. More importantly, it showed that up-dosing could be feasibly done at home (only the first step of each phase was supervised at a doctor's office), meaning that hospital supervision would no longer be required and large number of patients in the community could be treated with this method. We have now received a new grant from the Channel 7 Children's Research Foundation to conduct a Phase 2 clinical trial involving 66 peanut-allergic children in 2017 / 2018. The trial was registered and its details can be found at the Australian New Zealand Clinical Trials Registry (https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=373026&isReview=true).

The new trial differs from the pilot study in that: (a) it includes many cases of severe peanut allergy, (b) it is a 3-step procedure starting with 12-hour boiled peanut, then 2-hour boiled and finally roasted peanut, while the pilot study uses a 2-step procedure starting with 2-hour boiled peanut and then roasted peanut, (c) the commencing dose in each phase will be 1/16th of a peanut instead of 1/4th, further reducing the risk of AE, and (d) doses are administered twice daily instead of three times daily, making it more convenient for parents. This new study is expected to be completed in early 2019.

Stepping back and looking from a distance, desensitisation only forms a small part of the overall picture of peanut allergy treatment. It may take a full year or so for a child to go through the process but, in comparison, post-desensitisation management has a life span of many years. This is because desensitisation is not a permanent cure for peanut allergy and requires ongoing and regular consumption of peanuts in order to maintain its protective status. It is not exactly clear how many of desensitised children will drop out from eating peanuts after initial success, which most likely will climb over time, but there are not many such studies in the literature to clarify the situation. On the other hand, its significance cannot be overstated, because discontinuation of regular peanut ingestion after desensitisation will very likely cause a return of the allergy status, which means that all previous efforts and time will be wasted. Our team of investigators in the Phase 2 clinical trial are determined to continue monitoring these patients post-desensitisation, and work out a solution that will encourage the long term adherence to regular peanut ingestion and extend the benefit of sustained desensitisation.

To provide a glimpse of the task that we are facing, in a recent long term SLIT follow up study involving 37 subjects, 23 (62%) of them had chosen not to continue with treatment within 3 years. In another study, which involved the use of probiotic peanut oral immunotherapy, 24 subjects in the intervention arm were reviewed 4 years after desensitisation and 8 (33%) of them had discontinued eating peanuts. In my own experience with the pilot study, 11 treated patients were contacted by telephone approximately one year after completion (one of the original 12 could not be contacted), and only 5 of them were still eating peanuts, despite being told to continue eating peanuts on a regular basis after desensitisation.

We think the offering of a choice between daily and weekly maintenance regimens in a randomised and controlled fashion post-desensitisation may provide a practical framework to begin with in the study of adherence. It is envisaged that a combination of quantitative and

qualitative psychological analysis in focus groups (both children and their parents) would be useful in understanding why some of these subjects might choose to drop out rather than to continue with regular peanut consumption. Hopefully, the outcome of our psychological investigations may lead us to a more effective solution to the problem of long term adherence, and such a study has never been done before.

Appendix

1. Participant Information Sheet and Informed Consent
2. The biphasic (2-hour boiled to roasted) OIT protocol
3. Additional advice to participants
4. The new HYPES protocol (12-hour boiled to 2-hour boiled to roasted peanut OIT)

PARTICIPANT INFORMATION SHEET

Name of Organisations

Flinders Medical Centre and Flinders University

Title of the project

"A pilot study for a new oral immunotherapy (desensitisation) treatment for nut allergies"

Researchers

Dr Billy Tao, *Senior Visiting Paediatrician, Flinders Medical Centre; also PhD candidate, Flinders University*

Dr Tim Chataway*, Head, Flinders Proteomics Facility, Flinders University*

Professor Kevin Forsyth, *Professor of Paediatrics and Child Health, Flinders University*

Dr Michael Wiese, Senior lecturer, *Department of Pharmacology, University of South Australia*

Invitation to participate

In Australia about 1 in every 50 children are allergic to at least one nut, if not more, and in some serious cases the allergy can be life-threatening because of anaphylaxis. Currently there is no safe or effective treatment for this medical condition, but most specialist doctors who see nut-allergic patients regularly believe that one day we can cure nut allergies by a method called desensitisation, also known medically as immunotherapy. Desensitisation is a process to reduce or eliminate a patient's allergic reaction to a food or substance.

Traditional desensitisation method (e.g. when used to treat peanut allergy) involves the initial ingestion of a tiny amount of defatted peanut flour and then gradually increasing the dose over several months, until a daily maintenance dose is reached. The patient is then required to continue with this daily dose until desensitisation is complete. Because defatted peanut flour is as allergic as the original raw peanut it can cause a lot of allergic reactions during treatment, especially in the earlier stages when the amount of ingested peanut flour is being increased. They can affect as many as 90% of patients.

At Flinders Medical Centre we have devised a new oral immunotherapy method which can potentially overcome this problem. Our idea is to split the desensitisation process into two steps (or phases) instead of just one step, which is the traditional approach. In the first step we start desensitisation with a much weaker nut-allergic product (boiled nut), which we think will result in much fewer allergic reactions. In the second step we continue desensitisation with increasing doses of the original unprocessed raw nut until full immunotherapy is achieved, but since the patient is already partially protected after the first step, there will also be very few allergic reactions in the second step. In theory this argument seems logical and reasonable, but before we can embark on a full scale large clinical trial we must first do a smaller pilot study, which is the purpose of our current project.

In the past few months we have completed some very interesting experiments on boiled nuts (especially peanut) at our laboratory in Flinders Medical Centre, and the results showed that boiling does indeed reduce the allergic properties of nuts. More recently, we have also carried out skin prick tests on a number of peanut allergic patients, and the results showed that boiled peanut extracts produced much lower skin prick test readings than raw peanut, again supporting our laboratory findings. We are now ready to put our theory to test.

You are invited to participate in this research project but we would like to emphasize that involvement is completely voluntary. This means that you do not have to be involved, whether you wish to or not is entirely up to you, and whether you take part or not will not affect the medical care of your child or your relationship with any of the investigators.

Selection

Your child is selected because he or she is already known to us to be allergic to at least one nut, and has participated in a previous study of ours, called "Can controlled consumption of non-allergic nuts benefit children with nut allergy? A randomised controlled study". We know from our previous conversations with you that you are keen for your child to be cured of his or her nut allergies, so we think you might be interested to find out more about this project. A major attraction of our new approach is that it could mean fewer and milder allergic reactions from treatment in comparison to traditional desensitisation methods.

Aim of the project

The aim of this project is to show that desensitisation or immunotherapy is much safer if the program is split into two steps or phases, so that the chance of having an allergic reaction from treatment is greatly reduced. In the first phase we will use nuts that have been boiled for 2 hours for desensitisation, and in the second phase we will switch back to the use of original allergic raw nuts to complete the desensitisation process.

Summary of procedures

Dr Tao will send you an invitation letter together with this Participant Information Sheet and an outline of the desensitisation protocol, to give you an idea about how desensitisation in two phases is done and how your child and your family can be involved in the study.

You will have plenty of time to think about our research, and if you are interested in helping us by participating in the study you may contact Dr Tao by phone or email. A meeting will be arranged so that Dr Tao can sit down with you, explain the research and desensitisation protocol in more detail, and answer all your questions personally. The appointment will take approximately one hour, and is provided to you free of charge. If you are happy to join the study your child will be enrolled and informed consent will be collected from you. For your convenience you may enrol at this point, or if you prefer another time a separate appointment can be arranged.

At enrolment some medical history and demographic data will be taken, and a skin prick test will be performed if your child has not had one done already within the past 6 months. Afterwards, an appointment will be made for your child to have an oral nut challenge at the Paediatric Allergy Day Unit at Flinders Medical Centre, which will also be free of charge. The challenge test is important because we need to prove that your child is truly allergic to the "suspect" nut before we can give its boiled form to him or her for desensitisation. You can be assured that the challenge test will be stopped at the earliest sign of an allergic reaction, no matter how mild it may be. The Day Unit is situated within Flinders Medical Centre, and when the challenge is done there will be at least one, often two, specialist paediatric allergists and several allergy nurses in attendance. The Day Unit carries out all challenge tests at Flinders Medical Centre, averaging about 6 such tests per week for children and 2 – 3 tests for adults.

Once your child's allergy status is confirmed we will collect about 10 ml of blood so that we can measure his or her allergy antibodies to the allergic nut. We will supply you and your child with a local anaesthetic called EMLA, which will numb the skin at the needle site so that blood-taking becomes painless. Allergy antibodies are produced by your child's immune system after an allergic reaction has occurred, and they are quite "allergen-specific", meaning that they are <u>only</u> formed against the particular nut that your child is allergic to, so their measurements can help us determine any change of allergy status as a result of treatment.

We will also have to repeat skin prick and blood tests on two more occasions afterwards, which are (1) at the end of the first phase of desensitisation, approximately 8 months from commencement of treatment and (2) at the end of the second phase, which starts at 8 months and finishes at 12.5 months from the commencement of treatment. So there will be altogether 3 sets of skin prick and blood tests (you will be supplied with new EMLA patches at each blood test). These will coincide with your three visits for the study: at enrolment, at end of Phase 1, and end of Phase 2. These three visits are on top of the oral nut challenge test at Flinders Medical Centre Day Unit soon after enrolment (so total visits = 4).

The first dose of desensitisation at either Phase 1 or Phase 2 will be given at Dr Tao's private practice address called Allergy SA, where you have seen him before. If you have a look at the desensitisation protocol you will notice that it has two pages: the first page is for boiled nut in Phase 1, and the second page is for raw nut in Phase 2. On the first day of phase 1 your child

will ingest ¼ of a boiled nut, but this ¼ nut will be broken down into a number of even smaller pieces (starting with roughly 1/100 of a nut). As a result, it may take up to an hour before the whole ¼ nut is completely ingested. This is just a safety precaution at the start of the desensitisation program, and if your child does not show any reaction to the first ¼ nut he or she should be able to eat larger pieces in subsequent days.

You will also notice that at the start of the protocol your child will only be eating the boiled nut once a day, but as time goes on (the increments are made weekly) the frequency will be gradually increased to three times a day, and then the amount at each serve will be gradually increased afterwards (all serves are eaten at home, except the first dose on Day 1). The reason for making small increments on weekly basis and spreading the daily dose to three times a day is because we want to reduce the chance of allergic reactions to a minimum. As you gradually move down the desensitisation protocol from Week 1 to Week 18 you will notice that some of the weeks are shaded or highlighted. These weeks are called "milestones", and they give you a rough idea about how your child is progressing. So for example at week 6 your child will be eating 1½ nuts a day, and at week 9 he or she will be eating 3 nuts a day. If everything goes well according to plan your child should be able to eat 10 nuts a day by week 18. At enrolment Dr Tao will go over the protocol with you in even greater detail and answer all your questions until you are completely satisfied. He will also give you additional instructions on what you need to watch throughout the desensitisation program. Your contact with Dr Tao is not limited to the formal visits with him during the study – at any time when you have any question, or if your child has an adverse reaction, you can always contact him by phone, SMS or email.

Dr Tao will provide you with all the boiled and raw nuts that you need for the desensitisation program, but if you are interested in how the nuts are boiled he is more than happy to show you. Boiling nuts for two hours does not destroy their shape, so it is just as easy to count them as the raw nuts, and they still taste similar to the original nut too. They are simply less allergic than the raw nuts.

Commitments

Once your child is enrolled the desensitisation program will last 12.5 months, but it could be longer if progress is slowed. During desensitisation your child will be eating the boiled nuts in Phase 1 and raw nuts in Phase 2 at home, unless there is an adverse reaction that may interrupt desensitisation. Your child must also be prepared to eat the nuts in smaller doses but three times a day, which is designed to reduce the risk of allergic reactions.

Benefits

From your family's point of view, at the end of the desensitisation program, which is roughly 12.5 months, your child is expected to be able to eat 10, or at least 10 grams, of the original nut on any day and will not have an allergic reaction.

From our point of view, we have found a simple and effective way to treat all nut allergies, just like how we have treated your child. This pilot study will provide the foundation for a larger future study in which we will test our theory that a 2-step desensitisation procedure starting with boiled nuts is superior to the current one-step method. If we can show in this pilot study that all participants including your child do not have many allergic reactions during treatment, and can eat at least 10 allergic nuts after treatment, we have found a promising lead to the cure of all nut allergies.

Risks and adverse effects

Being an untested method we cannot guarantee that it is completely free from any adverse effects. However, we can reassure you that all efforts have been made to improve safety and reduce risk in this pilot study. We know from reviewing the literature that there have been 6 published reports on oral peanut immunotherapy and a lot of participants have minor non-life-threatening allergic reactions during treatment, including skin rashes, mild facial swelling, running nose, red eyes, vomiting or abdominal pains. Very rarely, one or two participants may need the treatment with an epipen, but this is very uncommon. As already mentioned, we have evidence to believe that boiled nuts are much less allergic than raw nuts, so we expect much less or much milder reactions from our treatment. We have further designed our desensitisation protocol in such a way that the total daily doses of boiled or raw nuts are given separately in three divided portions throughout the day, mainly to reduce the chance of having an allergic reaction from treatment. As well, all oral nut challenges will be carried out at the fully equipped Paediatric Allergy Day Unit at Flinders Medical Centre, which is staffed by at least one, often two, specialist allergists, supported by one advanced immunology trainee registrar and several allergy nurses on site. All initial doses of Phase 1 or Phase 2 immunotherapy are given by Dr Tao personally at Allergy SA, also fully equipped to deal with skin prick tests and oral immunotherapy, and the maximum (total) first-day dose of boiled or raw nut (depending on which phase your child is in) is only ¼ of a nut. Safety is always number 1 priority in our mind, so after we have demonstrated to both you and us at Allergy SA that your child can eat ¼ of a boiled nut in Phase 1 or ¼ of a raw nut in Phase 2 we will feel comfortable about allowing your child to eat the same dose at home afterwards.

The local anaesthetic EMLA designed to numb the skin at needle site and reduce pain is widely used in medical practice and does not have any significant adverse event, unless your child is allergic to it, and then he or she will develop a local reaction after application. If that is the case we will either not use it or try a different brand of local anaesthetic on your child.

Dr Tao will also be available 24 hours a day and 7 days a week to hear any report of allergic reactions from your child, and he will terminate the study if there is any concern of the safety of this treatment method.

Compensation

Participation in this study does not impact on your basic legal right to seek compensation; however, if you do suffer harm, you may receive compensation without litigation.
Participants in this study are insured under the medical indemnity of Dr Tao.

Confidentiality

All records containing personal information will remain confidential and no information which could lead to your child's identification will be released, except as required by law.

If the results of this study are published, for example in scientific journals, your child will not be identified by name. Your child's records and your right to them will be protected in accordance with Australian law.

Publication

The purpose of this pilot study is to show that boiled nuts can be used in conjunction with non-boiled (raw) nuts in a two-phase immunotherapy program to successfully desensitise nut allergies with very little adverse effect. We intend to publish the results in an international scientific journal when the study and data analysis are complete. The results of the research may also be used to support a future research proposal involving a full clinical trial.

Withdrawal

Your participation in this study is entirely voluntary and you have the right to withdraw from the study at any time without giving a reason. If you decide not to participate in this study, or if you withdraw from the study, you may do so freely, without affecting the standard of care or treatment you will receive.

Outcomes

You and your child will be fully informed of the outcome of this pilot study when it is finished.

You will be told of your child's skin test results immediately after they have been performed, and the blood test results will also be given to you when they become available, but they will not be passed on to any other person, including your referring doctor. The data will be "de-identified" (i.e. no name will be attached to the results, just an identification number) and then put away for analysis later. We plan to enrol 15 subjects for this pilot study.

Disclosure of incentives

There is no financial benefit to any of the investigators for enrolling you in this study.

Expenses and payments

You will be compensated for travelling and parking at the amount of $20 dollars per visit for the study, totalling 3 visits, plus $30 for the challenge test at Flinders Medical Centre. All tests and consultations related to the study will be conducted free of charge, including free local anaesthetics (called EMLA) for taking blood samples. Boiled nuts will also be supplied to you by Dr Tao. However, professional services provided by Dr Tao unrelated to this study will be bulk-billed as usual. In summary, there will be no out-of-pocket money for you to pay under any circumstances.

Contact

If you have any query about the results of the skin prick test, or indeed any other problem, please contact Dr Billy Tao (Work 83312711, Home 82788353, Mobile 0418802380).

Complaints

This study has been reviewed by the Southern Adelaide Flinders Clinical Human Research Ethics Committee. If you wish to discuss the study with someone not directly involved, in particular in relation to matters concerning policies, your rights as a participant, or should you wish to make a confidential complaint, you may contact the Executive Officer on 82046453 or by email at research.ethics@health.sa.gov.au.

Government of South Australia
SA Health

Flinders UNIVERSITY

SOUTHERN ADELAIDE HEALTH SERVICE
FLINDERS MEDICAL CENTRE

CONSENT BY A THIRD PARTY TO PARTICIPATION IN RESEARCH
(TO BE USED IN CONJUCTION WITH THE INFORMATION SHEET)

I, ... give consent

(first or given names) last name)

to ....`s involvement in the research

(first or given names) (last name)

"A pilot study for a new oral immunotherapy (desensitisation) treatment for nut allergies"

I acknowledge the nature, purpose and contemplated effects of the research project, especially as far as

they affect ....

(first or given names) (last name)

have been fully explained to my satisfaction by ....

(first or given names) (last name)

and my consent is given voluntarily.

I acknowledge that the details of the following have been explained to me:

1. The background and rationale for using a two-phase desensitisation program starting with boiled nuts
2. The potential benefit of this method is to reduce adverse event from treatment
3. The reason for completing the second stage with raw nuts is to achieve full protection at the end of desensitisation
4. The need for doing an oral nut challenge before the start of desensitisation, in order to prove that my child is truly allergic to the nut

5. Participation of the study also means that blood will be collected from my child on three occasions, and there will be a total of three skin prick tests performed on my child at about the same time as the blood tests.
6. The boiled and raw nuts will be supplied to me by Dr Tao
7. My child will eat the nuts in three separate daily doses during desensitisation.

I have understood and am satisfied with the explanations that I have been given.

I have been provided with a written information sheet.

I understand that ....'s involvement in this

(first or given names) (last name)

research project may not be of any direct benefit to him/her, and that I may withdraw my consent at any stage without affecting his/her rights or the responsibilities of the researchers in any respect.

I declare that I am over the age of 18 years.

I acknowledge that I have been informed that should he/she receive an injury as a result of taking part in this study, legal action may need to be taken to determine whether he/she should be paid.

Signature of parent, legal guardian or authorised person:

....

Date: ....

Relationship to participant: ....

I, ... have described to ....

the research project and nature and effects of procedure(s) involved. In my opinion he/she understands the explanation and has freely given his/her consent.

Signature: ....

Date: ....

Status in Project:..

BIPHASIC PEANUT ORAL IMMUNOTHERAPY PROTOCOL

PHASE 1 – 2-HOUR BOILED PEANUT (approximately 7 months):

WEEK NUMBER/ DAILY DOSE	BREAKFAST	AFTER SCHOOL	DINNER
1		**¼ boiled peanut***	
2		¼ boiled peanut	¼ boiled peanut
3	¼ boiled peanut	¼ boiled peanut	¼ boiled peanut
4	¼ boiled peanut	½ boiled peanut	¼ boiled peanut
5	¼ boiled peanut	½ boiled peanut	½ boiled peanut
6	**½ boiled peanut**	**½ boiled peanut**	**½ boiled peanut**
7	½ boiled peanut	1 boiled peanut	½ boiled peanut
8	½ boiled peanut	1 boiled peanut	1 boiled peanut
9	**1 boiled peanut**	**1 boiled peanut**	**1 boiled peanut**
10	1 boiled peanut	1½ boiled peanut	1 boiled peanut
11	1 boiled peanut	1½ boiled peanut	1½ boiled peanut
12	1½ boiled peanut	1½ boiled peanut	1½ boiled peanut
13	1½ boiled peanut	2 boiled peanuts	1½ boiled peanut
14	1½ boiled peanut	2 boiled peanuts	2 boiled peanuts
15	2 boiled peanuts	2 boiled peanuts	2 boiled peanuts
16	2½ boiled peanuts	2½ boiled peanuts	2½ boiled peanuts
17	3 boiled peanuts	3 boiled peanuts	3 boiled peanuts
18	**3 boiled peanuts**	**4 boiled peanuts**	**3 boiled peanuts**
18+	10 boiled peanuts per day	For a minimum of 2.5 more months	**End of Phase 1**

BIPHASIC PEANUT ORAL IMMUNOTHERAPY PROTOCOL

PHASE 2 – ROASTED PEANUT (approximately 5 months):

WEEK NUMBER/ DAILY DOSE	BREAKFAST	AFTER SCHOOL	DINNER
1		**¼ roasted peanut**	
2		¼ roasted peanut	¼ roasted peanut
3	¼ roasted peanut	¼ roasted peanut	¼ roasted peanut
4	¼ roasted peanut	½ roasted peanut	¼ roasted peanut
5	¼ roasted peanut	½ roasted peanut	½ roasted peanut
6	**½ roasted peanut**	**½ roasted peanut**	**½ roasted peanut**
7	½ roasted peanut	1 roasted peanut	½ roasted peanut
8	½ roasted peanut	1 roasted peanut	1 roasted peanut
9	**1 roasted peanut**	**1 roasted peanut**	**1 roasted peanut**
10	1 roasted peanut	1½ roasted peanut	1 roasted peanut
11	1 roasted peanut	1½ roasted peanut	1½ roasted peanut
12	1½ roasted peanut	1½ roasted peanut	1½ roasted peanut
13	1½ roasted peanut	2 roasted peanuts	1½ roasted peanut
14	1½ roasted peanut	2 roasted peanuts	2 roasted peanuts
15	2 roasted peanuts	2 roasted peanuts	2 roasted peanuts
16	2½ roasted peanuts	2½ roasted peanuts	2½ roasted peanuts
17	3 roasted peanuts	3 roasted peanuts	3 roasted peanuts
18	**3 roasted peanuts**	**4 roasted peanuts**	**3 roasted peanuts**
18+	10 roasted peanuts per day	Consult doctor on what to do next	**End of Phase 2**

Additional advice if your child is eating a boiled or roasted peanut for Oral Immunotherapy

1. You should try to adhere to the printed protocol as much as possible. If in doubt you can slow down the pace, but should never hasten it.

2. If your child has asthma that is not well controlled, do not start immunotherapy until it is fully treated to the satisfaction of both you and your child's doctor.

3. If your child is temporarily sick or unwell, stop giving him or her any nut (boiled or raw) until the sickness is completely resolved. If the recovery has taken only a few days you may resume safely at the same dose. However, if it is more than 7 days it will be safer to restart at half the amount of the previous dose, and only take one dose on the first day. If this is tolerated well, you may gradually work back to the original dose in a week or so, e.g. first increase the frequency to twice and then three times a day, and then doubling up the dose per serve until the original dose is achieved. If it is longer than a few weeks, it will be a good idea to consult Dr Tao and tailor-make a new schedule that suits your child.

4. Ring Dr Tao at any time if your child seems to have a reaction to the nut.

5. Also ring Dr Tao approximately one month after the start of desensitisation to report your child's progress, and tell him anything that may concern you.

6. When your child is eating either boiled or raw nuts, it is essential that he or she:

    - Does not eat the nut with an empty stomach. If you are not sure you should give him or her some food before eating the nuts.

    - Do not exercise at least one hour before or one hour after eating the nuts.

7. It is advisable that your child does not take aspirin or Nurofen during desensitisation. On the other hand, Panadol is safe to take.

8. When you child can eat 10 boiled nuts every day he or she will be ready for the start of Phase 2 in another 3½ months. Ring Dr Tao for an appointment, and while waiting for the appointment he or she must continue to eat the boiled nuts at full dose every day.

9. When your child has reached eating 10 raw nuts, which is the end of Phase 2, continue to eat the nuts every day until your next appointment with Dr Tao, who will then discuss with you a long term management plan.

The HYPES (Hypoallergenic Peanut Eaten Safely) Protocol:

(Desensitisation performed in three stages, peanuts eaten twice daily)

This is how the protocol works:

1. "**Three stages**" means eating 12-hour boiled peanuts for 12 weeks, then 2-hour boiled peanuts for 20 weeks, and finally roasted peanuts for 20 weeks, total 52 weeks (=12 months).

2. "**Twice daily**" because the peanuts are eaten twice a day: morning (at breakfast) and evening (at dinner), except in the first week of each stage, when peanuts are eaten only once a day.

3. The **starting dose** of each stage is always 1/16 of a peanut (whether 12-hour boiled, 2-hour boiled, or roasted).

4. The doses are **progressively increased every week** and follow a **simple pattern**. (note – the doses are given every day in the week, not once a week):

   First week 1/16
   Second week 1/16, 1/16
   Third week 1/16, 1/8
   Fourth week 1/8, 1/8
   Fifth week 1/8, 1/4
   Sixth week 1/4, 1/4
   Seventh week 1/4, ½ ….and so on.

5. To make things easy for participants in the **formal clinical trial**, these peanuts will be individually prepacked in clearly labelled containers and supplied to you regularly. You must hand back the used containers in order to exchange for the newly filled ones.

Date (Start of the week)	Week Number	A.M. Dose	P.M. Dose
Stage 1: 12-hour boiled peanut			
	1	None	1/16
	2	1/16	1/16
	3	1/16	1/8
	4	1/8	1/8
	5	1/8	1/4
	6	1/4	1/4
	7	1/4	1/2
	8	1/2	1/2
	9	1/2	1
	10	1	1
	11	1	2
	12	2	2

Stage 2: 2-hour boiled peanut			
	13	None	1/16
	14	1/16	1/16
	15	1/16	1/8
	16	1/8	1/8
	17	1/8	1/4
	18	1/4	1/4
	19	1/4	1/2
	20	1/2	1/2
	21	1/2	1
	22	1	1
	23	1	2
	24	2	2
	25	2	3
	26	3	3
	27	3	4
	28	4	4
	29	4	5
	30	5	5
	31	5	6
	32	6	6
Stage 3: Roasted peanut			
	33	None	1/16
	34	1/16	1/16
	35	1/16	1/8
	36	1/8	1/8
	37	1/8	1/4
	38	1/4	1/4
	39	1/4	1/2
	40	1/2	1/2
	41	1/2	1
	42	1	1
	43	1	2
	44	2	2
	45	2	3
	46	3	3
	47	3	4
	48	4	4
	49	4	5
	50	5	5
	51	5	6
	52	6	6

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