Epidemiology and Clinical Presentations of Seafood Allergy in the Asia Pacific

Thesis submitted by

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STATEMENT OF SOURCES

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given.

Thi Kieu Thu Le

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted in accordance with the National Statement on Ethical Conduct in Human Research (2007) - Updated 2018 and obtained the approval from the James Cook University Human Research Ethics Committee (HREC).

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"I hear and I forget. I see and I remember. I do and I understand."

-Confucious

ABSTRACT

Food allergy is defined as an adverse response of a human's immune system triggered by food antigens. Food allergy is not a new abnormal health phenomenon, but was observed and documented thousands of years ago. However, recently, food allergy has become a substantial and severe health concern in many populations worldwide with a dramatically increasing number of hospital admissions due to food-related allergic reactions and food-induced anaphylaxis. Substantial investigations have been conducted, mostly in developed countries in Europe, America, and Oceania, to estimate food allergy prevalence and define its possible negative impacts on population health. Despite enormous research efforts and advances in the field of food allergy, its pathogenesis and the disparities in the patterns of food allergens across regions are not fully understood. This thesis aims to investigate the prevalence of food allergy and its risk factors in Vietnam. Further investigations on the clinical presentations and immunological profiles of seafood allergic subjects in Vietnam and Australia were carried out in an effort to compare and identify crucial determinants of seafood allergy, which may enable the development of immunotherapy and improve allergy diagnosis.

A comprehensive review of the contemporary understanding and studies of food allergy worldwide is presented in **Chapter 1** of this thesis. The current advances in food allergy diagnosis and its pitfalls are discussed. An overview of seafood consumption and seafood safety, along with current gaps and needs in seafood allergy management in Vietnam, are also addressed.

Food allergy is reported to affect up to 10% of children and 5% of adults in the developed world, with this high prevalence often referred to as an emerging allergy epidemic. Given that these cases of food allergy are often assumed to be the consequence of an industrialized lifestyle, my research question is whether or not there has also been a food allergy epidemic in developing economies. Do people in other parts of the world suffer from allergy to the same type of foods as those already characterized in Western societies? These are the rationales for me to conduct the first population-based survey of food allergy in Vietnam; to evaluate the current situation of

food allergy in this developing country. A survey of Vietnamese preschool children is presented in **Chapter 2** and a survey of Vietnamese adults is provided in **Chapter 3** of this thesis. These studies estimate the frequency of doctor-diagnosed food allergy in preschool children and adults in Vietnam to be 6.7% and 4.6%, respectively. Vietnamese subpopulations have comparatively high incidence rates of food allergy compared to what has been reported in previous studies from Europe or America. This Vietnamese population showed a stark difference in the allergy-triggering food patterns exhibited, with adominance of crustacean, mollusk and fish allergy occurring in both children and adults. Allergy to beef was also identified; this being the first time this new food allergy type has been recognized in Asia. The variation in types of food allergy present was addressed across geographical regions in Vietnam. Food allergy associated risk factors were identified, underlying the interrelations of genetic and environmental determinants to food allergy incidence. These findings provide insights into the current food allergy situation in Vietnam and addresses the need for more effective allergy management initiatives in this country.

Food allergy studies remain limited in many parts of the world; thus, leading to a lack of food allergy management policies and medical readiness for appropriate interventions. This raises concerns about potential impacts of food allergy on population health. It is assumed that the paucity of food allergy epidemiologic data is due to the high financial costs of organizing conventional epidemiological studies of food allergy. **Chapter 4** of this thesis sought out and validated an alternative method for the traditional population-based survey, with the aid of internet tools. By comparing the study outcomes from two consistent and independent food allergy population-based surveys, using two different modes: paper-based and web-based surveys, we confirmed the applicability of web-based surveys as a reliable and low-cost alternative for future epidemiological studies, especially in developing countries.

Self-administered questionnaire surveys have been a major tool in estimating food allergy prevalence worldwide. A thorough clinical history is important in the diagnosis of food allergy, however misconceptions from the survey respondents regarding true food allergy and other types of food hyperactivity are likely. The discovery of Immunoglobulin E (IgE) antibody as the biomarker of type I food allergy subsequently plays a crucial role in the current diagnosis of food allergy. Acknowledging this

importance of applying multiple *in vitro* and *in vivo* diagnostics in food allergy, an investigation on the serum IgE reactivity among people with a history of seafood allergy to commonly consumed crustacean, mollusk and fish species was conducted. The analysis of the immunological profiles of people with seafood allergy in Vietnam is described in **Chapter 5** of this thesis. Generally, seafood allergic participants from Vietnam showed a diverse pattern of serum specific IgE reactivity to different crustacean and mollusk species. Multiple cross-reactivities between crustacean allergic patients and allergens from house dust mite, cockroach and mealworm were revealed. This finding once again confirmed the enormous contribution of environmental factors to the incidence of food allergy and was in line with the findings from the population-based surveys in the previous chapters.

Besides environmental factors, ethnicity and eating habits may play a role in developing a food allergy. The latter includes the availability of a food commodity in a region and local food preparation practices. A similar investigation on seafood allergy conducted in Australian adults was presented in **Chapter 6**. Participants were invited to an interview with food allergy specialists to collect clinical history. Participants' sera were collected and screened for the serum specific IgE reactivity in the laboratory to a panel of typical local crustacean, mollusk and fish species. In general, seafood allergic participants from Australia demonstrated diversified species-specific IgE reactivity to crustacean, mollusk and fish species. Prawns appeared to be the most allergenic crustacean. Mite exposure seems to be common among participants with a history of shellfish allergy. Shellfish allergic subjects reacted to fish allergens and vice versa. Besides tropomyosin, the contribution of other allergens is possible and needs further investigation.

Following on from the findings in the previous chapters, **Chapter 7** of this thesis focuses on the identification and characterization of putative crustacean allergens utilizing the participants from Vietnam and Australia. Crustacean protein extracts were separated by their molecular weight (SDS-PAGE), and immunoblotting techniques were applied to identify the participants' specific IgE recognition pattern to the different crustacean proteins. The protein bands that displayed IgE reactivity were cut out and digested with trypsin for the identification of the allergen by mass spectrometry (Bio21, Melbourne, Australia). The mass spectral interpretation of the proteins was performed

by uploading the data to Mascot (Matrix Science, London, UK). The crustacean allergen profiles among the two populations were compared and discussed.

By conducting a consistent investigation of seafood allergy in two distinct populations, the investigator was able to characterize and compare the phenotypes and allergen reactivity profiles between the two representative populations. This study provided evidence into the clinical characteristics of seafood allergy, giving crucial insights for the development of more reliable food allergy diagnostics for the local populations. All the outcomes, as well as future directions, are discussed in **Chapter 8**.

In summary, this thesis provides an extensive analysis of food allergy and seafood allergy in Vietnam. Seafood-including crustacean, mollusk, and fish-is the most common type of allergy-triggering food in Vietnam. Regarding allergy risk factors, both child and adult participants with a family history of food allergy were significantly more prone to developing food allergy. The study demonstrated and compared distinct species-specific IgE binding patterns among seafood allergic patients in Vietnam and Australia. The cross-reactivity of seafood allergic participants to insect and indoor allergens was revealed. The findings from this thesis provide an important contribution towards the current gaps and needs in the national-scale management of food allergy in Vietnam, and initiate the development of advanced, more precise diagnosis of seafood allergy, not just in Vietnam but also in Asia-Pacific regions in general. Several directions for future work involve following-up investigations on other major food allergies that were identified from the population-based survey (i.e. red meat allergy), investigating food allergy prevalence in other subpopulations (e.g. infants, adolescents) in Vietnam to determine the overall food allergy frequency and the variation of food allergy over the life course. The clinical and immunological data from the seafood allergy study in this thesis is paving the groundwork for the future development of region-specific *in vitro* diagnostic tests.

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LIST OF ABBREVIATIONS

AK – Arginine kinase

APC – Antigen-presenting cell

BAT – Basophil activation test

CI – Confidence Interval

DC – Dendritic cell

EAACI - European Academy of Allergy and Clinical Immunology

FA – Food Allergy

FAO – Food and Agriculture Organization

HC – Hemocyanin

HDM – House dust mite

IgE – Immunoglobulin E

IQR – Interquartile range

IUIS – International Union of Immunological Societies

OAS – Oral allergy syndrome

OFC – Oral food challenge

OR – Odd ratio

PBS – Paper-based survey

PV – Parvablumin

SCP – Sarcoplasmic calcium-binding proteins

SDS – PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SPT – Skin prick test

TM – Tropomyosin

WBS – Web-based survey

WHO – World Health Organization

CHAPTER 1. AN OVERVIEW OF FOOD ALLERGY AND CURRENT SITUATION OF SEAFOOD CONSUMPTION AND SEAFOOD ALLERGY IN VIETNAM

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1.1 Introduction

Food allergy is defined as an abnormal immune response to food proteins and the prevalence of food allergy is on the rise in many populations worldwide. It is estimated that up to 10% of children and 5% of adults in the developed world are suffering from some type of food allergy (1). The eight food groups that account for 90% of food allergic reactions are milk, egg, peanut, tree nut, soy bean, wheat, shellfish and fish.

The study of seafood allergy has become a priority with the rise in the prevalence of seafood allergy across the world (2). Generally, high consumption of seafood is associated with a higher prevalence of seafood allergy (3). Allergic reactions to seafood are directly linked to allergenic proteins in the different species (4). Among these allergenic proteins is the major fish allergen parvalbumin, and the major shellfish allergen tropomyosin (5).

Hypersensitivity to seafood is reported in both children and adults (2). The symptoms are often lifelong and cross-reactivity to different seafood species are often evident. The immunological mechanism of this disease is highlighted by the production of specific immunoglobulin E (IgE) antibodies, generated against the allergenic proteins. Other toxic and non-toxic components in seafood can also trigger immunologic disorders such fish parasites which may be present in the fish as an infection for example Anisakis (6). This makes the study of seafood allergy more complex.

Vietnam is a small country in Southeast Asia. Nevertheless, Vietnam is the main seafood producer for the world's market, with more than 4 million workers directly involved in this industry (7). Seafood is preferred by most local people as an affordable and easily available food source. The Vietnamese seafood consumption per capita per year is much higher than the world's average: 33 kg as compared to 21 kg (8). However, little is known about the status of seafood allergy in this population. It is hypothesized that allergic disorders caused by seafood are a significant public health concern in Vietnam, especially among seafood processing workers.

Thus, this chapter aims to provide an overview of the food allergy epidemic and its aetiology, especially type I (IgE-mediated) food allergies. Foundation knowledge and updated scientific findings of seafood allergy will be summarized for later studies in this thesis.

1.2 Overview of food allergy

1.2.1 Definition of food allergy

Humans consume food to provide the body with nutrients and energy to sustain life. With time, the food we eat and the way we prepare and cook food have significantly changed. The food consumed is intact and well-tolerated by most individuals. However, certain food groups might contain components that could induce undesirable adverse reactions in sensitive individuals. Hypersensitive reactions to food have been documented with a broad spectrum of clinical presentations from mild skin reactions to life-threatening events (9). According to the World Allergy Organization/European Academy of Allergy and Clinical Immunology (WHO/EAACI), food allergy is defined as an abnormal immunologic disorder triggered by food components (10). If the allergic responses have the participation of specific Immunoglobulin E - IgE, it is called IgE-mediated food allergy or type I IgE-mediated hypersensitivity.

1.2.2 Food allergen

Allergen is a general term used for any substance that could trigger an allergic response (11). Allergens can be categorized by their origins such as environmental allergens (e.g., indoor allergen, pollen allergen) and plant and animal food allergens. Most food allergens are proteins, often containing carbohydrate side chains (12). To date, eight food groups – the 'Big Eight' – contain allergens and are responsible for most allergic disorders include cow's milk, soya, egg, tree nuts, fish, shellfish, wheat and legumes (13).

Food allergens are distributed across different food sources and conveniently categorized under several protein families. The majority of protein families of the plant food allergens are in the Cupin superfamily, the Prolamin superfamily (soybean), Bet v 1-related protein and Profilin (14), while those of animal source are casein, tropomyosin and EF-hand proteins (15). The classification of allergens into protein families facilitates the study of their allergenicity and the prediction of the cross-reactivity between different food commodities.

Majority of known food allergens are proteins with a molecular weight range from 10 to 100 kDa. They often feature complex structure, heat stability and even proteolysis resistance (16). Lipid transfer protein in maize allergen maintained their IgE binding

capacity after heat treatment (17). *In vitro* test in birch pollen allergen Bet v 1, under the presence of trypsin and pepsin, showed that gastrointestinal enzymes demolished its histamine-releasing capacity but not T cell–activating property (18). The advanced processing practices like high pressure or pulsed electric field failed to induce any significant effects on the secondary structure of allergens in peanut and apple (19).

There are several key factors attributed to the allergenicity of a food component. First, allergic food proteins often have an amino acid sequence identity of less than 62% to human homologs (20). This means that the more “foreign” a food protein is to human proteins, the more likely it could trigger an allergic reaction. Secondly, the stability of the protein under the preparation/cooking practices or hostile conditions in the host’s gastrointestinal tract. Apart from that, the allergenicity of a food protein also depends on intrinsic factors such as the specific molecular properties of a protein, the number of its isoform (21), the expression level of the allergen (22) and the concentration of the allergen in food (23).

Currently, to assess whether a food component is an allergen, it is prerequisite that the investigated component could elicit immunological responses in allergic individuals (24). Registered allergens are named and listed in the WHO/IUIS Allergen Nomenclature Sub-committee official website www.allergen.org.

1.2.3 Routes of exposure

A human might expose to food allergens via three main pathways: ingestion, inhalation and skin contact. The gastrointestinal tract is considered the most common entrance for food allergen exposure. However, people might contact or inhale food allergens unconsciously, especially in the occupational setting. It is not known whether the route of exposure contributes to the potency of a food allergen.

1.2.3.1 Ingestion

Via the ingestion process, food allergens are exposed to different tissues/organs along the alimentary tracts. Immediate adverse reactions can be recognized around the local oral cavity (itching and/or swelling of the mouth, lip) within a few minutes after

consuming the offending food. Oral itching or oral pruritus manifesting in oral allergy syndrome (pollen-food-related syndrome) is frequently reported among subjects with fruit allergy (25).

In the gastrointestinal tract, digestive enzymes (i.e. pepsin, trypsin) break down food proteins into smaller fragments. The physical and biochemical activities in the stomach may alter the structure of food proteins and lead to the change of their allergenicity (26-28). Yet, most food allergens are known to be stable under the gastrointestinal digestion impact (27, 29). The alimental tracts themselves have complex barriers to prevent the invasion of harmful substances from the gut lumen penetrating into the circulation, such as gut epithelial cells, innate immunity (natural killer cells, macrophages, and toll-like receptors) or acquired immunity (specific IgA and cytokines) (16). The gut microbiota seems to play a key role in modulating of the manifestation of food allergy in children and adults (30, 31). The association between the gut microbiome and the development of food allergy has aroused the great interest of researchers worldwide (32, 33).

1.2.3.2 Inhalation

Food sensitization can be triggered by long-term exposure against aerosolized food proteins from the environment (34). Reported clinical symptoms occur in respiratory tracts such as nasal (rhinorrhea, sneezing, and nasal congestion), ocular (tearing, redness, and irritation), or lower respiratory (cough and wheeze) (35, 36). Cooking practices such as boiling, steaming or frying transform food allergens into the aerosols and circulate in the air (37). The air samples from the investigated seafood processing plants contained high levels of allergic proteins than the normal one (38) or soy processing sites were reported to present high levels of airborne soybean hull proteins (39). These are the reasons to provoke occupational allergy in food industry workers (36, 40). It is estimated that the prolonged inhalation of dust particles in the working setting accounted up to 25% of occupational rhinitis and/or occupational asthma (41).

Inhalation of food allergens could occur everywhere. Air travel passenger elicited allergic responses due to the presence of peanut in the aircraft's air filter system (42). Children developed specific IgE responses to allergens from cooking fume at home

(43). Food allergy via inhalation is thought to associate with occupational asthma and food allergy asthma (37, 44, 45). Co-exiting food allergy and asthma increases the risk of severe asthmatic episodes and may put the patient into the life-threatening condition (46).

1.2.3.3 Cutaneous

Sensitization to food allergens may occur through the skin, the primary barrier of the immune system. The immunological function of the skin is contributed by the Langerhans cell network comprising of antigen-presenting cells (APCs) distributed throughout the body (47). Sensitization to food allergens via skin exposure is frequently seen in infants, young children (48) and occupational group (49). As a result of the sensitivity of the skin barrier to food antigens, skin prick tests (SPT) and patch test have been applied widely in food allergy diagnosis (50).

1.2.4 Mechanism of food allergy

Immunologic mechanism of IgE-mediated food allergy involves different functional cells and organs of the immune system. First, allergens are recognized by APCs such as macrophages and dendritic cells (DCs). APCs then process and present the antigen peptides on the APCs' surface (51). Once, the major histocompatibility complex (MHC) molecules of T cells recognize and bind to these antigenic determinants (epitopes), T cells will stimulate B cells to produce allergen-specific IgE antibodies by secreting chemical signals including IL-4, IL-10, and IL-13 (Figure 1.1). This phase occurs typically silently, without any specific symptoms.

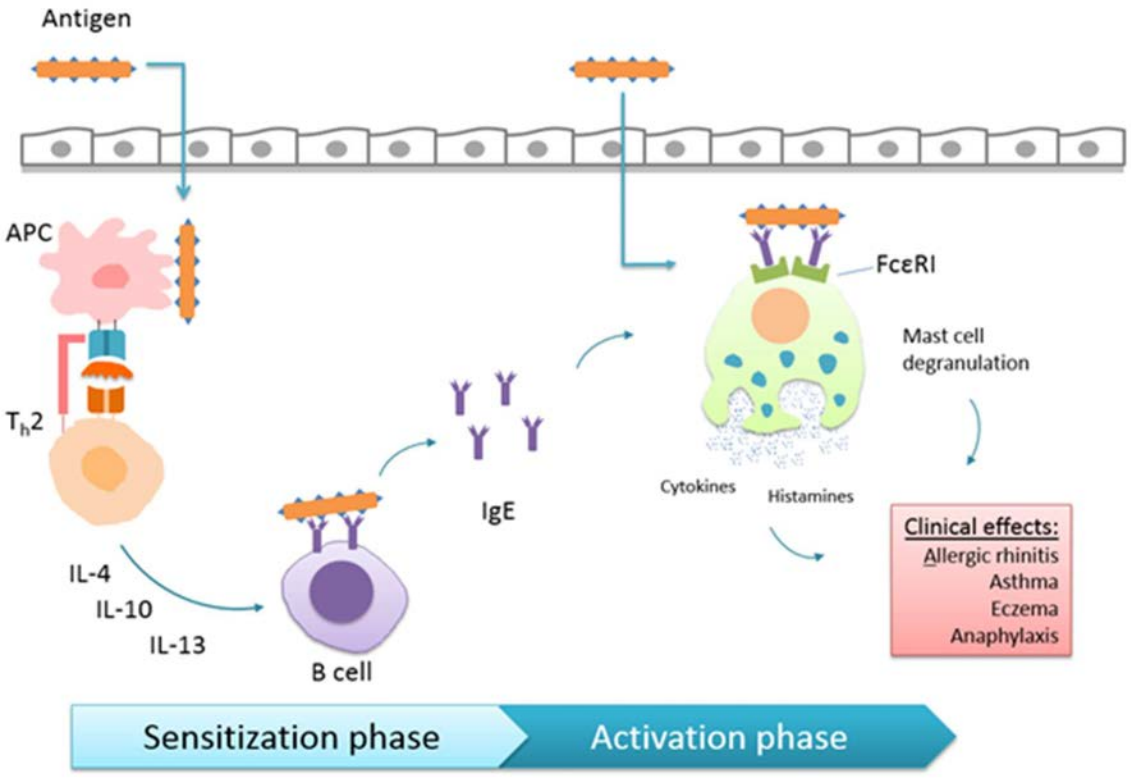

Figure 1.1 The overview of the induction and effector mechanisms of type I food allergy.

In the secondary and subsequent exposure to the same allergen, the immunogenic response occurs much faster and stronger due to the pre-existing allergen-specific IgEs distributed around the body. These antibodies bind to high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils or might be found as free antibodies in the bloodstream. The cross-link of the allergen to receptor-bound IgE antibodies activate mast cells/basophils and lead to the secretion of in-cell granules and mediators such as histamine, prostaglandins, and leukotrienes. These biological compounds contribute to the clinical manifestations of food allergy in the affected individuals.

1.2.5 Clinical presentations of food allergy

Food allergy has a broad spectrum of clinical presentations affecting multiple organs. Food allergic symptoms might be attributed to the type of food allergens and the exposure types. However, clinical manifestation of food allergy may occur through any

route of contact. This section summarizes and discusses common clinical symptoms of food allergy by the affected organs.

1.2.5.1 Local oral reactions

Food allergy reactions can be identified easily with typical symptoms at local oral and orbital such as mouth and lips swelling, mouth and tongue itching, eye itching, redness and watering (52). IgE-mediated food allergy often has quick onset in which the adverse symptoms occur within two hours after the food ingestion. Oral allergy syndrome (OAS) is the common term referring to a typical allergic syndrome characterized by symptoms in the oropharyngeal mucosa. OAS results from the cross-reactivity of allergic plant proteins to pollen proteins in the environment and often happens in season (25). Recently, OAS is used to describe the clinical symptoms of individuals with mite allergy cross-react to the pan-allergen tropomyosin in shellfish (53).

1.2.5.2 Gastrointestinal symptoms

Food allergens that come in contact with the gastrointestinal mucosa can provoke localized inflammation expressed by an array of symptoms: nausea, vomiting, abdominal cramping, and diarrhea (54). The upper gastrointestinal tract can provoke immunological manifestations within several minutes up to two hours of food ingestion while the onset at the lower gastrointestinal organs might take longer to occur. Noticeably, gastrointestinal symptoms due to type 1 food allergy might be misleading with other non-immunological food allergy problems or vice versa (55), especially among subjects with delayed onset (56); thus, it complicates the accurate diagnosis of food allergy.

1.2.5.3 Dermatological symptoms

Cutaneous manifestations including hives/urticaria and angioedema are the most common symptoms caused by immediate food allergy responses (54). Acute urticaria was found in 80-90% of subjects in workplace settings who frequently come in contact with food allergens (49). Food allergens with implications for atopic dermatitis in

children are cow's milk, hen's egg, peanut, wheat, soy, nuts, and fish (57). Skin problems were found to be the most common implicated food allergy symptoms from population-based surveys (48, 58).

1.2.5.4 Respiratory symptoms

Food allergy triggered by the inhalation of food allergens can provoke inflammatory reactions in the upper and lower airways. The symptoms range from mild to severe reactions including nasal itching, nasal obstruction, sneezing, wheezing to asthma (52). Food allergy asthma is an atopic disorder characterized by episodes of reversible airway narrowing, bronchial hyper-responsiveness and chronic pulmonary inflammation (59). The symptoms of an asthma attack can be mild coughing, and wheezing to more severe such as shortness of breath, chest tightness and rapid heart rate (59). Children with food allergy in infancy are more likely to develop asthma at a later age (60, 61). All food allergens in the 'Big Eight' could trigger a food allergy asthma in sensitized subjects (62).

1.2.5.5 Anaphylaxis

According to the World Allergy Organization, anaphylaxis is defined as 'a severe, life-threatening generalized or systemic hypersensitivity reaction' (10). The exact term 'IgE-mediated allergic anaphylaxis' is used when the reaction is caused by an immunological mechanism. Anaphylaxis might include one or a combination of the following symptoms: vomiting, nausea, rapidly progressing urticarial, respiratory distress, vascular collapse, systemic shock and possibly leading to death (63).

Anaphylaxis due to food allergens is more common but varies with each food triggers and the age groups. Milk and egg are the most frequently reported food-induced anaphylaxis in children, whereas in adults, peanut, nuts, fish, fruits, and shellfish are the main offenders (64-66).

The accountability for inducing a food allergy anaphylaxis is used to assess the allergenicity of a food allergen. The current estimation of the anaphylaxis rate in the developed countries was 0.14 to 0.21 per 100 person-years (66). Whereas, little is

known about this rate in the developing world. A higher anaphylaxis rate was described in young children as compared to adults (67). There has been a steady increase in the hospital admission rates due to food anaphylaxis over the last decades in many developed countries (68, 69), raising an enormous concern about the safety of food consumption and the emerging impact of food allergy to the quality of life of food allergy sufferers.

1.3 Diagnosis of food allergy

1.3.1 Self-reported clinical presentations and family history

Clinical manifestations are primary cues for the diagnosis of food allergy. Affected subjects might start noticing clinical symptoms that persistently occur when they expose to the same suspected food. Self-reported clinical history is an important source of information for the food allergy diagnosis. These data can help the physician/clinician to narrow down suspected food allergens and support in selecting appropriate diagnostic tests. Symptom onset is another crucial clue to pinpoint the type of food hypersensitivity. Further details of health status (e.g., atopic conditions) of the subjects and their family history of food allergy are of benefit to the diagnosis. Currently, many westernized countries conducted national surveys on food allergy based on the self-administrated questionnaires (58, 70, 71). However, with the advance of food allergy diagnosis, it is recommended that the self-reported food allergy data need to be confirmed by *in vivo* and *in vitro* tests to minimize misdiagnosis (1).

1.3.2 In vivo tests

1.3.2.1 Skin tests

SPT has been used widely as a primary predictor for IgE-mediated hypersensitivity (72, 73). The test is designed to test for the interaction of cutaneous effector cells (i.e., mast cells and basophils) with the suspected allergens. If positive, histamine and other mediators will be released and lead to the presentation of a visible weal and flare reaction peaking. The test is conducted on the patient's back or volar aspect of the forearm. Histamine and saline are used as positive and negative controls, respectively. A positive SPT result is commonly defined as a weal ≥ 3 mm diameter (12). The advantages of this method are the rapid outcome delivery and the rich variety of

commercial allergen extracts and SPT devices in the market (74, 75). The test has been regarded as a safe procedure with minor undesired adverse effects (73). The drawbacks are the inconsistence of test result due to technical issues in measuring the weal diameter or interpreting the test results or the variability of test reagents (52). SPT can be applied to test for allergen extracts or allergen components from numerous food commodities (72). The biggest concern of SPT remains in its testing reagent panels. The variation of protein concentrations and allergen concentrations among different commercial SPT reagents to a specific food/allergen has been demonstrated (76). Further SPT guidelines and recommendations are available from the EAACI position paper (50).

Other skin tests include intradermal skin test, prick to prick test and atopic patch test (77). They are occasionally used in the clinical settings for food allergy diagnosis but less common than SPT. The prick to prick test appears to be less safe due to numerous anaphylaxis incidences reported after the test (78, 79).

1.3.2.2 Oral food challenge

Further to the SPT, investigation of food allergy can be done by using oral food challenge (OFC) test. There are three types of challenge test: open, single-blind placebo-controlled (SBPCFC) or double-blind placebo-controlled food challenge (DBPCFC). As its name suggests, in the open food challenge, both the physician and patients know of food being tested and its dose. In the SBPCFC, the patient is not aware of the food being tested but the physician. Finally, with the DBPCFC, neither the physician nor the patient knows of the being tested food. DBPCFC is considered as the gold standard for food allergy diagnosis as it minimizes possible bias and provides valuable and accurate data (80).

OFC provides an accurate diagnosis of food allergy but requires elaborate preparation and attention. The detailed protocol of the food challenge test is mentioned elsewhere (81). Normally, the test is prescribed after reviewing self-reported clinical history or/and skin test result or/and blood test result. The implementation of OFC requires carefully prepared for the worst situation (anaphylaxis). Only trained health professionals can conduct this test and often in the clinic settings with high readiness of emergency

procedures. The DBPCFC is often suggested when the blood test and SPT come up with negative results (82). Patients prior to the OFC is required to avoid suspected foods and any antihistamine medication at least two weeks. The protocol for the food challenge test can be obtained from the EAACI guidelines (81).

1.3.3 In vitro tests

Food allergy can be diagnosed by applying numerous *in vitro* assays for the qualitative and quantitative determination of the allergen-specific IgE (sIgE) in human blood sera. sIgE measurement demonstrates the sensitization to offending foods. Theoretically, allergic subjects present a higher level of allergen-specific IgE antibodies than the tolerant group (83). Within the allergic group, sIgE level might fluctuate according to their demographics (e.g., age, ethnicity, atopic conditions). Due to the close correlation between serum IgE and cell-bound IgE (84, 85), immunoassays can be designed to determine the level of sIgE to allergen extracts, allergen components (purified natural allergen or recombinant allergen) or even to allergen peptides to predict the sensitization. In the laboratory settings, further sophisticated examinations such as T-cell assays can be employed for research purposes (86).

Several commercial immunochemical assays are currently available such as ImmunoCAP® test kits developed by Phadia (ImmunoCAP Rapid, ImmunoCAP ISAC multiplexing) or PROTIA™ Allergy-Q® (87) or multiple allergens simultaneous test chemiluminescent assay® (MAST CLA) (88). These test kits are limited to certain areas, mostly in Europe and North America.

Basophil activation test (BAT) and mast cell activation test (MAT) are the two diagnostic options but more frequently applied in research. Mast cells and basophils play a dominant role as the effector cells in the immediate hypersensitivity. Thus, these cells can be applied as the target cells for the *in vitro* assays to detect the immediate sensitization. Santos et al. (89) reported the successful application of BAT to differentiate between peanut allergy and peanut tolerant group, whilst Bahri et al. (90) suggested MAT as a robust tool with the superior discrimination performance compared with existing allergy diagnostics in peanut allergy diagnosis. Reviews on the protocols and the effects these methods are described elsewhere (91, 92).

In general, there are multiple tests that can be applied to diagnose a food allergy. The review of patients' clinical history and the skin tests should be the first-line approach to screen for suspected triggers. Serum sIgE quantification can be performed to confirm the clinical relevance and elucidate the likelihood of cross-reactivity. OFC is the option when there is a disagreement among diagnostic tests (93).

The sIgE test and SPT are frequently performed in the clinical setting to confirm the sensitization status to suspected food triggers. However, neither SPT nor sIgE tests are sufficient to diagnose FA on their own. It is essential to address the limitations of these methods, especially when the test outcomes are contradictive. For instance, when employing SPT and sIgE test in the diagnostic evaluation of suspected cow's milk and hen's egg allergy in 395 children in Germany, Mehl et al., (94) reported a low concordance between the two tests on an individual basis. In another investigation among 137 French young children, the SPT results and sIgE tests indicated a poor agreement to 13 aeroallergens and five food allergens being investigated (95). SPT seemed to be more sensitive, quicker and simpler than sIgE test (96). However, the sensitivity and specificity of both tests depend largely on the defined cut-off values of the sIgE level and the skin test weal size, and can therefore vary between studies.

In general, a food allergy diagnostic routine should begin with the review of patients' clinical history to primarily determine the cause and/or nature of the disorders. Simple tests like SPT or sIgE measurement can be applied to screen for suspected triggers. However, it is important to take into consideration the limitations of these tests, especially when the test results are different; OFC is therefore still the gold standard to diagnose food allergies.

1.4 Prevalence of food allergy worldwide

Food allergy is an emerging public health concern in many industrialized countries. Numerous studies have been conducted in Europe, North America and Australia with the efforts to evaluate the accurate prevalence of food allergy as well as its impact on population health. It is estimated that food allergy affects up to 5% of adults and 10% of children worldwide (97). However, due to the complex nature of the disease and somewhat the lack of a well-designed guideline and protocol for food allergy surveys,

the current epidemiological food allergy data remain a considerable debate. Only 15/193 countries have population-based data on food allergy derived from OFC (98). The overview of the challenge-proven food allergy prevalence in young children is presented in Figure 1.2. At the moment, Australian children present the highest food allergy prevalence in the world (10%) (99).

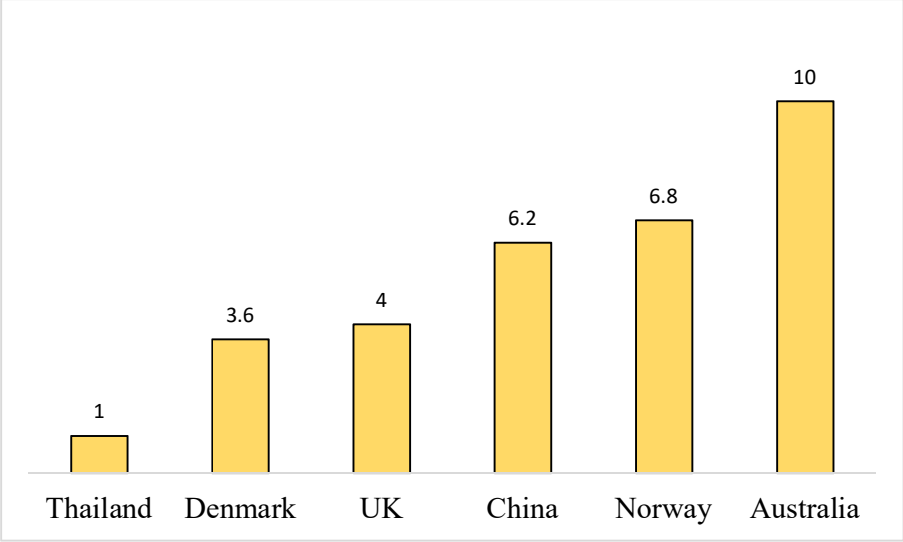

Figure 1.2 Food allergy prevalence defined by oral food challenge in children less than 5 years (%).

When stratifying into a food group, the food allergy frequency varies significantly across geographic regions. Eggs, cow's milk, and peanut are the most common triggers for food allergic reactions (52), in which peanut is the leading cause for food-induced anaphylaxis (100). In the US, the top four frequent food allergens are egg, seafood, milk and peanut (101), while those in Canada are peanut, fish, shellfish, and sesame (80). Some communities have their unique food allergen patterns such as the common of fruit allergy in Europe (102) or the bird's nest allergy in East Asian (103). The distribution of the food allergy prevalence in European countries by eight food allergens from two survey methods: self-reported and oral food challenge is described in Figure 1.3 (104).

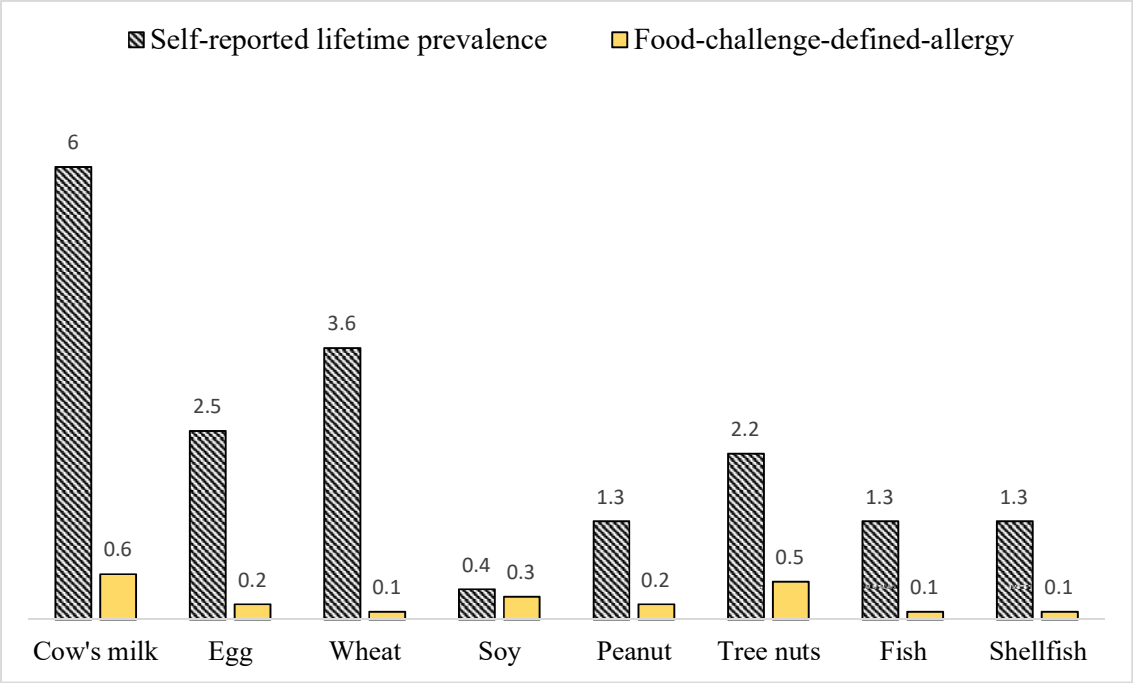

Figure 1.3 The prevalence of food allergy in Europe derived from different study designs (%).

The association of food allergy prevalence with certain age groups has been revealed in many studies. In general, children are more likely to develop food allergy than adults. Mailhol, Giordano-Labadie (105) demonstrated that young infants are more vulnerable to food allergens than the older groups. Similar findings were found in a randomized telephone survey among ten European nations (102). An example of the variation of food allergy prevalence among children and adults within a region (i.e. Canada) is presented in Figure 1.4 (11). As we can see that peanut and tree nuts are the most frequent allergy-inducing foods in children but not in adults. Most of the Canadian adults reported allergic reactions to fish and shellfish and have lower rates of peanut and tree nut allergy. Children may outgrow certain food allergies after their childhood such as milk and egg allergy; thus, this might contribute to the decrease of this food allergy prevalence in the older population (106). Besides, there are some types of food allergy that might have an adult-onset (107) or the sensitization to food allergen may be developed after a long time exposure to allergens from a working environment (108).

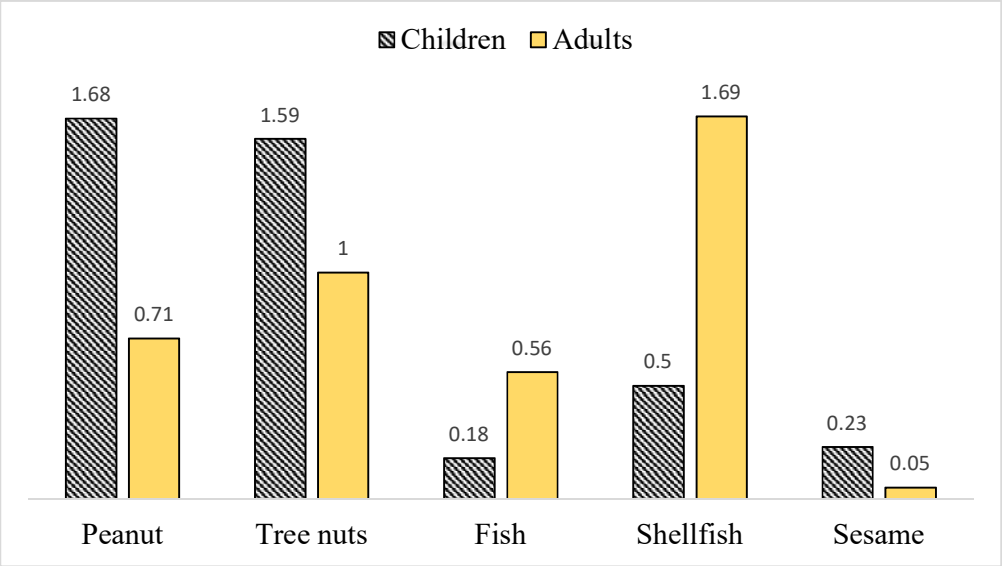

Figure 1.4 The comparison of food allergy prevalence by food allergens among children and adults in Canada (%).

1.5 Introduction to seafood allergy

Seafood is a highly nutritious food commodity with many health beneficial properties. Seafood is currently trading worldwide, and the world's consumption has been increasing over the last decades. The study of seafood allergy has become a priority with the rise of seafood allergy occurrence. Generally, fish and shellfish allergy affect about 0.3% and 0.6% of the world's population, respectively (2).

Hypersensitivity to seafood can be found in children and adults. The symptoms are lifelong and the cross-reactivity to different seafood species are often evident. Several seafood species including prawn and crab are known as the leading causes for the food-induced anaphylaxis (3). The IgE-mediated seafood allergy, a subclass of food hypersensitivity regulated by the production of specific IgE antibodies to allergic seafood proteins (3).

1.5.1 What is seafood?

Seafood is a general term used to name all creatures living in seawater that is used for human consumption. Seafood can also be subcategorized into fish and shellfish.

The latter includes mollusks and crustaceans. Nowadays, substantial seafood species have been introduced into intensive aquaculture production to meet the increasing demand of a growing world's population. The term "seafood" also includes other aquatic life such as freshwater fish or brackish-water fish. Figure 1.5 illustrates the classification of seafood and the species used in the following-up studies in this thesis.

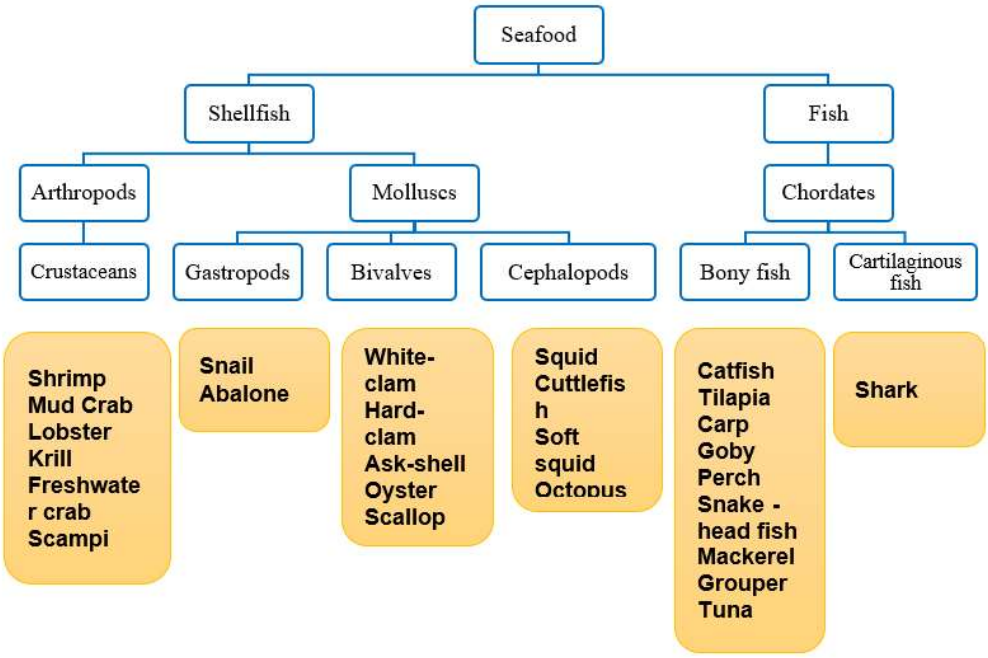

Figure 1.5 Classification of seafood and the most common seafood species in Vietnam.

1.5.2 The prevalence of seafood allergy

It is estimated that up to 2.5% of the general population suffering from adverse reactions to seafood (97). Seafood allergy incidence varies substantially across regions and is more frequently found in adults than in children (2). The seafood allergy epidemiological data seems to vary dramatically by the survey methods with a much higher rate in the self-reported data than the doctor-confirmed one. Thus, the comparison between different studies may be inappropriate if a different survey tool and method was applied. The epidemiological seafood allergy data worldwide are summarized in Table 1.1.

Fish and shellfish allergy appear to dominate in regions where seafood contributes as a staple food (109). The highest rates of the self-reported fish allergy were 5% of Finnish preschool-children (110) and 2.29 % (95% CI, 2.02-2.56) of young Filipino (111). The highest shellfish allergy was found at 5.5% (95% CI, 4.3-7.1) among French children (5-17-year-old). In the adult group, the highest fish allergy prevalence was reported in the US (2.04%, 95% CI, 1.7-2.38) (112) and up to 9.0% (95% CI, 6.7-11.9) among the American adults (2).

However, the population-based surveys that employed confirmed allergy diagnostic tests indicated much lower rates of fish and shellfish allergy. Confirmed fish allergy rate was 0.7 % (95% CI, 0.5-1.2) in 4-year-old group in Sweden (113) and 0.6% (95% CI, 0.3-1.3) among 2- to 6-year-old German children (114). The highest fish sensitization rate in adults was reported at 0.8% (95% CI, 0.2-2.5) (115) whilst crustacean allergy was reported at 0.2-0.3 % among Italian adults confirmed with SPT (116). The food allergy occurrence varied from 0.0 to 0.3 % to shrimp, crab, and fish in children and adults from Europe and Southeast Asia when confirmed by OFC (2, 117). Mollusk allergy was more common in Southern Europe and Asia (2, 118), but its prevalence was less common when confirmed with available *in vitro* and *in vivo* tests (119, 120).

Table 1.1 The prevalence of seafood allergy worldwide

Country	Year of study	Age(years)	Population (n)	Shellfish (%)	Fish (%)	Mollusk (%)	Methodology	Reference
Asia								
Singapore	2007-2008	14-16	9,570	-	0.26		Convincing history	(111)
	2007-2008	4-6	4,115	1.19			Convincing history	(121)
	2007-2008	14-16	6,342	5.23			Convincing history	(121)
Thailand	2007-2008	14-16	2,536	-	0.29		Convincing history	(111)
	2010	3-7	452	0.88	0.22		Self-response, SPT, OFC	(122)
	2005	3 mon - 6	656	0.30			Self-response, SPT, OFC	(123)
Philippines	2007-2008	14-16	13,989	-	2.29		Convincing history	(111)

	2007-2008	14-16	11,158	5.12			Convincing history	(121)
Taiwan	2004	<3	813	1.1	0.49		Convincing history, SPT, IgE	(118)
	2004	4-18	15,169	7.71	1.49		Convincing history, SPT, IgE	(118)
	2004	>19	14,036	7.05	1.17		Convincing history, SPT, IgE	(118)
Japan	2001-2002	0-80	3,882	6.2	4.4		Self-response questionnaires	(124)
	2004-2007	0-6	101,322	0.14	0.09		Food avoidance	(103)
Hong Kong	2006-2007	2-7	3,677	0.90	0.25		Self-administered questionnaire, doctor-diagnosed	(125)
	2005-2006	0-14	7,393	1.80	0.19		Face-to-face interview, self-	(54)

							administered questionnaires	
China	2009-2010	0-2	1,604	0.17-0.42	0.17-0.21		Questionnaires, SPT, Food elimination, oral food challenge	(126)
America								
US	2004	0-17	2,707	0.66	0.22		Convincing history or doctor diagnosed	(127)
US	2007-2010	adults	20,686	2.04	0.46	-	Self-reported	(112)
		children	20,686	0.87	0.43	-	Self-reported	(112)
Canada	2008-2009	>18	9,667	0.71	0.12%		Convincing history, SPT/IgE	(128)
Canada	2008-2009	0-18	9,667	0.06	0.0%		Convincing history, SPT/IgE	(128)

Europe								
Portugal	2004	>39 years old	659	0.5%	0.9%	-	Self-reported	(129)
Denmark	-	>18	843	0.3% (shrimp)	0.2% (codfish)	-	Questionnaire, SPT, Histamine release test, sIgE, OFC	(116)
Denmark	-	3 years old	486	0.0%	-	-	Questionnaire, SPT, Histamine release test, sIgE, OFC	(116)
Denmark	-	22 years old	1,272	0.2% (shrimp)	0.1% (cod fish)	0.1% (octopus)	Questionnaire, SPT, Histamine release test, sIgE, OFC	(130)
Finland	2001-2006	0-4 years old	3,899		5%		Self-reported, physician diagnosis, SPT, sIgE, OFC	(110)

UK	-	11-15	1,532	0.1%	1.3%	-	Report, SPT, DBPCFC	(131)
UK	-	8 years old	1,029		0.5%		Self-reported, SPT, sIgE, OFC, DBPCFC	(132)
France	2000	9-11 years old	7,781	-	0.7%	-	Self-reported, SPT	(133)
France	-	2-14 years old	2,716	1.5%	-	-	Self-reported	(134)
Turkey	2008	>18 years old	17,064	-	0.0%	-	Self-reported, SPT, sIgE, DBPCFC	(135)
Turkey		6-9 years old	2,739		0.0%		Self-reported, SPT, sIgE, DBPCFC	(136)
Iceland		1 year old	1,341	0.1%	0.2%		Self-reported, SPT, sIgE, DBPCFC	(137)

Greenland		5-18 years old	1,068	-	0.7%		sIgE	(138)
Sweden		13-21 years old	1,488		1.0%		Self-reported	(139)
Sweden	1999-2000	4 years old	2,563		0.7%		Self-report, sIgE	(113)
Germany	-	6 years old	1,082		0.6%		Self-report, sIgE	(114)

1.5.3 Seafood allergen

Of all 870 allergens which are registered in the systematic allergen nomenclature of the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (www.allergen.org), there are 92 food allergens, and 50 seafood allergens identified. The major allergen in fish is parvalbumin, whilst tropomyosin is predominant in most shellfish species.

Most seafood allergens are proteins with the molecular weight range of 8-100 kDa. They are normally present in complex conformations of secondary and tertiary structures and multiple isoforms (140). Some seafood proteins are heat-sensitive; thus, they might be broken into smaller fragments or downgraded into a lower structure from heat treatments. Besides, a seafood allergen may have multiple IgE binding epitopes, the regions in the protein structure that IgE antibodies could recognize and bind to. These epitopes can be in linear (sequential) or the conformational (discontinuous) form. For example, the allergen arginine kinase from blue swimmer crab has four conformational epitopes (141). The investigation on the number of IgE binding epitopes and their structure of a protein could help to predict its allergenicity (142), assess the cross-reactivity likelihood and advance the development of the accurate immunotherapies in seafood allergy management.

1.5.4 Fish allergen

Fish proteins that are responsible for mounting an adverse immune reactions in humans include parvalbumin (143), enolases and aldolases (144), fish collagen (143), beta-prime-component of vitellogenin (145-148) and recently the report of tropomyosin in tilapia (149). The identified fish allergens are presented in Table 1.2.

Parvalbumin (MW: 10-12 kDa, pI: 3.9-5.0) is the major fish allergen that has been identified in many different fish species. It is found abundantly in different orders of bony fish class: perciformes, gadiformes, clupeiformes, salmoniformes, pleuronectiformes, cypriniformes, anguilliformes and scorpaeniformes (150). It is also first identified in many temperate water fish species: carp, cod, and salmon (151, 152), then in tropical fish: threadfin (*Polynemus indicus*), Indian anchovy (*Stolephorus indicus*), pomfret (*Pampus chinensis*) and tengirri (*Scomberomorus guttatus*) (153).

Parvalbumin was found unevenly in fish species from both northern (154) and southern hemisphere seawater zones (155). Fish are ubiquitous throughout aquatic environments worldwide. However, the correlation of the natural habitats' effects on the concentration and distribution of putative allergens in fish is limited so far.

Table 1.2 Allergenic proteins from fish registered and deposited in the WHO/IUIS database (www.allergen.org).

Allergen	Species	Scientific name	Allergen ID	MW (kDa)
Beta-parvalbumin	Atlantic herring	*Clupea harengus*	Clu h 1	12
	Common carp	*Cyprinus carpio*	Cyp c 1	12
	Baltic cod	*Gadus callarias*	Gad c 1	12
	Atlantic cod	*Gadus morhua*	Gad m 1	12
	Barramundi	*Lates calcarifer*	Lat c 1	11.5
	Whiff	*Lepidorhombus whiffiagonis*	Lep w 1	11.5
	Rainbow trout	*Oncorhynchus mykiss*	Onc m 1	12
	Indian mackerel	*Rastrelliger kanagurta*	Ras k 1	11.3
	Atlantic salmon	*Salmo salar*	Sal s 1	12
	Pacific pilchard	*Sardinops sagax*	Sar sa 1	12
	Yellowfin tuna	*Thunnus albacares*	Thu a 1	11

	Swordfish	*Xiphias gladius*	Xip g 1	11.5
	Ocean perch, redfish, snapper	*Sebastes marinus*	Seb m 1	11
Beta-prime-component of vitellogenin	Chum salmon	*Oncorhynchus keta*	Onc k 5	18
Tropomyosin	Mozambique tilapia	*Oreochromis mossambicus*	Ore m 4	33
Aldolase A	Atlantic cod	*Gadus morhua*	Gad m 3	40
	Atlantic salmon	*Salmo salar*	Sal s 3	40
	Yellowfin tuna	*Thunnus albacares*	Thu a 3	40
Beta-enolase	Atlantic cod	*Gadus morhua*	Gad m 2	47.3
	Atlantic salmon	*Salmo salar*	Sal s 2	47.3
	Yellowfin tuna	*Thunnus albacares*	Thu a 2	50

Parvalbumin is a heat-stable and highly water solubility protein (156). It has two subgroups (alpha and beta) but the beta lineage is predominant in fish. Parvalbumin distributes unevenly in fish (157, 158). Recently, a measurement of parvalbumin content in 22 species reconfirmed the considerable variation of parvalbumin expression from fish to fish (154). For instance, within a fish individual, the white muscle contained a higher amount of parvalbumin than the dark muscle; more parvalbumin expression found in the dorsal white muscle than the ventral white muscle. Cod, carp, and salmon have a high ratio of parvalbumin in the total soluble

proteins whilst the large-sized pelagic fish like tuna and swordfish contain a very low level of parvalbumin (157). Furthermore, fish parvalbumin has a highly conserved amino acid sequence and the clinical cross-reactivity has been characterized (159). Participants with a fish allergy could also express clinical reactions to parvalbumin from other distant related species, such as in the case of the fish-chicken syndrome (160). The likelihood of cross-reactivity due to parvalbumin among fish species is about 50% (161).

1.5.5 Shellfish allergen

Shellfish allergy is an umbrella term for allergic responses caused by protein compounds from crustacean and mollusk. Besides tropomyosin which is considered as the major allergen in shellfish (162, 163), other putative allergenic proteins have been identified. For instance, arginine kinase, myosin light chain and sarcoplasmic calcium-binding protein in crustacean and myosin heavy chain, hemocyanin and amylase in mollusk (164). Table 1.3 displayed allergens from crustacean species that have been characterized and registered with the WAO/IUIS.

Table 1.3 Allergenic proteins from crustacean registered and deposited in the WAO/IUIS database (www.allergen.org).

Allergen	Species	Scientific name	Allergen ID	MW (kDa)
Tropomyosin	North Sea shrimp	*Crangon crangon*	Cra c 1	38
	Crab	*Charybdis feriatus*	Cha f 1	34
	American lobster	*Homarus americanus*	Hom a 1	34
	White shrimp	*Litopenaeus vannamei*	Lit v 1	36
	Giant freshwater prawn	*Macrobrachium rosenbergii*	Mac r 1	37
	King prawn	*Melicertus latisulcatus*	Mel l 1	38
	Shrimp	*Metapenaeus ensis*	Met e 1	34
	Northern shrimp	*Pandalus borealis*	Pan b 1	37
	Spiny lobster	*Panulirus stimpsoni*	Pan s 1	34
	Shrimp	*Penaeus aztecus*	Pen a 1	36
	Black tiger shrimp	*Penaeus monodon*	Pen m 1	38

	Blue swimmer crab	*Portunus pelagicus*	Por p 1	39
Arginine kinase	North Sea shrimp	*Crangon crangon*	Cra c 2	45
	White shrimp	*Litopenaeus vannamei*	Lit v 2	40
	Black tiger shrimp	*Penaeus monodon*	Pen m 2	40
	Red swamp crayfish	*Procambarus clarkii*	Pro c 2	40
	Mud crab	*Sylla paramamosain*	Scy p 2	40
Myosin, light chain 1	Brine shrimp	*Artemia franciscana*	Art fr 5	17.5
	North Sea shrimp	*Crangon crangon*	Cra c 5	17.5
	Red swamp crayfish	*Procambarus clarkii*	Pro c 5	17.5
Myosin, light chain 2	American lobster	*Homarus americanus*	Hom a 3	23
	White shrimp	*Litopenaeus vannamei*	Lit v 3	20
	Black tiger shrimp	*Penaeus monodon*	Pen m 3	20

Sarcoplasmic calcium-binding protein	Black tiger shrimp	*Penaeus monodon*	Pen m 4	20
	North Sea shrimp	*Crangon crangon*	Cra c 4	25
	White shrimp	*Litopenaeus vannamei*	Lit v 4	20
	Narrow-clawed crayfish	*Pontastacus leptodactylus*	Pon l 4	24
	Mud crab	*Sylla paramamosain*	Scy p 4	20
Troponin C	Black tiger shrimp	*Penaeus monodon*	Pen m 6	16.8
	North Sea shrimp	*Crangon crangon*	Cra c 6	21
	American lobster	*Homarus americanus*	Hom a 6	20
Troponin I	Narrow-clawed crayfish	*Pontastacus leptodactylus*	Pon l 7	30
Triosephosphate isomerase	Crustacean species	*Archaeopotamobius sibiriensis*	Arc s 8	28
Ovary development-related protein	Eriocheir sinensis	*Eriocheir sinensis*	Eri s 2	28.2

Tropomyosin is a coiled-coiled secondary structure, highly conserved myofibrillar protein with a molecular weight of 34-39 kDa. It has a slightly acidic isoelectric point and water-soluble. Tropomyosin can be found in muscle and non-muscle cells of invertebrate and play essential roles in multiple biological processes. Tropomyosin is stable under different heat and chemical treatments (165). Tropomyosin is predominant in crustacean and mollusk and commonly used as a biomarker for shellfish allergy diagnosis. However, clinical reactivity of tropomyosin from shellfish seems to vary within study populations. Studies in Singapore claimed tropomyosin as the major trigger for shrimp allergy in children (166). Tropomyosin was also identified as the major allergen of tropical oyster in Malaysia (167). Whereas, a study in Japan revealed that tropomyosin is a minor but distinct allergen in patients with shrimp allergies (168) and a similar finding was reported in another investigation of shrimp allergic population in China (169). Thus, the shellfish allergy diagnosis needs to take into consideration of all putative allergens present in crustacean and mollusk species.

Arginine kinase (AK) is an enzyme involving in energy metabolism in the invertebrates. Recently, AK was reported as a pan-allergen in crustacean (141). AK Lit v 2 (MW: 40 kDa) was first identified from the muscle of the Pacific white shrimp *(Litopenaeus vanname*i) (170) and has a 96% identity to previous AK Pen m 2 in black tiger prawn (*Penaeus monodon)* (171). Apart from prawns, AK from mud crab (*Scylla paramamosain*) (172) and octopus (*Octopus fangsiao*) (173) was identified and characterized.

Two other heat-stable allergens found in shrimp are sarcoplasmic calcium-binding protein (SCP) Lit v 4 of 22 kDa (174) and myosin light chain (MLC) Lit v 3 of 20 kDa (175). Both SCP and MLC are associated with clinical reactivity to shrimp (164) and their IgE recognition sites were characterized (176). Several novel shellfish allergens were identified recently including hemocyanin (HC) in the giant freshwater prawn *Macrobrachium rosenbergii* (177), Troponin C in American lobster (178), triosephosphate isomerase in the Northern Sea shrimp *Crangon crangon*, fatty acid-binding protein (FABP), and alpha-actinin, beta-actin and ubiquitin in red shrimp *Solenocera melantho* (179).

Many mollusk species are nutritious food with high economic value; however, little is known about the allergenicity of mollusk proteins to seafood sensitized patients. Table

1.4 below summarized allergens from mollusk that deposited in the IUIS database. Tropomyosin is so far the most reported allergen found in three edible mollusk classes: gastropods, bivalves, and cephalopods (180). Mollusk tropomyosin was identified with a molecular weight of about 31-49 kDa. Other allergens reported in mollusk are paramyosin (MW: 100 kDa) (181), myosin heavy chain, hemocyanin and amylase (109).

Table 1.4 Allergenic proteins in mollusk registered and deposited in the WAO/IUIS database (www.allergen.org).

Allergen	Species	Scientific name	Allergen ID	MW (kDa)
Tropomyosin	Brown garden snail	*Helix aspersa*	Hel as 1	36
	Squid	*Todarodes pacificus*	Tod p 1	38
	Abalone	*Haliotis midae*	Hal m 1	49
	Pacific oyster	*Crassostrea gigas*	Cra g 1	18
	Sydney rock oyster	*Saccostrea glomerata*	Sac g 1	38

1.5.6 Cross-reactivity

In food allergy, cross-reactivity is the circumstance that a subject develops allergic responses to one food group can express similar allergic symptoms to other foods of phylogenic relation. Proteins like parvalbumin present in fish muscle can be found in different fish species and different animal meats (i.e. chicken) (182). Thus, individuals allergic to fish parvalbumin are likely to be allergic to other fish of phylogenetical relation (183). This is explained by the highly conserved amino acid sequence of parvalbumin between fish species and consequently, the similarity of specific IgE binding sites (143). The possibility for cross-reactivity within fish is up to 50% (150).

The cross-reactivity occurred more common among shellfish allergic patients (184). Tropomyosin is abundantly distributed in crustacean and mollusk and has a more conservative amino acid sequence than parvalbumin; thus, the likelihood of cross-reactivity climbs up to 75% among shellfish species (161). Also, the clinical cross-reactivity was found between shellfish and mites or cockroach (185, 186). Mite

sensitization has been considered as the primary sensitizer for later shellfish allergy, especially among populations in the tropics (166).

1.5.7 Occupational allergy to seafood

The concern of occupational health risks in seafood processing workers was first raised in 1988 (187). The incidence of developing seafood hypersensitivity was reported among individuals working with squid (36), octopus (188), crustacean (crab, lobster, and shrimp) (189) and fish (190). Raw fish aeroallergens were detected from an open-air fish market (191) and a high level of seafood allergens was recorded inside seafood processing sites (192). Food processing activities such as boiling, cooking, frying, and drying are believed to contribute to the elevated aerosolized seafood proteins in the working environment (37) and the persistent exposure to these allergens elicit sensitization (193). The frequently reported clinical manifestations of the occupational seafood allergy are asthma, rhinitis, conjunctivitis, oral allergy syndrome (194) and contact urticaria (189).

1.6 Seafood allergy in Vietnam

Located in the South-East of Asia, Vietnam is one of the main seafood producers for the world market with more than 4 million workers directly involved in this industry (7). Seafood is preferred by local consumer as a reasonable and available food source. Local people consume about 33 kg fish per capita per year which is much higher than the world's average of 21 kg (8). Although the safety of seafood consumption has been raising lots of attention from the public, no studies on seafood allergy have ever been conducted in this population so far. With the predominance of seafood allergy in neighbor nations, this present work aims to review the current situation of seafood production and consumption in Vietnam; identify seafood species preferably consumed by the locals and review recent seafood-related outbreaks in this country. This literature mentions the background and the rationale for the upcoming study on seafood allergy in the next chapters of this thesis.

1.6.1 Commonly consumed seafood species in Vietnam

The diversity of natural habitats including freshwater ponds, rivers, channels, mangroves and stretched coastal areas benefit for the development of aquatic production in Vietnam. There are about 3,500 seafood species, of which 135 common seafood species are available for regular catching and consumption (195). The Red River delta in the North and Mekong river delta in the South are the main location for fish and shellfish production and processing. Offshore seafood capture activities are focused on the coastal provinces of the country. The distribution of seafood species in Vietnam is presented in Table 1.5 (freshwater fish species), Table 1.6 (marine fish species), Table 1.7 (Crustacea) and Table 1.8 (Mollusca), respectively (196-198).

Table 1.5 Major **freshwater fish** species in Vietnam

Order	Trading name, common name	Vietnamese name	Scientific name
Perciformes	Climbing perch, Anabas	Cá rô đồng	*Anabas testudineus*
	Snakehead, Mudfish, Snakehead fish	Cá lóc đen	*Ophiocephalus striatus*
	Snakehead, Mudfish, Snakehead fish	Cá lóc bông	*Ophiocephalus micropeltes*
	Giant gourami	Cá tai tượng	*Osphronemus goramy*
	Red Tilapia, Tilapia	Cá diêu hồng	*Oreochromis sp*
	Nile Tilapia	Cá rô phi	*Tilapia* sp
	Marble goby, sleepy goby	Cá bống tượng	*Oxyeleotris marmoratus*
	Sand goby	Cá bống cát	*Glossogobius giurus*
Siluriformes	Basa fish, Bocourti catfish	Cá basa	*Pangasius bocourti*
	Striped catfish, Pangas catfish	Cátra	*Pangasius hypophthalmus*

	Mekong catfish	Cá bông lau	*Pangasius krempfi*
	Catfish, Pangasius	Cá hú	*Pangasiusconchophilus*
	Walking catfish	Cá trê	*Clarias spp*
Synbranchiformes	Spiny eel	Cá chạch	*Macrognathus aculeatus*
	Armed spiny eel	Cá chạch chấu, cá chạch sông	*Mastacembelus armatus*
Cypriniformes	Grass carp	Cá trắm cỏ	*Ctenopharyn godonidellus*
	Common carp	Cá chép	*Cyprinus carpio*
Osteoglossiformes	Grey feather back, feather back fish	Cá thát lát	*Notopterus*

Table 1.6 **Marine fish** species in Vietnam

Order	Trading name, common name	Vietnamese name	Scientific name
Perciformes	Pirapitinga	Cá chim trắng	*Piaractus brachypomus*
	Common ponyfish	Cá liệt lớn	*Leiognathus equulus*
	Splendid pony fish	Cá liệt xanh	*Leiognathus splendens*
	Jack mackerel, Japanese horse mackerel	Cá sòng	*Trachurus japonicus*
	Red bigeye	Cá bã trầu, cá trác	*Priacanthus tayenus*
	Yellow tail scad	Cá ngân	*Atule mate*
	Parrot fish	Cá mó	*Scarus spp*
	Grouper	Cá mú	*Epinephelus*
	Yellowtail scad	Cá nục gai	*Decapterus maruadsi*
	Marlin, black marlin	Cá cờ gòn	*Makaira indica*
	Golden threadfin bream	Cá đổng cờ	*Nemipterus virgatus*
	Hairtail, Hairfish	Cá hố	*Trichiurus lepturus*

	Indian mackerel	Cá bạc má	*Rastrelliger kanagurta*
	Red snapper	Cá hồng	*Lutjanus sanguineus*
	Bonito tuna	Cá ngừ bông	*Sarda orientalis*
	Skipjack tuna	Cá ngừ vằn	*Katsuwonus pelamis*
	Spanish mackerel	Cá thu vạch	*Scomberomorus commerson*
	Barramundi, giant sea perch	Cá chẽm	*Lates calcarifer*
Carangiformes	Cobia	Cá bớp biển	*Rachycentron canadum*
	Greater amberjack, amberjack fish	Cá cam	*Seriola dumerili*
	Mahi-mahi	Cá dũa	*Coryphaena hippurus*
	Yellow stripe trevally	Cá chỉ vàng	*Selaroides leptolepsis*
	Black pomfret	Cá chim đen	*Formio niger*
Siluriformes	Pangasius polyuranodon	Cá dứa	*Pangasius polyuranodon*
Gonorynchiformes	Milkfish, bony salmon	Cá măng biển	*Chanos chanos*
Aulopiformes	Lizard fish	Cá mối	*Saurida undosquamis*

Clupeiformes	Anchovy	Cá cơm	*Stolephorus commersonii*
Mugiliformes	Mullet fish	Cá đối	*Mugil cephalus*
Myliobatiformes	Stingray fish	Cá đuối	*Aetobatus narinari*
Pleuronectiformes	Sole fish, Tongue fish	Cá lưỡi trâu	*Cynoglossus robustus*
Syngnathiformes	Goatfish	Cá phèn	*Parupeneus barberinus*

Table 1.7 **Shrimp and crab** species in Vietnam

Trading name, common name	Scientific name	Vietnamese name	Natural habitats
Black tiger shrimp	*Penaeus monodon*	Tôm sú	Inland aquaculture
Cat tiger shrimp	*Parapenaeopsis hardwickii*	Tôm sắt	Marine, wild capture
Pink shrimp	*Metapenaeus affinis*	Tôm chì	Wild capture
White shrimp, banana shrimp	*Penaeus merguiensis*	Tôm thẻ	Wild capture
Yellow ring spiny lobster	*Panulirus ornatus*	Tôm hùm xanh	Inland aquaculture
Slipper lobster	*Thenus orientalis*	Tôm mũ ni	Wild capture
Krill shrimp, baby shrimp	*Acetes japonicus*	Ruốc	Wild capture
Baby shrimp	*Macrobrachium lancestery*	Tép xanh	Wild capture
Mantis shrimp	*Squilla spp*	Tôm tít	Wild capture
Fresh water prawn	*Macrobrachium nipponensis*	Tôm càng	Inland aquaculture

Scampi, giant freshwater prawn	*Macrobrachium rosenbergii*	Tôm càng xanh	Marine aquaculture
White leg shrimps,	*Penaeus vannamei*	Tôm thẻ chân trắng	Marine aquaculture
Blue swimming crab	*Portunus pelagicus*	Ghẹ xanh	Marine aquaculture
Three spot swimming crab	*Portunus sanguinolentus*	Ghẹ ba chấm	Wild capture
Musk crab	*Charybdis cruciate*	Ghẹ lửa	Wild capture
Red swimming crab	*Portunus haani*	Ghẹ đỏ	Wild capture
Mud crab, mangrove crab	*Scylla serrata*	Cua biển	Marine aquaculture
Red frog crab, king crab	*Ranina Ranina*	Cua huỳnh đế	Wild capture

Table 1.8 **Mollusk** species in Vietnam

Class	Species	Local names (Vietnamese)	Scientific name	Natural habitats
Cephalopoda	Cuttlefish	Mực nang	*Sepia spp*	Marine, wild capture
	Cuttlefish	Mực nút	*Sepiella spp*	Marine, wild capture
	Squid	Mực ống	*Loligo edulis*	Marine, wild capture
	Broad squid, soft squid	Mực lá	*Sepioteuthis lessonniana*	Marine, wild capture
	Octopus	Bạch tuộc	*Octopus spp*	Marine, wild capture
Bivalvia	Clam, white clam, hard shell clam	Nghêu trắng, nghêu Bến Tre	*Meretrix lyrata*	Marine aquaculture
	Yellow clam	Nghêu lụa	*Paphia undulata*	Marine aquaculture
	Red Arkshell, Blood cockle	Sò huyết	*Arca granosa*	Marine aquaculture
	Scallop	Điệp rang lược	*Chlamys nobilis*	Marine, wild capture
	Silverlip pearl Oyster	Hàu	*Pinctada maxima spp*	Marine, wild capture

	Clam	Hến	*Corbi culidae*	Fresh water, wild capture
Gastropoda	Apple snail	Ốc bươu	*Pila polita*	Inland capture
	Periwinkle, maculated ivory whelk	Ốc hương	*Babylonia areolate*	Marine aquaculture
	Abanone	Bào ngư	*Haliotis diversicolor*	Marine aquaculture
	Snail	Ốc gạo	*Assiminea lutea*	Brackish water
	Common periwinkle	Ốc mỡ	*Littorina litorea*	Marine, wild capture

1.6.2 Seafood consumption in Vietnam

On average, Vietnamese people consume about 33 kg seafood per capita per year (8). However, a recent study of fish and fish products consumption based on household surveys explored the fluctuation of seafood consumption throughout different regions of Vietnam (199). The average amount of fish and fish product consumed per capita per year from this report was 14.6 kg, much lower than the data published by Food and Agriculture Organization (FAO) at the same period (8). Local people from Mekong delta consume the highest amount of seafood (24.4 kg/capita/year), followed by people in the Northern and Coastal Central Region (16.5 kg/capita/year). The lowest rates were found in the Midlands and Northern mountainous areas (6.8 kg/capita/year).

1.6.3 Reported adverse reactions due to seafood consumption

It is estimated that about 1,000 people are taken to hospitals each year due to food-induced adverse reactions (200). Of which, seafood accounted for up to 11.0% of the cases and was the leading cause of fatalities (201). Table 1.9 summarizes recently seafood-induced events reported from local agencies.

Table 1.9 Recently reported seafood-induced adverse reactions in Vietnam

Incidence	Implicated species	Onset	Symptoms	References
3 fishermen died after eating snail and crab at sea	Undefined snail	20 mins to 3 hours,	Signs of dizziness, nausea, loss of muscle control	(202)
1 fisherman died after eating boiled sea snail	*Nassarius spp*	1 hour	Blurred vison, dizziness, nausea, vomiting, weakness	(203)
1 died after eating snail	*Nassarius spp*	30 mins	Tongue numbing, difficulty breathing, comma	(204)
After eating a sea snail, 2 children out of 3 in a family were hospitalized, one 7-year-old girl died few hours later	Undefined sea snail	3 hours	High fever, coma	(205)

Eating sea snail, 4 people in a family were hospitalized, 6-year-old girl died	*Nassarius papilosus*	30 mins	Vomiting, limbs twitching, tongue numbing, cyanosis	(206)
26 people hospitalized after eating seafood	Undefined crab species	After 8 hours of food ingestion	Abdominal cramps, nausea, vomiting, diarrhea	(207)
A woman hospitalized after eating sea crab	Undefined species	3 hours	Anaphylaxis	(208)
17 fishermen hospitalized	Barracuda fish *(Sphyraenidae spp)*	2 hours	Headache, vomiting, diarrhea, taste disturbances, hypotension, no feeling in the fingers and toes	(209)
1 dead, 4 hospitalized after eating crab	Mangrove horseshoe crab	1 hour	Jaw stiffness, redness in face, burning, unable to speak, dizziness, vomiting	(210)

	(Carcinoscorpius rotundicauda)

1.6.4 Current studies on food allergy and seafood allergy in Vietnam

There is a paucity of food allergy investigation in Vietnam. The only report can be found in the literature is a preliminarily unpublished survey of allergy among under 5-year-old children and 7 to 12- year-old students conducted by the Nutrition Centre of Ho Chi Minh City (no data of the study sample size). According to the survey results, food allergy occurred at a frequency of 20.4% of these subpopulations. The most implicated seafood species were seawater fish (37%), beef meat (22.2%), prawn (20.4%) and crab (16.7%) (211). In 2009, there was a food outbreak suspiciously an acute allergic reaction to fresh milk supplemented with galacto-oligosaccharides affected many children from Ho Chi Minh City (212). Unfortunately, no further investigation of the implicated food allergen was conducted.

1.7 Conclusions

With rapid urbanization, many Asian communities are suffering from food allergy epidemics. Neighbor countries like the Philippines, Singapore, and Thailand reported high rates of seafood allergy and food-induced anaphylaxis. In Vietnam, seafood is an important food commodity and the seafood-related health incidence has been increasing and raising considerable concern to the local people. There is a need to investigate the incidence of food allergy and the impacts of this health condition to the general population. To fill the current gap, this thesis aims to estimate the distribution of food allergy in the Vietnamese children and adults by a population-based survey. From the survey outcomes, further investigations on the implicated food allergens will be conducted. My aim is to further identify the correlation between clinical manifestations and allergen-specific IgE level among allergic participants. These data are promising to aid in evaluating the effectiveness of the current food allergy diagnosis tests and developing new immunotherapies for better management of food allergy in Vietnam and the Asian community.

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CHAPTER 2. A POPULATION-BASED SURVEY OF FOOD ALLERGIES IN VIETNAMESE CHILDREN

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2.1 Introduction

Food allergy, an adverse immune reaction to food proteins, has a wide spectrum of clinical presentations, ranging from mild skin problems to severe systematic reactions. In the most severe case, a food allergy can lead to anaphylaxis and might result in death within minutes. Food allergy is estimated to affect about 8% of children and 5% of adults in the general population worldwide (1).

Children are more likely to develop food allergies than adults due to the remaining controversial causes, including the immature immune system in childhood and/or the inappropriate food introductory practices (2, 3). Eight food groups often referred as the "Big 8", account for over 90% of food allergic reactions and include cow's milk, egg, peanut, tree nuts, soy, wheat, fish and shellfish (1). Except for cow's milk and egg allergy which are often outgrown, most other food allergies often persist for life (1). So far, no cure is available and childhood food allergy imposes a substantial health and economic burden for children and their caregivers (4, 5).

The common food commodities accounting for food allergy in children are cow's milk, egg, peanut, tree nuts and fish (6); the first three foods are the leading triggers for pediatric anaphylaxis in Western countries (7). In Asia, the prevalence of pediatric food allergy seems to vary between 1.11-7.65% (8), and the patterns of food allergy showed marked differences from other parts of the world (1, 8). Recent studies among 2-7-year-old children from Singapore, Thailand, the Philippines, and Hong Kong demonstrated that shellfish allergy was dominant, but not milk, egg or peanut allergy (9, 10). Furthermore, fish was reported to be the predominant allergen in adolescents in the Philippines, Singapore, and Thailand (11). Within Asia, studies from Japan and Korea showed different food allergy patterns to most available food allergy data, with wheat allergy particularly common in East Asian countries (8). These data were supported by a recent study from Australia, where significant differences in allergy/anaphylaxis risk and trigger were demonstrated between migrant children born in Australia and those born in Asia (12). The variation of food allergy patterns throughout Asia indicates that region-specific and accurate data on food allergy

prevalence and clinical patterns are crucial for an effective food allergy management program in any community.

In Vietnam, about 4.4 million children aged 2-6-year-old attend kindergartens, accounting for over 90% of all children at this age group in 2016 (13). No population-based data on food allergy have been reported in this country. In 2009, a milk-related outbreak in children was recorded and suspected to be acute allergic reactions caused by galacto-oligosaccharides in a dairy product (14). However, the study remained as a case report without further investigations on the possible allergy-triggers or milk allergy incidence. In this chapter, we sought to evaluate the epidemiologic and clinical features of food allergy in Vietnamese preschool children. The possible variations of childhood food allergy prevalence and its associated risk factors in socio-economically different regions in Vietnam were also investigated.

2.2 Aims of this chapter

- To estimate the prevalence of food allergies in Vietnamese preschool children.
- To identify the distribution of ‘Big 8’ food allergen in Vietnamese children.
- To identify the clinical manifestations of food allergy.
- To identify the contribution of gender, geographic location, family history of food allergy, atopic conditions to food allergy incidence in children.

2.3 Methods

2.3.1 Study design and subjects

A cross-sectional, population-based study was conducted in preschool children aged 2 to 6 years in 2016. Survey participants were randomly selected using the cluster sampling method from a list of 25 kindergartens in Hue city and 14 kindergartens in Cai Be district, Tien Giang province, representing a total of 104,602 preschool children in two regions (13). The paper-based questionnaires were distributed to parents/guardians of children at their kindergartens. Most of the answer sheets were collected on the same day. The response rate was calculated based on the number of returned answer-sheets divided by the total distributed questionnaires.

2.3.2 Sample size calculation

To obtain a statistical estimation of the prevalence of food allergy, the minimum sample size was calculated based on the current estimated prevalence of food allergy in children (8%) in the general population (15); the chosen precision of the estimation d = 1/5p was calculated with a statistical confidence of two standard errors of the mean z = 1.96 (95% Confidence Interval (CI), $P < .05$). The minimum necessary sample size calculated for children was 1,825 participants.

2.3.3 Study locations

The study was conducted in two different regions of Vietnam: Hue City and Cai Be District of Tien Giang Province. Hue City is in the Central region of Vietnam with a population density of 5,011 per square kilometer. The main economic activities in Hue are tourism, industry and aquaculture. Urbanization has quickly taken placed in this city due to the rapid development of tourism. Hue has an average temperature of 25.4^{o}C, average humidity of 87% and a total of 1,754.2 hours of sunshine per year.

Cai Be District is a rural area in the Mekong Delta of southern Vietnam. This river-land mixed town has a population density of 657 per square kilometer. The major economic activities in Cai Be are aquaculture, rice and fruit farming. Cai Be-Tien Giang has an

average temperature of 28.2°C, an average humidity of 80.4% and a total of 2,104.6 hours of sunshine per year (16).

In this study, taking into consideration the effects of population density, living lifestyle and environmental conditions, we defined participants in Hue City as living in urban areas and participants from Cai Be District as living in the rural area.

2.3.4 Questionnaire design

The questionnaire, modified from published studies in the US and Asia (9, 11), had two parts: part I asked the participant demographic information, and part II contained ten questions on food allergy (Appendix B1). The questionnaire was translated into Vietnamese. The content of the questionnaire and its translation were reviewed and approved by the Human Research Ethics Committee at James Cook University (Approval ID: H6437–Appendix A1). By answering the questionnaire, the parents/guardians gave informed consent to the study and the permission to use obtained child health information for research publications and reports.

2.3.5 Definitions

We established a set of criteria to define self-reported and doctor-diagnosed food allergy in this survey based on the most recent European Academy of Allergy and Clinical Immunology (EAACI) guidelines on food allergy and anaphylaxis (17). In specific, the suggestive symptoms of food allergy were considered including persistent symptoms towards food ingestion and the co-occurrence of two or more different clinical presentations (18). The typical symptoms for Immunoglobulin E (IgE)-mediated food allergy included hives/urticaria or angioedema or vomiting or gastrointestinal symptoms or anaphylactic reactions (i.e. reduced blood pressure, loss of consciousness, chest pain, and weak pulse) after food intake. In this study, children with only one symptom of hives/angioedema were also defined as food allergic.

Self-reported food allergy was the group of participants who fulfilled the above criteria and reported having a food allergy.

Doctor-diagnosed food allergy was a group of participants with self-reported food allergy, which was clinically confirmed by a medical practitioner.

Food-induced adverse symptoms: any abnormal clinical response that occurs following the ingestion of a food or food component.

Family history of food allergy was defined when the participant had in their immediate family a member with food allergy.

Coexisting other allergic diseases was defined when the participant had any other allergic diseases including pollen allergy, antibiotic allergy, asthma, eczema, etc.

2.3.6 Statistical analysis

The survey data were analyzed and plotted using the IBM SPSS Statistics for Windows, version 24.0 and GraphPad Prism version 7.03. Continuous variables were presented as the median and interquartile range (IQR). Categorical data were compared by using either Fisher's exact test or Chi-square test with a 2-tailed *P*-value. The Wilson/Brown method was performed to provide a 95% CI of proportions. Multiple logistic regression model was used to study the association between multiple risk factors and the incidence of having doctor-diagnosed food allergy. A *P*-value of $< .05$ was considered statistically significant for all tests.

2.4 Results

2.4.1 Participants

A total of 8,620 questionnaires were completed and returned from the two survey sites (response rate of 81.5%). The survey in Hue gained a higher response rate (93.5%) than in Tien Giang (69.5%). Minimal difference in gender distribution was observed across the two survey sites. The age median (IQR) of the participants was 4 (2-6) years in Hue and 6 (2-6.5) years in Tien Giang. The demographic characteristics of participating children are presented in Table 2.1.

Table 2.1 Demographics of participating children in Hue and Tien Giang.

Variable, *n* (%)	**Hue**	**Tien Giang**	**Difference, *P***	**Total study population**
Total	4,443	4,177		8,620
Female	2,206 (49.6)	2,120 (50.8)	.2860	4,326 (50.2)
Male	2,239 (50.4)	2,055 (49.2)	.2860	4,294 (49.8)
Age group (years)			< .0001	1,192 (13.8)
2 to <3	1,140 (25.7)	52 (1.3)	<.0001	2,020 (23.4)
3 to <4	1,365 (30.7)	655 (15.7)	<.0001	5,407 (62.7)
4 to 6	1,940 (43.6)	3,467 (83.0)		
Age, median (IQR)	4 (2-6)	6 (2-6.5)		
Reported adverse reactions to food	911 (20.5)	1,994 (47.8)	<.0001	2,905 (33.7)
Self-reported FA	433 (9.8)	330 (7.9)	.0026	763 (8.9)
Seeking medical advice for FA[‡]	394 (91.6)	250 (76.7)	<.0001	644 (84.4)
Doctor-diagnosed FA	373 (8.4)	207 (5.0)	<.0001	580 (6.7)
FA to 1 food group	328 (87.9)	125 (60.4)	<.0001	453 (78.1)
FA to 2 different food groups	40 (10.7)	36 (17.4)	.9084	76 (13.1)
FA to more than 2 different food groups	4 (1.1)	37 (17.9)	<.0001	41 (7.1)

[‡] among subjects with self-reported FA. FA, food allergy. The Fisher's exact test was performed using GraphPad Prism for Windows (GraphPad Software, La Jolla California USA) to obtain *P* -values.

2.4.2 Comparison of reported food-induced adverse symptoms between children in Hue and Tien Giang

Children in Tien Giang were reported to have twice the food-induced adverse symptoms than children in Hue (47.8% vs. 20.5%) (Table 2.1). However, self-reported food allergy in Hue (9.8%) was higher than in Tien Giang (7.9%) (Table 2.2). In the perceived food allergy group, more children in Hue presented to doctors for medical advice, 91.6% compared to 76.7% in Tien Giang. Overall, the prevalence of life-time doctor-diagnosed childhood food allergy in Hue was 8.4%, nearly double the rate of 5.0% in Tien Giang ($P < .0001$).

Suspected food allergy children in Hue reported less concurrent episodes than those in Tien Giang (an average of 1.4 episodes compared to 2.0 episodes, respectively). Hives, diarrhea and nausea or vomiting were the most predominant clinical presentations reported. Ten participants (0.2%) in Tien Giang experienced severe symptoms (i.e. loss of consciousness, drop in blood pressure, chest pain and weak pulse) due to food allergy while in Hue, only one case was reported (0.02%) (Figure 2.1).

Table 2.2 Comparison of the prevalence of self-reported FA and doctor-diagnosed FA in two survey populations.

	Self-reported FA				Doctor-diagnosed FA			
	Hue	**Tien Giang**	**Difference, *P***	**Entire study population**	**Hue**	**Tien Giang**	**Difference, *P***	**Entire study population**
Any food	9.75 (8.91 – 10.65)	7.90 (7.12 – 8.76)	**.0027**	8.85 (8.27 – 9.47)	8.40 (7.62 – 9.25)	4.96 (4.34 – 5.66)	**< .0001**	6.73 (6.22 – 7.28)
Crustacean	5.22 (4.61 – 5.92)	4.29 (3.71 – 4.94)	**.0415**	4.77 (4.34 – 5.24)	4.79 (4.20 – 5.46)	2.80 (2.34 – 3.35)	**< .0001**	3.83 (.344 – 4.25)
Fish	1.55 (1.23 – 1.96)	1.70 (1.35 – 2.14)	.6097	1.62 (1.38 – 1.91)	1.37 (1.07 – 1.76)	1.10 (0.83 – 1.47)	.2845	1.24 (1.03 – 1.50)
Mollusk	0.90 (0.66 – 1.22)	2.13 (1.73 – 2.61)	**< .0001**	1.50 (1.26 – 1.78)	0.72 (0.51 – 1.01)	1.36 (1.05 – 1.76)	**.0038**	1.03 (0.84 – 1.27)
Beef	0.34 (0.20 – 0.56)	2.32 (1.91 – 2.82)	**< .0001**	1.30 (1.08 – 1.56)	0.27 (0.15 – 0.47)	1.46 (1.14 – 1.87)	**< .0001**	0.85 (0.67 – 1.06)
Milk	0.81 (0.59 – 1.12)	0.26 (0.15 – 0.47)	**.0006**	0.55 (0.41 – 0.72)	0.70 (0.49 – 0.99)	0.22 (0.11 – 0.41)	**.0012**	0.46 (0.34 – 0.63)
Egg	1.15 (0.87 – 1.51)	1.10 (0.83 – 1.47)	.9187	1.13 (0.92 – 1.37)	0.95 (0.70 – 1.28)	0.74 (0.52 – 1.05)	.3471	0.85 (0.67 – 1.06)
Wheat	0.07 (0.02-0.20)	0.50 (0.33 – 0.77)	**.0001**	0.28 (0.19 – 0.41)	0.07 (0.02 – 0.20)	0.38 (0.24 – 0.62)	**.002**	0.22 (0.14 – 0.34)
Peanut	0.47 (0.31 – 0.72)	0.36 (0.22 – 0.59)	.5046	0.42 (0.30 – 0.58)	0.27 (0.15 – 0.47)	0.31 (0.18 – 0.53)	.7226	0.29 (0.20 – 0.43)
Soy	0.18 (0.09 – 0.35)	0.26 (0.15 – 0.47)	.4934	0.22 (0.14 – 0.34)	0.16 (0.08 – 0.32)	0.17 (0.08 – 0.35)	>.9999	0.16 (0.10 – 0.27)
Tree nuts	0.07 (0.02 – 0.20)	0.43 (0.27 – 0.68)	**.0006**	0.24 (0.16 – 0.37)	0.02 (0.00 – 0.13)	0.31 (0.18 – 0.53)	**.0007**	0.16 (0.10 – 0.27)
Other foods	0.41 (0.26 – 0.64)	0.53 (0.35 – 0.80)	.4314	0.46 (0.34 – 0.63)	0.25 (0.14 – 0.44)	0.29 (0.16 – 0.50)	.8354	0.27 (0.18 – 0.40)

All data were analyzed using GraphPad Prism for Windows. The Wilson/Brown method was used to calculate the 95% CIs. Fisher's exact test and *Chi*-square test were used (where appropriate) to compare the prevalence in two study groups. A *P* value of < .05 was denoted as statistical significance and highlighted in bold.

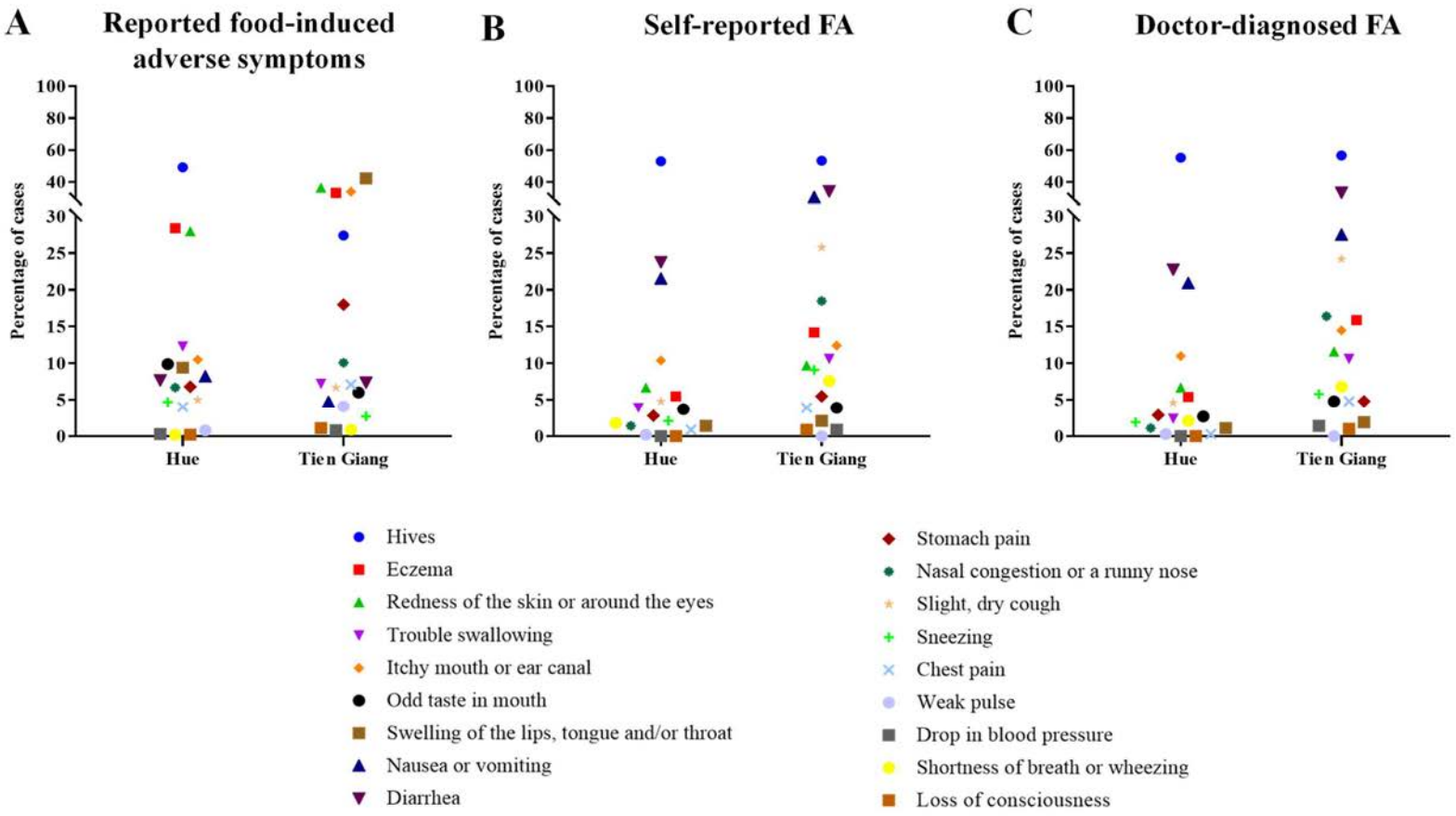

Figure 2.1 Proportion of reported clinical symptoms in participating children in Hue and Tien Giang. A. Reported food-induced adverse symptoms (n = 2,905). B. Reported adverse symptoms in self-reported FA participants (n = 763). C. Reported adverse symptoms in doctor-diagnosed FA participants (*n* = 580).

2.4.3 Distribution of the major food allergens in FA children in Hue and Tien Giang

Most of the affected subjects (78.1%) reported food adverse symptoms to only one food item; 13.1% reported adverse reactions to two different food items and 7.1% had reactions to more than two different food groups. Crustacean was the most predominant allergy-causing food type in both Hue (50.1%) and Tien Giang (30.6%), while the distribution of the remaining 'Big 8' food groups was very different (Figure 2.2). Statistically significant differences were seen in the prevalence of crustacean, mollusk, beef, milk, wheat and tree nut allergies between children in Hue and Tien Giang ($P < 0.05$) (Table 2.2).

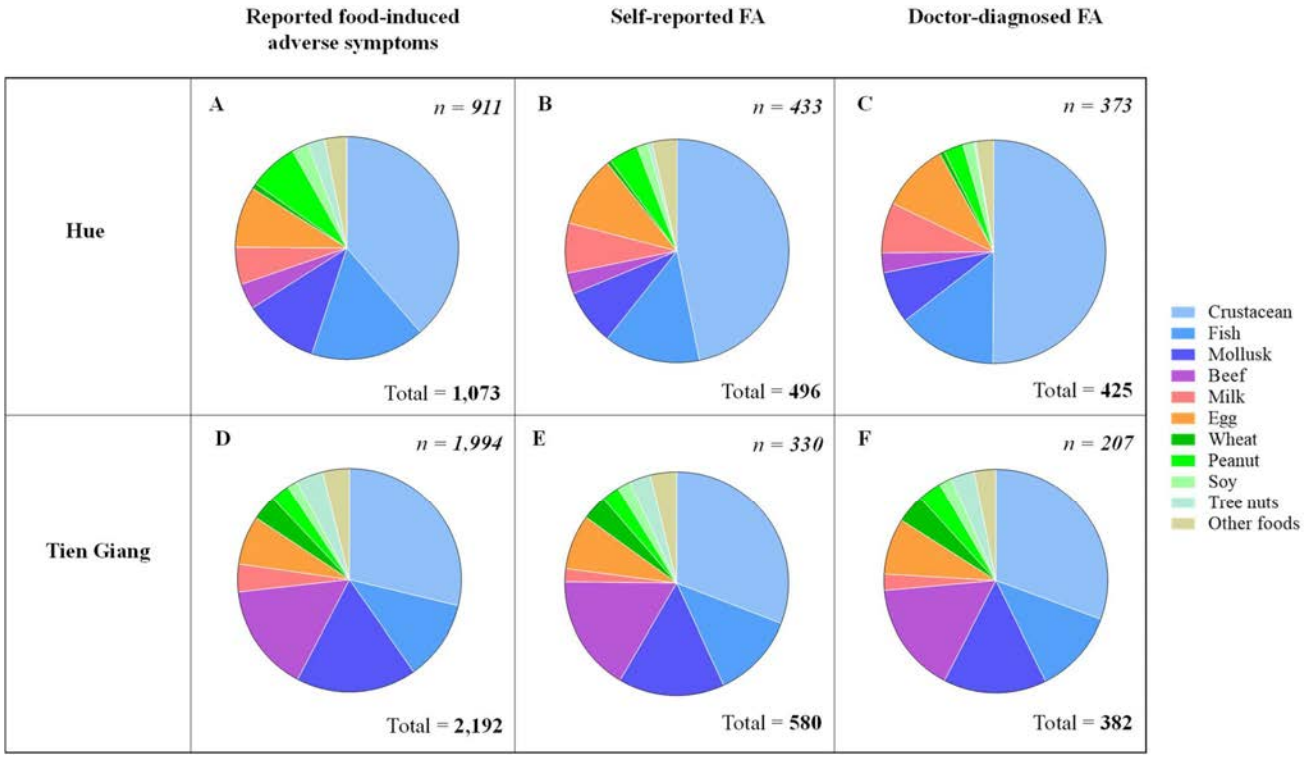

Figure 2.2 Comparison of the distribution of reported food groups eliciting clinical reactions in participating children in Hue and Tien Giang.

A. Reported food-induced adverse symptoms in Hue (number of participants *n* = 911); B. Self-reported FA in Hue; C. Doctor-diagnosed FA in Hue; D. Reported food-induced adverse symptoms in Tien Giang; E. Self-reported FA in Tien Giang; F. Doctor-diagnosed FA in Tien Giang. The total number of reported food groups is presented for each study area and symptom group. FA, food allergy.

2.4.4 Contribution of environmental factors to FA incidence

Genetic and environmental factors are reported to play a role in the development of food allergy (1, 19). In this study, we analyzed the contribution of geographical location, gender and family history of food allergy as well as coexisting other allergic diseases to the food allergy incidence by using a multivariable logistic regression model. A strong influence of participant location and atopic conditions to food allergy risk was observed in this study. Children living in Hue (urban area) have a higher risk of having food allergy than children living in Tien Giang (*Odds Ratio* (*OR)*: 3.902, *P* < .001). The food allergy rate was found to be 3.428 times higher in participants with other existing allergic diseases (*P* < .006). Gender and family history of food allergy showed no impact on food allergy risk in this study population (Table 2.3).

Table 2.3 Multivariable logistic regression analysis of demographic factors for FA.

Risk factor	OR	*P*-value
Gender (*Female/ Male*)	1.567	.172
Family history of FA (*Yes/ No*)	1.018	.961
Co-existing other allergic diseases (*Yes/ No*)	3.428	**.006**
Participant location (Hue/ Tien Giang)	3.902	**< .001**

Binary logistic regression was performed in SPSS Statistics for Windows to generate ORs. A *P* value of <.05 was considered as statistical significance. FA, food allergy. OR, odds ratio.

2.5 Discussion

This population-based survey is the first to establish the prevalence of self-reported food allergy (8.9%) and doctor-diagnosed food allergy (6.7%) in Vietnamese children. Our findings indicate significant variations of food allergy prevalence between two survey sites with different socio-economic backgrounds. The population living in the urban area presented a higher prevalence of food allergy but also had a higher rate of doctor consultation to diagnose a food allergy. Most participants (78.1%) reported adverse symptoms to only one food group, with crustacean the dominating food allergen. Hives and gastrointestinal tract problems were the most commonly reported clinical symptoms for both regions.

We observed a higher rate of self-reported food allergy (8.9%) than doctor-diagnosed food allergy (6.7%), consistent with previous assessments of questionnaire-based food allergy rates in Asian populations (1.11-7.65%) (8). This variation appears to be determined by the complex pathophysiology of adverse reactions to food and the perception of respondents of this disease. Common etiology in pediatrics with food-related adverse symptoms are immune-mediated food allergies and non-immune mediated food intolerance (20). There is a lack of strong evidence to differentiate food allergy from food intolerance exclusively based on reported clinical history, especially in Asian communities. Among doctor-diagnosed milk allergic participants, two-thirds of participants presented gastrointestinal symptoms which might imply the contribution of other food-induced disorders rather than true food allergy. A food outbreak, suspected to be an acute allergic reaction to a new formula product, was recorded in 19 out of 229 hospitalized children in 2009 (14). Unfortunately, no allergens were identified due to the constraint of diagnostic capacity in Vietnam. Further investigations will exclude other non-IgE-mediated food allergies, such as Food Protein-induced Enterocolitis Syndrome and eosinophilic esophagitis in the pediatric population to give an accurate estimation of true food allergy prevalence (21).

Patient's clinical history of food allergy is the initial motive for further diagnostic analysis, however, only 4 to 5% of the self-reporting food allergy population is generally confirmed as true food allergy (6). Parent-reported food allergy in Thai children was found to be 9.3% but reduced to 1.1% when confirmed by oral food challenge (OFC)

(22). A survey of Singapore-born children aged 4-6 years showed the variation of self-reported food allergy to shellfish with 7.22 % as compared to a rate of 1.19 % with convincing history food allergy (9). As it was consistently concluded in previous studies, an accurate diagnostic procedure of IgE-mediated food allergy must comprise of multiple tests including skin prick testing, measurement of serum specific IgE and OFC (23). However, only limited services are available in Vietnam for diagnosing food allergy, particularly in rural areas. Most commercial diagnostic tests that are readily available in Western countries, including IgE quantification and skin prick tests, are not registered or partially available to private patients and in specialized clinics. In the presented study, data could not be collected for the onset of adverse symptoms that might have better supported differentiating between IgE-mediated and non-IgE mediated food allergy. This is one of the biggest challenges in studying the prevalence of food allergy in a country where only a few people have access to correct food allergy diagnosis. This paper-based survey on health conditions was thought to be a rather new practice for most Vietnamese, so we aimed and succeeded at keeping the questionnaire as simple as possible to achieve a high response rate (81.5%).

This study revealed a distinct distribution of the “Big 8” food allergens in Vietnamese children. Unlike the patterns of childhood food allergy from Western populations, previous studies in Asian populations showed the predominance of shellfish and fish allergy rather than egg, cow’s milk and peanut (8, 9, 11), and this tendency was also determined in this survey. Children from rural and urban Vietnam reported higher adverse reaction rates to seafood, then beef, milk, and egg. The predominance of seafood allergy in Asia might be claimed for the availability and high consumption of this food commodity (24). In Vietnam, the average fish consumption is with 33 kg per capita per annum much higher than the world’s average consumption of 21 kg (24). The impact of ethnic characteristics to seafood allergy in Asian communities was validated in a study among expatriate and local Singaporean children, revealing the predominance of shellfish allergy in local children compared with expatriate children (9).

Considering ethnic characteristics and cultural dietary practices, we found considerable variations of food allergy prevalence among urban and rural populations in Vietnam. Crustacean and milk allergy are predominant in children in Hue (urban area). However, there was insufficient data on the consumption of these commodities between the two areas to postulate food allergy risk. The high incidence of shellfish allergy in urban children might be related to higher exposure to indoor allergens as discussed in the current literature (25). For instance, indoor mites were documented to cross-react with the major shellfish allergen tropomyosin (26), and storage mites were identified in indoor environments in the north of Vietnam (27). In contrast, children in the Tien Giang province showed a much higher prevalence of mollusk, wheat, tree nuts, and beef. Recent studies in the US and Sweden documented the association of red meat allergy to tick bites (28, 29), which was explained by the cross-reactivity of a carbohydrate oligosaccharide galactose-alpha-1,3-galactose in mammalian meat and a similar component found in the saliva of tick. Children from rural areas are more likely to have tick bites than those in the city (30), and therefore environmental factors might contribute to the high rate of beef allergy in children in Tien Giang. Similarly, the high incidence of wheat and tree nut allergy in this subpopulation might be explained by the possible cross-reactivity of these food allergens with other aeroallergens abundant in the rural area. It should be noted that wheat is not a staple food in Vietnam and no data on gluten intolerance or coeliac disease have been reported so far in this population. This will be of interest to further investigate the influence of environmental factors on food allergy.

The data from the multivariable logistic regression analysis of demographic risk factors (gender, family history of food allergy, coexisting other allergic diseases and geographic location) demonstrated a strong contribution of coexisting other allergic diseases (OR = 3.428, P < .006) to food allergy incidence, but not a family history of food allergy (OR=1.018, P = .961). Food allergy is thought to run in a family (19). However, the contribution of a family history of food allergy to the risk of food allergy development remains inconsistent among studies (31, 32). In the present study, we did not apply any additional logistic regression models to further assess individual risk factors for food allergy.

The strengths of this study are the large population-based dataset (*n* = 8,620) collected at two different socio-economical survey sites and the high response rate (81.5%). The limitations of this study are the self-administered data on food allergy and therefore the response might contain recall bias. Our target population was children aged from 2-6 years and the information on children outside this age group with potentially different food allergy rates have not been included. There are several factors such as the disparity of the medical facilities among rural and urban areas in Vietnam and the economic circumstances of participants that might contribute to the variation on reported food allergy rates among the two study sites.

In conclusion, this study contributes to the current paucity of food allergy data in the broader Asian population and is the first to profile this emerging epidemic in Vietnam. Our study clearly showed that food allergy is prominent in Vietnam, but unexpected patterns of food allergies are perceived. A large variation of food allergy incidence was observed in subpopulations from rural and urban regions, implying possible impacts of living conditions. Further investigations are necessary to confirm the true prevalence of food allergy and the possible cross-reactivity between different allergen sources for a precise diagnosis and better management of this serious childhood illness.

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2.7 Chapter 2 summary

From this chapter, the research aims were met as below:

- Estimate the prevalence of food allergies in Vietnamese preschool children. The overall prevalence of self-reported food allergy in Vietnamese preschool children are 8.9% whilst the prevalence of doctor-diagnosed food allergy is much lower, at the rate of 6.7%. This is a common phenomenon in population-based surveys and has been reported in previous studies. The accuracy of a self-administrated food allergy survey depends largely on the knowledge and understanding of the participants about food allergy definition and its clinical symptoms. Thus, it is crucial to combine the self-reported data about clinical history with another evidence-based diagnosis such as skin prick tests or measurement of serum specific IgE or performing oral food challenge to confirm true food allergy. These limitations will be partly addressed in Chapter 5, where serum specific IgE reactivity to a diversity of crustacean, mollusk and fish species among Vietnamese participants with a history of seafood allergy was measured and analyzed.

- Identify the distribution of 'Big 8' food allergen in Vietnamese children. The most common allergy-triggering foods in Vietnamese children in this study are crustacean, mollusk, fish and beef. Less common food allergens are milk and egg; whilst a very low rate of participants reported adverse reactions to peanut, wheat and tree nut. The food allergy pattern in Vietnamese is completely different from previous investigations in preschool children in developed countries, for instance in Australia, the US, and many European countries. Seafood has been reported as the most common allergen in children's population in Singapore, the Philippines, and Thailand. In Singapore, prawns are reported to be the leading cause of food-induced anaphylaxis. Thus, this study confirmed the dominance of crustacean, mollusk and fish allergy in Southeast Asian communities. As seafood was reported as the leading food allergens in this population, seafood was selected for further investigation of IgE reactivity and clinical profiles. The findings of this investigations are discussed in **Chapter 5** of this thesis.

- Identify the clinical manifestations of food allergy. Food allergy has a broad spectrum of clinical presentations affecting different organs such as the skin barrier, the gastrointestinal, and the respiratory or systemic reactions. In the present study in Vietnamese children, hives/urticaria was the most commonly reported allergic reaction. Hives/urticaria is one of the typical symptoms for IgE-mediated food allergy according to EAACI guidelines on food allergy and anaphylaxis. Thus, it is assumed that most participants in this survey have IgE-mediated food allergy. Also, most participants in this study reported concurrently multiple symptoms of suspected food allergens. To the author's best knowledge, food allergy is not well-defined in Vietnam. At present, there are no guidelines on the diagnosis and treatment of food allergy in Vietnam. People with food allergic symptoms may be misdiagnosed with other health problems such as food poisoning or food-borne illness. This is, we believe, the first study to present the prevalence rate of people with food allergy in Vietnam and their typical clinical manifestations. The study provides evidence for policymakers, patients, clinicians, and the food production industry about the current situation of food allergy in the Vietnamese population.

- Identify the contribution of gender, geographic location, family history of food allergy, atopic conditions to food allergy incidence in children. By conducting the food allergy surveys in different regions in Vietnam, we could evaluate and identify contributing factors to having food allergy in this subpopulation. Excluding gender and family history of food allergy, geographic location and atopic condition are significant risk factors of food allergy in preschool children in Vietnam. The pattern of food allergens was different between rural and urban subpopulations. Children in urban areas have more allergy to crustacean, fish, and milk whilst children in rural have more beef, wheat, and tree nut allergy. It can be seen that within a specific country, the pattern of food allergy can vary significantly by geographical locations, therefore it is essential to have region-specific preventive and management programs to effectively control this health condition.

CHAPTER 3. A POPULATION-BASED SURVEY OF FOOD ALLERGIES IN VIETNAMESE ADULTS

Manuscript in revision:

Thu T.K. Le, Thuy T.B Tran, Huong T.M Ho, An T.L. Vu, Emma McBryde and Andreas L. Lopata. The predominance of seafood allergy in Vietnamese adults: results from the first population-based questionnaire survey. World Allergy Organization Journal. Submitted on 03/06/2019. Submission ID: waoj-19-00074.

3.1 Introduction

Food allergy is defined as abnormal reactions of the human's immune system triggered by food components following food ingestion and/or food exposure processes. Food allergy presents with a wide range of clinical manifestations, from mild skin problems to acute and severe systemic reactions. Food allergy occurs in both children and adults, and is among the most common cause of food-induced anaphylaxis (1). Food allergy impacts on the quality of life and imposes a substantial financial burden to its sufferers (2, 3); thus, it has been considered a major public health problem in many westernized countries.

Approximatly 10% of children and 5% of adults in developed countries experience food allergy, and this incidence is reported to be on the rise (4). However, very little is known about this epidemic in other parts of the world, especially in developing economies. In Asia, most food allergy studies have focused on children, reporting prevalence rates of 1.11% to 7.65%. Epidemiological studies on food allergy among adults have only been conducted in a few Asian countries, revealing a prevalence of 18% in China (5), 6.4% in Taiwan (6), 1.2% in India (7) and 0.21% for wheat allergy in Japan (8). Major food triggers also varied between Asian countries and differed greatly to the food allergy patterns seen in the West (9).

Food allergy in adults may be initiated from an early sensitization during childhood, such as is the case with peanut allergy and seafood allergy (10); however, new sensitizations to food allergens in adulthood is also reported (11). It is well evidenced that long-term exposure to allergens in the environment could trigger the development of food allergy later on (12, 13). As a result, adults might have different patterns of allergen sensitizations and clinical manisfestation, compared to the childhood food allergy phenotype. Thus, the study of food allergy in adults is of importance to providing valuable insight into the nature and development of food allergy over the course of life.

In Vietnam, there has been food allergy studies completed on the adult population. Extended from the population-based survey of food allergy in Vietnamese children in Chapter 2, we sought to estimate the prevalence of food allergy in Vietnamese adults

and identify the pattern of offending food allergens and their clinical presentations. Cohort demographics such as gender, family history of food allergy, comorbidities of other allergic diseases and living location were collected and statistically analyzed to predict their associations to food allergy incidence in this population.

3.2 Aims of this chapter

- To determine the prevalence of food allergies in Vietnamese adults.
- To identify the distribution of the ‘Big 8’ food allergens in Vietnamese adults.
- To identify the clinical manifestations of food allergy.
- To identify determinants to the incidence of food allergy.
- To compare the prevalence of food allergy between children and adults in the Vietnamese population.

3.3 Methods

3.3.1 Survey design

A cross-sectional, randomized paper-based survey was conducted from March to December 2016 among university students across four different regions of Vietnam. Questionnaires were distributed to the target populations, and most of the answer sheets were collected on the same day. By accepting to answer the questionnaire, a participant consented to the study. The response rate was calculated by dividing the number of returned questionnaires by the total distributed questionnaires. This study was approved by the Human Research Ethics Committee at James Cook University (Approval ID: H6437 – Appendix A1).

3.3.2 Participant recruitment

A minimum sample size of 1,963 participants were required to obtain a precision level of 20%, with a confident level of 95%. From a list of 516 classes of all participating universities, 150 classes were randomly selected to distribute the questionnaire. The participating universities included Nong Lam University, Nha Trang University and the University of Food Industry (Figure D3.1 – Appendix D). These are multi-discipline universities with a wide diversity of student age ranges and backgrounds. The survey at Nong Lam University was conducted at its three different campuses in Kon Tum province, Ninh Thuan province and Ho Chi Minh City. Participants in the South East part of the country were considered to be living in an urban area. Participants from the remaining regions were considered to be living in a rural area. The cluster sampling method was applied to recruit a relatively equivalent number of participants from each geographical region, feasible for further analysing the contribution of living location to food allergy incidence.

3.3.3 Questionnaire

The same questionnaire that had been used for the population-based survey of food allergy in Vietnamese children in Chapter 2 was used for this study with minor

modifications (Appendix B2). By answering the questionnaire, the participants gave their informed consent to the study with permission to use obtained information for research publications and reports.

3.3.4 Definitions

The definitions of self-reported food allergy and doctor-diagnosed food allergy used in this study had been described in Section 2.3.5 in Chapter 2 of this thesis.

3.3.5 Statistical analysis

For the analysis of generated data, the IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, N.Y., USA) was used. A sampling design without replacement was chosen for the estimation of prevalence. The ratio of male to female participants was weighted to fit the natural gender ratio in Vietnam (14). Continuous variables were presented as median and interquartile range (IQR). Categorical data was compared using Chi-square tests with a 2-tailed *P*-value. The prevalence was calculated to provide a 95% CI of responses to each criterion. A multiple logistic regression model was used to study the association between multiple risk factors and the incidence of having doctor-diagnosed food allergy. The significance level was accepted at a *P*-value of $< .05$ for all tests.

3.4 Results

3.4.1 Demographic features

Table 3.1 presents the demographic features of the survey. The questionnaire was distributed to 14,500 subjects, with 9,039 subjects responding (response rate 62.4%). The median age and IQR of participants were 20 and 2 years. The survey recruited participants from five different regions of Vietnam. There are more participants from South Central Coast (3,753 participants) and South East (4,249 participants) than the remaining areas: North Central Coast (91 participants), the Central Highlands (617 participants), and Mekong Delta (329 participants). Female participation (67.3%) was much higher than male participation in all survey sites.

Table 3.1 Demographic features of participants in this survey.

Variable	***n* (%)**
Total questionnaire distributed	14,500 (100)
Number of respondents	9,039 (62.4)
Sex distribution	
Male	2,955 (32.7)
Female	6,084 (67.3)
Age median (years)	20
Interquartile range	18-20
Age range (years)	
16 – 20	6,802 (75.3)
21 – 25	2,064 (22.8)
26 – 30	88 (1.0)
31 – 35	41 (0.5
Over 35	44 (0.5)
Number of participants by regions	
North Central Coast	91 (1.0)
South Central Coast	3,753 (41.5)
Central Highlands	617 (6.8)
South East	4,249 (47.0)
Mekong Delta	329 (3.6)
Distribution of health service approach in this study by region ‡	
North Central Coast	3 (15.0)
South Central Coast	364 (22.3)
Central Highlands	78 (26.4)
South East	513 (27.5)
Mekong Delta	45 (28.7)
Doctor-diagnosed FA	
FA to 1 food group	264 (50.1)
FA to 2 different food groups	117 (22.2)
FA to more than 2 different food groups	146 (27.7)

‡ among participants with food-induced adverse symptoms. Percentage was calculated by dividing the number of participants with food allergy symptoms visiting health care services for allergy diagnosis by the total number of participants with food-induced adverse reactions in this survey. FA, Food allergy.

3.4.2 Reported food-induced adverse reactions and offending food groups

There were 6,563 (72.6%) respondents who experienced adverse clinical symptoms after food intake, with an average of 3.7 symptoms per respondent (Table 3.2). Symptom re-occurrences were reported in 48% of participants (Table D3.1 – Appendix D). Gastrointestinal symptoms were the leading complaint with the contribution of diarrhea (16.7%), followed by nausea or vomiting (12.2%) and stomach pain (10.6%) (Table 3.2). Systemic reactions and skin problems were the most common reasons for medical service visits/ hospital admission (Table 3.3). The study reported different rates of participants using medical services for their allergy problems, across studied regions (Table 3.3).

Table 3.2 Reported clinical adverse reactions caused by food consumption in adults (*n* = 6,563) in descending order of prevalence.

Symptom	**Response**	
	n	**%**
Diarrhea	**4,153**	**16.7**
Nausea or vomiting	**3,047**	**12.2**
Stomach pain	**2,650**	**10.6**
Hives	2,317	9.3
Sneezing	1,954	7.8
Odd taste in mouth	1,795	7.2
Nasal congestion or a running nose	1,708	6.9
Slight, dry cough	1,655	6.6
Trouble swallowing	1,299	5.2
Itchy mouth or ear canal	1,056	4.2
Chest pain	831	3.3
Shortness of breath or wheezing	600	2.4
Drop in blood pressure	426	1.7
Eczema	402	1.6
Redness of the skin or around the eyes	349	1.4
Swelling of the lips. Tongue and/or throat	307	1.2
Weak pulse	224	0.9
Loss of consciousness	142	0.6
Total	**24,915**	**100.0**

Table 3.3 The number of participants utilizing health services by clinical symptoms and the percentage of participants seeking medical advice by clinical symptoms (in descending order) among participant reported clinical symptoms caused by food consumption (*n* = 6,563).

Symptom	Number of participants seek medical advice (n)	Percentage of participants seek medical advice
Loss of consciousness	**51**	**94.4**
Redness of the skin or around eyes	**119**	**84.4**
Eczema	**130**	**75.1**
Weak pulse	71	73.2
Drop in blood pressure	116	64.8
Swelling of the lips, tongue and/or throat	87	63.5
Shortness of breath or wheezing	148	57.1
Hives	609	55.9
Chest pain	159	43.7
Itchy	203	39.3
Trouble swallowing	225	38.5
Stomach pain	435	38.1
Slight, dry cough	273	38.1
Nausea or vomiting	515	37.5
Nasal congestion or a runny nose	285	36.1
Sneezing	319	35.6
Odd taste in mouth	262	31.5
Diarrhea	578	30.9

The top three causative food items for allergic reactions belong to seafood groups: crustacean (28%), fish (15.2%) and mollusk (15.1%). Milk (9.5%) and beef (6.8%) were more common offending foods as compared to peanut (5.0%), wheat (5.0%), tree nut (4.6%), egg (3.8%) and soy (3.3%). Other reactive foods, besides beef, included animal meats (i.e., chicken, duck, dog and cat), fruits (i.e., mango, papaya and strawberry), vegetables (mostly chilli and mushroom) and alcoholic drinks (i.e., beer and wine), accounting for the remaining 10.2% (Table 3.4).

Table 3.4 Causative food groups evoking adverse reactions in this survey reported from 6,563 affected participants in descending order of prevalence.

Food group	n	% among food groups	% among participants
Crustacean	**1,835**	**28.0**	**24.9**
Fish	**995**	**15.2**	**13.5**
Mollusk	**994**	**15.1**	**13.5**
Other foods	750	11.4	10.2
Milk	701	10.7	9.5
Beef	504	7.7	6.8
Wheat	372	5.7	5.0
Peanut	371	5.7	5.0
Tree nut	337	5.1	4.6
Egg	279	4.3	3.8
Soy	241	3.7	3.3
Total	**7,379**	**100.0**	**100.0**

In this survey, of the 1,629 (18.0%) participants who perceived food allergy, only 617 subjects (37.9%) sought medical services for their health condition. Of the 617 medical services-seeking participants, 527 (85.4%) were diagnosed to have food allergy, indicating that 14.6% of the remaining adults might manifest food-induced adverse reactions (e.g. by food toxins) or could not be confirmed due to unavailable diagnostics. Among the doctor-diagnosed food allergy group, half of the participants reported adverse reactions to only one food item; 22.2% had reactions to two different food groups and the remaining 27.7% of food allergic patients had allergic reactions to more than two different food groups (Table 3.1).

3.4.3 Prevalence of self-reported and doctor-diagnosed food allergy

The survey data was weighted by gender according to the current distribution of male and female adults aged below 50 years in Vietnam (14) to estimate a more accurate prevalence of food allergy (Table D3.2 – Appendix D). As anticipated, the overall prevalence of food allergy for all survey food groups was more than twofold in self-reported than in doctor-diagnosed participants (11.8% *vs.* 4.6%) (Table 3.5).

Crustacean, fish and mollusk were the top three allergy-triggering foods. The pattern of allergy-offending foods was the same for both the self-reported and doctor-diagnosed groups, except for milk. Combining the data from crustacean and mollusk allergy indicated a prevalence of 10.0% (95% CI: 9.4-10.6) and 4.2% (95% CI: 3.8-4.6) for shellfish allergy in the self-reported and doctor-diagnosed groups, respectively.

Table 3.5 Weighted prevalence of FA in study population

	Self-reported FA	Doctor-diagnosed FA
Any food	11.80 (11.14-12.47)	4.55 (4.12-4.98)
Crustacean	6.88 (6.36-7.40)	2.95 (2.60-3.30)
Fish	3.71 (3.32-4.10)	1.58 (1.32-1.84)
Mollusk	3.09 (2.73-3.44)	1.27 (1.04-1.50)
Beef	2.09 (1.80-2.39)	0.95 (0.75-1.15)
Milk	1.66 (1.40-1.92)	0.46 (0.32-0.60)
Egg	1.04 (0.83-1.25)	0.65 (0.49-0.82)
Wheat	1.06 (0.85-1.27)	0.37 (0.24-0.49)
Peanut	0.89 (0.69-1.08)	0.32 (0.20-0.44)
Soy	0.81 (0.62-0.99)	0.31 (0.20-0.42)
Tree nut	0.77 (0.59-0.96)	0.25 (0.15-0.36)
Other foods	2.05 (1.75-2.34)	0.66 (0.50-0.83)

Value reported as % (95% CI). FA, food allergy.

‘Any food’= any food groups other than listed in the questionnaire including ‘other foods’. ‘Other foods’ = other food groups not listed in the questionnaire. Other food commodities reported in the survey are animal meat (i.e. chicken, duck, dog and cat), fruits (i.e. mango, papaya and strawberry), vegetables (mostly chili and mushroom) and alcoholic drinks (i.e. beer and wine).

3.4.4 Clinical features of food allergy

Clinical features of doctor-diagnosed food allergic participants are presented in Figure 3.1. Allergic subjects presented with multiple adverse symptoms involving different organs (an average of 5.5 symptoms per subject). Cutaneous symptoms (hives/urticaria, eczema) were dominant, present in 87.8% of all confirmed food allergic subjects, followed by gastrointestinal symptoms (diarrhea, nausea/vomiting and

stomach pain). Manifestations of severe reactions (i.e. loss of consciousness, weak pulse, drop in blood pressure, chest pain) was not rare among these subjects, accounting for up to 38.9% of all affected participants.

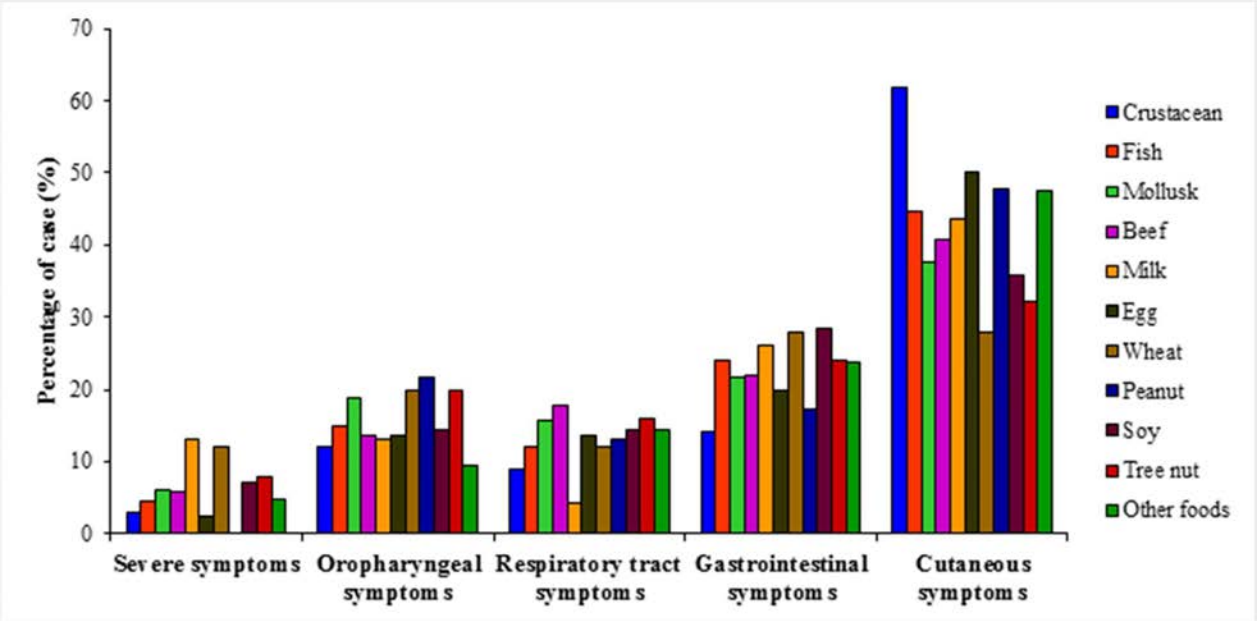

Figure 3.1 The distribution of clinical manifestations among doctor-diagnosed food allergic participants (*n* = 506) by food allergen type.

Clinical symptoms are divided into five categories: severe symptoms (loss of consciousness, weak pulse, drop in blood pressure, chest pain); oropharyngeal symptoms (trouble swallowing, itchy mouth or ear canal, odd taste in mouth, swelling of the lips, tongue and/or throat, redness of the skin or around eyes); respiratory tract symptoms (sneezing, nasal congestion or a runny nose, coughing); gastrointestinal symptoms (nausea or vomiting, diarrhea, stomach pain) and cutaneous symptoms (hives, eczema). 'Other foods': other food groups that were not listed in the questionnaire. Other food commodities reported in the survey are animal meat (i.e. chicken, duck, dog and cat), fruits (i.e. mango, papaya and strawberry), vegetables (mostly chili and mushroom) and alcoholic drinks (i.e. beer and wine).

3.4.5 Influence of demographic factors on the risk of having food allergy

The influence of demographic factors on food allergy was analyzed by multivariable logistic regression (Table 3.6). Predictor variables were gender, family history of food allergy and co-existence of other allergic diseases, while the outcome variable was doctor-diagnosed food allergy. Family history of food allergy was shown to be the strongest predictor of doctor-diagnosed food allergy (*OR,* 8.0, *P* < .001), while co-existance of other allergic diseases (*P* = .734) and gender (*P* = .082) did not show any significant associations with doctor-diagnosed food allergy rate.

Table 3.6 Multivariable logistic regression analysis of demographic factors to food allergy.

	Risk factor, *OR* (95% CI)	***P* - value**
Sex (*Female/Male*)	1.2 (1.0 - 1.5)	**.082**
Family history of FA (*Yes/No*)	8.0 (6.2 - 10.4)	**< .001**
Co-existing other allergic diseases (*Yes/No*)	1.0 (0.8 - 1.3)	.734

Binary logistic regression was performed in SPSS Statistics for Windows to generate *ORs*. A *P*-value of <.05 was considered as statistically significant and highlighted in bold. FA, food allergy.

The relationship between living location and the incidence of having a doctor-diagnosed food allergy was analyzed using Chi-square tests. The difference in overall food allergy incidence was recorded between the South Central Coast and the South East (*P* < .001), between the Central Highlands and the Mekong Delta (*P* < .05) and between South East and Mekong Delta (*P* < .001) (Figure 3.2). Specifically, the prevalence of food allergy in the Mekong Delta (9.7%) was much higher than in the other study sites: South East region (7.1%), South Central Coast (4.3%), North Central Coast (3.3%) and Central Highlands (4.7%). Taking into consideration the impacts of population density, lifestyle and living environment, participants from the South East - mostly residing in Ho Chi Minh City, the biggest city in Vietnam (14)- were defined as people living in an urban area, and participants from other survey sites were considered as living in a rural area. We observed a higher prevalence of crustacean,

fish, mollusk, beef and other food allergies ($P < .001$) in the South East, as compared to the other study sites (Figure 3.3).

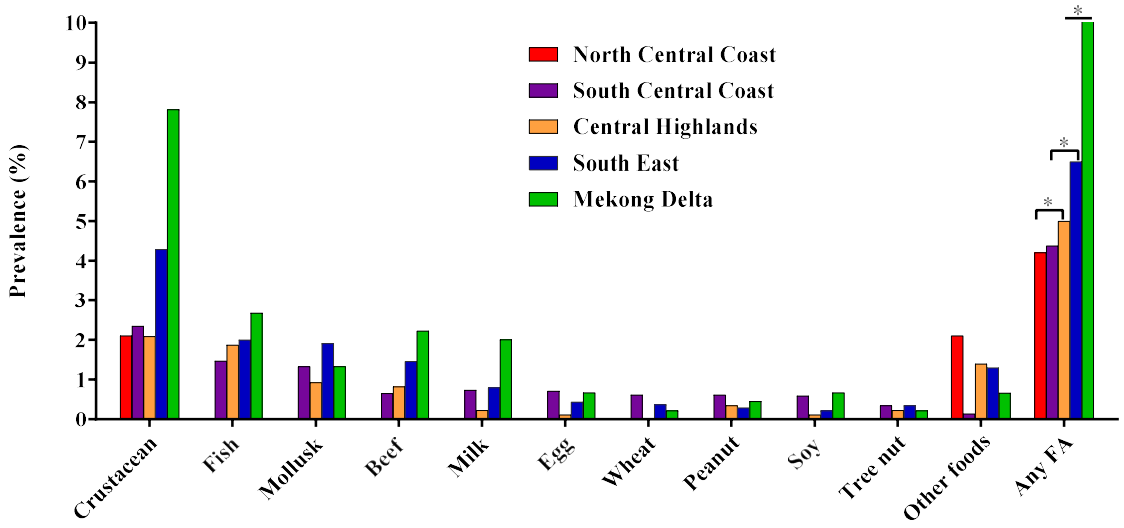

Figure 3.2 Distribution of the prevalence of doctor-diagnosed food allergies across different regions in Vietnam. Statistical significance was recorded between North Central Coast (n = 91) and Central Highlands (n = 617) ($P < .001$), between South Central Coast (n = 3,753) and South East (n = 4,249) ($P <.001$); between South East (n = 4,249) and Mekong Delta (n = 329) ($P < .001$), and between Central Highlands (n = 617) and the Mekong Delta (n = 329) ($P < .05$). 'Other foods': other food groups that were not listed in the questionnaire. Other food commodities reported in the survey are animal meat (i.e. chicken, duck, dog and cat), fruits (i.e. mango, papaya and strawberry), vegetables (mostly chili and mushroom) and alcoholic drinks (i.e. beer and wine). 'Any FA': any food groups other than listed in the questionnaire including 'other foods'. '*' denotes statistical significance ($P < .001$).

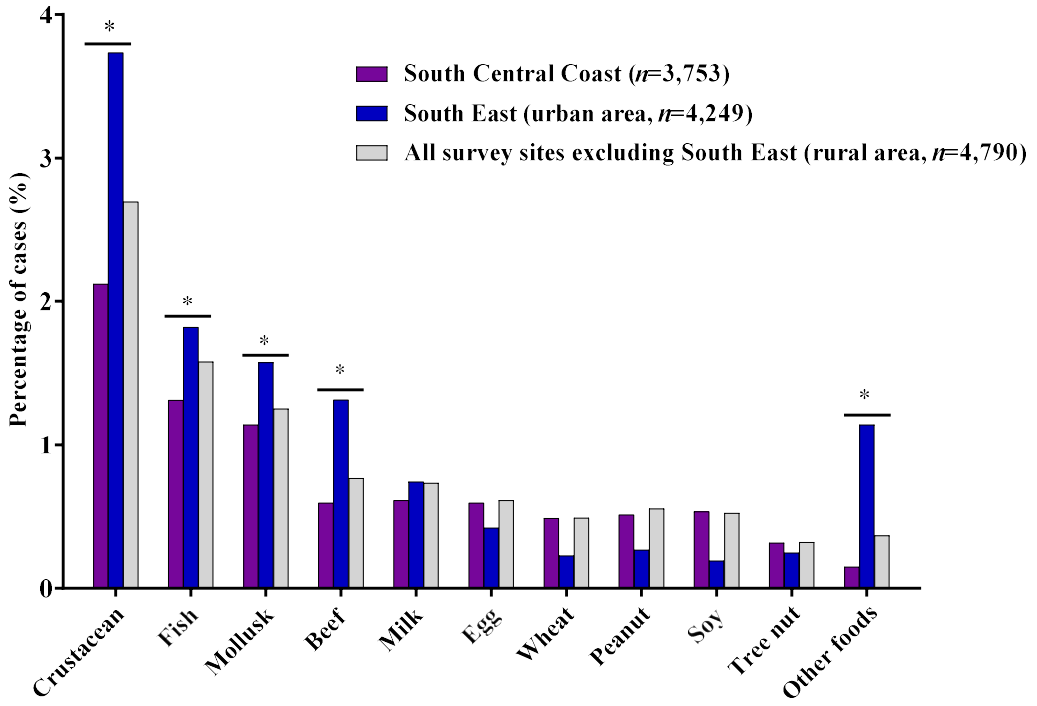

Figure 3.3 Distribution of the prevalence of doctor-diagnosed food allergies among two major survey sites: South Central Coast (*n* = 3,753) and South East (*n* = 4,249). Survey data were combined to generate the prevalence of food allergies for the population living in the rural areas of Vietnam (*n* = 4,790). Taking into consideration the population density and lifestyles, participation from the South East (mostly residing in Ho Chi Minh City, the largest city in Vietnam) were considered to be living in an urban area. The urban population demonstrated a higher risk of being sensitized to seafood, beef and some other foods (*P* < .001). 'Other foods': other food groups that were not listed in the questionnaire. '*' denotes statistical significance (*P* < .001).

3.5 Discussion

This study determined the lifetime prevalence of doctor-diagnosed food allergy among Vietnamese adults to be 4.6% (95% CI, 4.1-5.0), which is lower than the frequency of 6.4% previously reported in Taiwanese adults (6). The pattern of food allergies observed revealed seafood to be the most common food allergy culprit, consistent with findings in Korean adults (15) and the current trend among US adults (16). Our study demonstrated the disparity of food allergy across geographic locations ($P < .001$), implying the possible influence of environmental exposures and dietary habits to allergy risk. Additionally, a family history of food allergy was strongly associated with food allergy incidence (*OR,* 8.0; 95% CI, 6.2-10.4) but not for other allergic comorbidities or gender. These findings would be of great interest to local clinicians, researchers and policy makers and benefit towards a better management of food allergy in this country.

We noted a wide gap between people with suggestive food allergy symptoms and those who approached medical advice for food allergy diagnosis. Specifically, less than half of the self-reported food allergic subjects in this survey ever visited doctors for their medical condition. While most people who visit a medical practitioner are confirmed to have food allergy, there is a high proportion of people with food allergy who do not seek advice. These people remain undiagnosed and untreated, leaving them at risk of unexpected food allergy reactions, which could be fatal. In the current context of Vietnam, the low rates of presentation for suspected allergy symptoms may be explained by insufficient awareness in the general public about food allergy, and/or the possible shortage of medical services providing allergy testing.

Manifestations of food allergy among adults in the study varied according to the causative allergen (Figure 3.1). Among food allergy events, the major manifestation of food allergy in Vietnamese adults involved cutaneous symptoms (42.7%). Hives was the major indicator of an allergic condition for all food allergens in the study, and is consistent with previous studies (15) and the EAACI guidelines (17). The second most frequent food allergy manifestation was gastrointestinal symptoms, induced more by foods of plant-based origin than animal-based origin, in this study. We also noticed that plant-origin foods were the major cause for oral allergy syndrome in the doctor-

diagnosed food allergy group. However, milk and wheat were the leading causative food items that evoked severe food allergy events/anaphylaxis; milk and wheat allergy were reported at 0.46% and 0.37%, respectively. Previous studies showed that the majority of food-induced anaphylaxis in adults was caused by plant foods such as wheat, peanut and tree nut (18, 19). Thus, presenting severe milk-inducing food allergic reactions is rather unusual in adults. Lactose intolerance is common in the Asian population (20) and is undoubtedly presumed to be the major reason for any adverse symptoms evoked by milk consumption. However, in a recent investigation of food allergy in Israeli patients, milk-induced anaphylaxis was reported and confirmed in adults who reported to previously tolerate that food (11). This finding is of importance for clinicians and food allergy specialists, as well as adult patients with milk allergy, in addressing the significant risk of anaphylactic and possibly fatal reactions.

In our study, seafood allergy clearly accounted for more than half of all food allergy cases. The rate of perceived shellfish allergy in Vietnamese adults (10.0%) is higher than the rate previously reported in Taiwanese counterparts (7.05%) (6). We also demonstrated a doctor-diagnosed shellfish allergy prevalence of 4.2%, which is the highest rate of shellfish allergy in adults reported worldwide (21). Shellfish is a common food source in the Asia Pacific and has been claimed to be the leading allergic food in this region (22). A retrospective survey in Korean patients demonstrated seafood, including crustacean, cephalopod and fish, to be the most frequent cause of food allergy and seafood-induced anaphylaxis in adults (51.1%) (15). Similar findings were reported in both Taiwanese children and adults with food allergy (6). Although there are limited robust studies to investigate the evolution of seafood allergy throughout a life course, we noticed a strikingly high rate of shellfish allergy in both children and adults in Asia (23). Shellfish allergy was reported in very young children aged 3 months to 6 years in Thailand (0.3%) (24), and appears to increase in other older children. School-age children from Vietnam showed a prevalence of 3.83% to crustacean and 1.03% to mollusk allergy (25), while shellfish allergy rates were 5.12% and 5.23% in Filipino and Singaporean adolescents, respectively (26). There are several hypotheses in circulation to explain the elevated of shellfish allergy in the Asia Pacific, the main one implicating the high abundance and consumption of this food commodity (9). Furthermore, the tropical climate might play a role in favoring the abundance of indoor

creatures (e.g. house dust mites and cockroaches) (27) that can cause clinical cross-reactivity of indoor allergens with the allergens in shellfish (e.g. tropomyosin) (28, 29).

Similarly, we found a higher rate of doctor-diagnosed fish allergy (1.58%) in this cohort than previously reported in the US (0.8%) (16) and Canada (0.56%) (30). The self-reported fish allergy in Vietnamese adults (3.71) is much higher than in Taiwan (1.17%) (6). The identified prevalence of seafood allergy in Vietnamese adults appears to surpass the highest rates established in any published study from Northern America, Europe and Asia (i.e. Taiwan) (31). One plausible explanation is the availability and abundance of this food commodity in Vietnam as a major source of animal protein (32). The Vietnamese consume an average of 33 kg seafood per capita per year in comparison to 22 kg in North America and Europe (33). A correlation between seafood consumption rate and the prevalence ofseafood allergy across different survey sites was observed (Figure 3.2) (34). Another potential cause might be the allergic reaction to *Anisakis,* a food-borne parasitic nematode frequently contaminating fish (35). Although no specific case of *Anisakis* infection has been reported in Vietnam, parasite infection via seafood vectors are commonly reported (36, 37). The presence of this food-borne allergen seems to be particularly common in raw and undercooked fish, and was reported to cause infection and allergic reactions in Thailand, Korea and Japan (37-39).

The current study identified beef as the fourth most common allergy-inducing food. A strong correlation of beef allergy with previous tick bites has been previously identified in Australia, Europe and the US (40, 41). The observed anaphylactic reactions were explained by the production of specific IgE antibodies to galactose-α-1,3-galactose (α-Gal), a carbohydrate present in red meat. While no reports of tick bites in Vietnam have been published, ticks are very common in the region (42) and could be a new, unidentified cause of beef allergy in Asia.

Food allergy is thought to be controlled, at least in part, by the interaction between genetic and environmental factors. When family history, atopy, sex and living location were considered, we observed that a family history of food allergy was the strongest predictor for food allergy in adults. This finding is in line with previous population-based studies in infants where the investigators revealed that having two or more allergic

family members increased the risk of having food allergy in the child (*OR,* 1.8; 95% CI 1.5-2.3) (43). Furthermore, the geographical location can have a profound impact on allergen exposure, thus increasing the risk of developing atopic conditions (44). In this study, we noted the variation of food allergy incidence among different geographic regions of Vietnam, with a higher incidence of food allergy among people living in urban areas compared to rural areas ($P<0.001$). This observation supports the hypothesis that there are possible protective influences in the rural environment, and postulated mechanisms include the hygiene hypothesis (45).

The major limitation of this study is that the information for doctor-diagnosed food allergy was self-reported. Furthermore, it is not known if the physicians diagnosing food allergy in this study group utilized the currently available food allergy diagnostic tests. It would be ideal to confirm the allergic responses in suspected participants with diagnostic methods, including allergen specific serum IgE quantification and oral food challenge. However, the initial scope of this study was to evaluate the current situation of food allergy in Vietnam and to approach affected food allergy patients. The manifestation of true food allergy among Vietnamese patients is currently under investigation by the authors, using established *in vivo* and *in vitro* diagnostics.

This survey gained a slightly lower response rate (62.4%) than previous studies on food allergy in other Asian countries: 67.9% in Singapore, 81.1% in the Philippines and 80.2% in Thailand (46). We did not conduct any further investigations on the non-response group. We assume that paper-based questionnaire surveys might be unpopular with many Vietnamese. In addition, limited information and/or awareness of food allergy in the public might influence the response rate. We are also aware that the selection of university students might misrepresent the general Vietnamese adult population. A weak correlation of education level to the incidence of food allergy was demonstrated in US adults (*OR,* 1.06; 95% CI, 1.03-1.09) (47). Furthermore, three different universities in five different geographical regions participated, including over 50,000 students from different age groups and diverse cultural backgrounds. Therefore, the sample selection enabled this study to gain objective and representative data on food allergy in Vietnam.

In conclusion, this study provides the first population-based data on food allergy in the adult Vietnamese population. Our findings revealed the dominance of seafood allergy and the commonality of beef allergy as a new allergen source to be reported among adults in the Asian population. This study also suggests that under-diagnosis and under-treatment of food allergy may occur, owing to low rates of presentation to medical services for food allergy in Vietnam.

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3.7 Chapter 3 summary

From this chapter, there were several findings, as listed by the study aims below:

- Determine the prevalence of food allergies in Vietnamese adults.
  - ➔ The overall prevalence of self-reported food allergy in Vietnamese adults is 11.8% whilst the prevalence of doctor-diagnosed food allergy is less than half the perceived rate (4.6%). This phenomenon was seen in the similar population-based survey in Vietnamese children (Chapter 2) and has been reported in previous studies. This observation suggests the need to combine clinical history with other *in vitro* and *in vivo* tests to identify true food allergy. In Chapter 5 of this thesis, sera from participants with a history of seafood allergy were collected and analyzed for specific IgE reactivity to a panel of commonly consumed crustacean, mollusk and fish species.

- Identify the distribution of the 'Big 8' food allergens in Vietnamese adults.
  - ➔ This study identified crustacean, mollusk, fish and beef as the most common allergy-triggering foods in Vietnamese adults. Less common food allergens were egg and milk; whilst a very low rate of participants reported adverse reactions to wheat, peanut, soy and tree nut. A similar allergen pattern was seen among Vietnamese children and adults, especially the top four leading food allergens as mentioned above. Seafood is the most predominant food allergy type in Vietnamese adults and this finding is consistent with previous reports from population-based surveys in adults in Taiwan and Korea. More interestingly, beef allergy is a new, unreported food allergy in Asia.

- Identify the clinical manifestations of food allergy.
  - ➔ Food allergy has a broad spectrum of clinical presentations and occurs in different organs. In the present study, skin problems including hives/urticarial or eczema were the most reported allergic reactions caused by all investigated food items. The second most common clinical manifestation was gastrointestinal symptoms such as nausea/vomiting, diarrhea or stomach pain. Most of the participants reported to be suffering from multiple

symptoms at the same time. Milk and wheat were reported to be the top triggers for severe adverse reactions among study population.

- Identify determinants to the incidence of food allergy.
  ➔ By conducting the food allergy survey across different regions in Vietnam, contributing factors for having food allergy were evaluated and identified. In this subpopulation, we observed that people who have an intermediate family member with food allergy are eight times more likely to have a food allergy. Other demographic factors such as gender and atopic condition did not show any associations. People in urban areas have a higher rate of seafood allergy and beef allergy than those in rural areas.

- Compare the prevalence of food allergy between children and adults in Vietnam
  ➔ In general, Vietnamese children have higher rates of doctor-diagnosed food allergy (6.7%) than adults (4.6%), which is consistent with previous publications on food allergy. It has been postulated that the immature immune system in children and the loss of many types of food allergy at a later age may account for this difference (48).

CHAPTER 4. VALIDATION OF FOOD ALLERGY SURVEY OUTCOMES FROM TWO SURVEY MODES: WEB-BASED SURVEY AND PAPER-BASED SURVEY

Published in:

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4.1 Introduction

Food allergy is a growing public health concern worldwide, affecting the wellbeing and quality of life of up to 5% adults and 10% children in the general population (1). Food allergy has received much attention in Western countries due to the high prevalence and severity of food-related anaphylaxis, especially in young children (2, 3). Many of these countries have comprehensive healthcare initiatives to help manage food allergy, for instance the HealthNuts Birth Cohort in Australia (4), EuroPrevall in the European community (5) and National Health Interview Survey in the US (6). These national/multinational programs have contributed enormously to improve the quality of life of affected people as well as raise public awareness of food allergy.

In other parts of the world, food allergy studies remain limited (7). For example, in Asia, only a few countries have available data on food allergy (8). Though food allergy has been considered as a problem resulting from modern lifestyles, recent studies in Asian communities revealed high prevalence rates of food allergy compared to findings in Europe and US, along with unique food allergen patterns (8). For instance, allergies to peanut and tree nut are the most common cause of food-induced anaphylaxis and death in children from Western countries (9), whereas these allergies are very low in Singapore and the Philippines (10). Furthermore, many developing countries lack food allergy management policies and medical readiness for appropriate interventions (11). This raises concerns about potential impacts of food allergy on population health in developing countries and emerging economies.

The paucity of food allergy epidemiologic data in developing countries is likely due to monetary constraints. Conventional epidemiological study methods such as telephone surveys, postal surveys or interview surveys often require a good infrastructure and substantial capital funding for implementation (i.e. employment of executive staff, development of survey programs and logistics) (12). In addition, population-based surveys are often a prolonged process, normally requiring from one to five years to yield the desired outcomes. The recent information technology explosion concomitant with an increase in internet penetration worldwide has resulted in the advent of web-based survey (WBS) as a new, cost-saving survey mode (13, 14). In the field of food

allergy, the first WBS was conducted in Greece in 2006 with the participation of 3,673 adult subjects (15). The survey data was collected after three months of implementation with low investment costs. However, one of the biggest concerns with WBS is the validation of its generated data compared to traditional survey methods. Many comparative studies have been conducted assessing the benefits of the WBS in the context of cost efficiency and time management. Yet, no studies have been implemented to validate the quality of WBS data over other traditional survey types.

In the present study, we assessed the data collected from two survey modes: WBS *versus* paper-based survey (PBS) on food allergy in Vietnamese adults. The surveys were conducted at different locations throughout Vietnam to determine the contribution of environmental factors (i.e. rural *vs.* urban) to food allergy incidence in this developing country. The main outcomes of the two independent surveys were compared, including demographic features of participants, distribution of food-induced adverse reactions, prevalence of self-reported food allergy, doctor-diagnosed food allergy and IgE-mediated food allergy, distribution of food allergens and the association of demographic factors with food allergy. This study sought to evaluate the possible application of WBS for future epidemiological studies, especially in developing countries.

4.2 Aims of this chapter

- Compare the food allergy survey outcomes from two population-based survey modes: web-based survey and paper-based survey.

4.3 Methods

4.3.1 Study design

Two population-based surveys (WBS and PBS) were conducted in an identical population aged 16-50 years to evaluate the current prevalence and pattern of food allergy in Vietnamese adults. Both survey modes used the same questionnaire to collect data. Study populations were randomly selected by cluster sampling method from a list of university students in two main regions: Khanh Hoa province and Ho Chi Minh City. Furthermore, these students were also divided based on specific areas they originally came from, to assess the possible impacts of environmental factors on food allergy incidence. Participants were invited to one survey mode only. The surveys were anonymous and voluntary for all participants. The study design and survey procedure were reviewed and approved by the Human Research Ethics Committee at James Cook University (Approval ID: H6437 – Appendix A1).

4.3.2 Paper-based food allergy survey

The paper-based food allergy survey was conducted from March to December 2016. Questionnaires were distributed to the target population and most of the answer sheets were collected on the same day. By accepting to answer the questionnaire, the participant gave their consent to the study. The response rate was calculated by dividing the number of returned questionnaires by the total distributed.

4.3.3 Web-based food allergy survey

Students' email addresses were randomly selected from a list of more than 35,000 participating students. These email addresses were assigned by participating universities (Gmail, supplied by Google). Official approvals for using the students' email in this study were obtained before conducting the survey.

An invitation letter with detailed information about the study was randomly sent to 6,000 email addresses from March to May 2016. By clicking an email link to the questionnaire, participants gave their consent to the study. The waiting period for collecting the first response was two weeks. Another reminder email was automatically

sent to the participant after two weeks to complete the survey, with an additional waiting time of two more weeks. Participants were invited to the survey once only and asked to disregard the reminder emails if they had already completed the questionnaire.

The WBS was designed by using Google Forms. The Google account foodallergy.vn@gmail.com for this study was set up and managed by the lead investigator to collect survey responses. Each IP address could only access the questionnaire once. Survey responses were collected anonymously and saved in the designed platform. The survey responses were backed up in Microsoft Excel for further analysis.

4.3.4 Questionnaire design

Both WBS and PBS used the same set of questionnaires that had been described in Section 3.3.3 in Chapter 3 of this thesis (Appendix B2).

4.3.5 Definition of food allergy in the surveys

The definitions of self-reported food allergy, doctor-diagnosed food allergy, IgE-mediated food allergy were used in this study had been mentioned in Section 2.3.5 in Chapter 2 of this thesis. In brief, participants who answered ‘yes’ to questions 1 to 4 in part II of the questionnaire were considered to have self-reported food allergy; participants who answered ‘yes’ to questions 1 to 6 were identified as the individuals with doctor-diagnosed food allergy; and participants who exhibited the typical symptoms for IgE-mediated food allergy, including hives/urticaria or angioedema or anaphylaxis reactions (i.e. drop in blood pressure, loss of consciousness, chest pain and weak pulse) after food intake (16), and answered ‘yes’ to questions 2 to 6 were considered to have IgE-mediated food allergy. The lifetime prevalence of self-reported food allergy, doctor-diagnosed food allergy and IgE-mediated food allergy was estimated.

4.3.6 Statistical analysis

Survey data were imported to the IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, N.Y., USA) for statistical analysis. Continuous variables were expressed as mean ± SD. Categorical data were calculated to generate prevalence rates. The prevalence rate was calculated to provide a 95% CI of responses to each criterion.

Comparative analysis of the same variables (i.e. food allergy prevalence, distribution of clinical symptoms, allergy-triggering food groups and multivariable logistic regression analysis results) between the two survey modes was performed by either two-tailed *t*-test or *z*-test. 95% CIs were calculated to interpret the difference in proportion or ORs. Statistical significance was considered at a *P* value of < .05 for all tests.

4.4 Results

4.4.1 Comparing the demographical data between two survey modes

1,854 adult participants answered the questionnaire from the WBS compared to 9,039 responses from adult participants in the PBS (Figure 4.1).

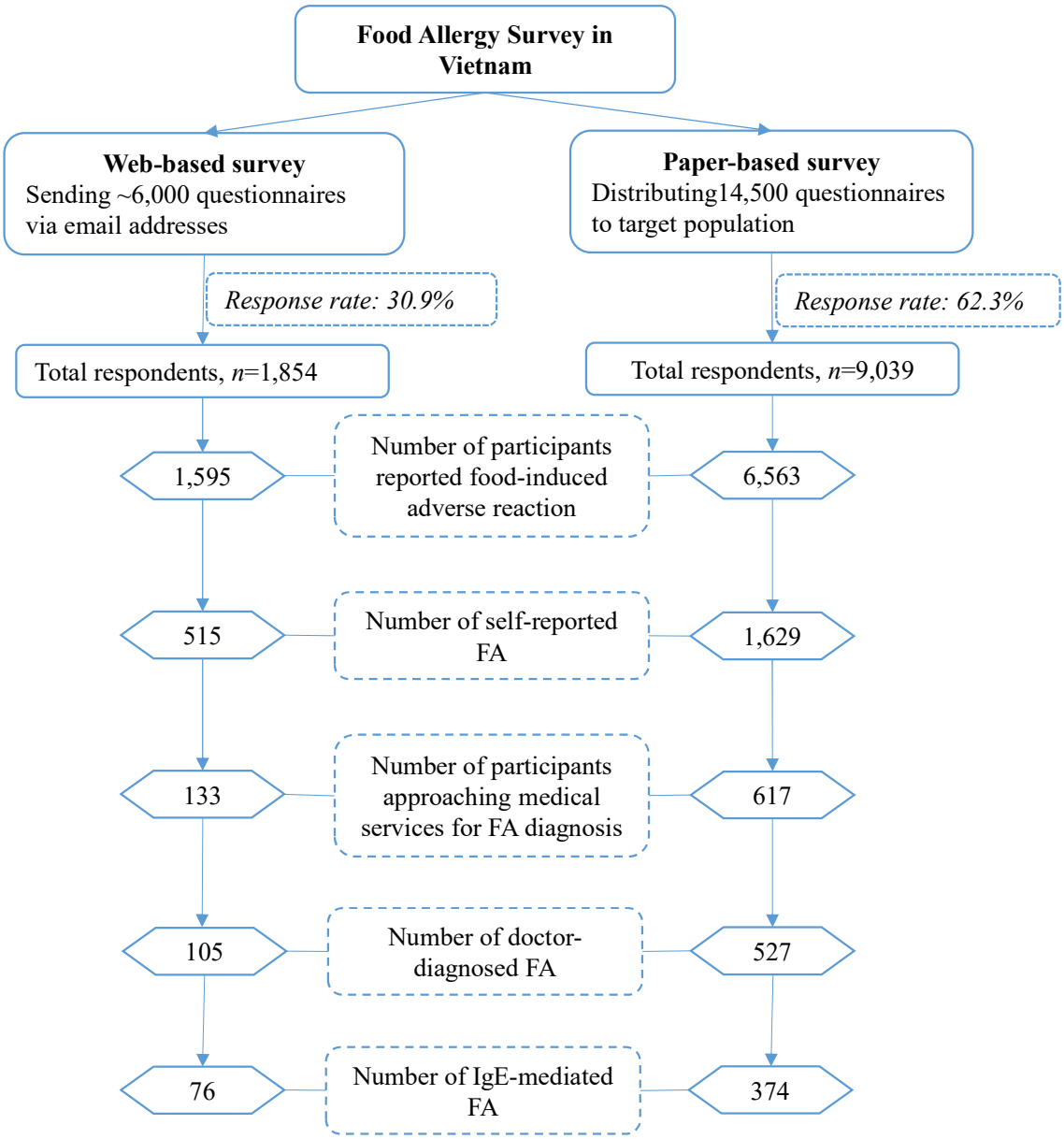

Figure 4.1 Flow diagram showing the surveys on food allergy in Vietnam. The survey was performed by two modes: web-based survey and paper-based survey.

Overall, PBS gained a higher response rate than WBS (62.3% *vs*. 30.9%). The two survey modes showed the predominance of female participants: 61.7% in the WBS and 67.3% in the PBS. The average age of participants was 21.6±3.4 years (WBS) and 19.8±2.5 years (PBS) (Table 4.1).

Table 4.1 Demographic features of adult participants in the two survey modes.

	Web-based survey	**Paper-based survey**	**Difference, *P* value**
Number of respondent, n	1,854	9,039	-
Sex distribution, n (%)			
Male	711 (38.3)	2,955 (32.7)	< .001
Female	1,143 (61.7)	6,084 (67.3)	
Age, mean ± SD	21.6±3.4	19.8±2.5	< .001

4.4.2 Comparing the distribution of clinical manifestations and food triggers between the two survey modes

There were more people suffering from food-induced adverse reactions in the WBS (86.0%) than in the PBS (72.6%). The difference was seen in the number of perceived food allergy: 27.8% (WBS) *vs*. 18.0% (PBS) and the number of participants with perceived food allergy seeking medical advice: 25.8% (WBS) *vs*. 37.9% (PBS) between the two survey modes. However, the two surveys had very similar prevalence of doctor-diagnosed food allergy (WBS: 5.7%; PBS: 5.8%) and IgE-mediated food allergy (4.1% for both WBS and PBS) (Figure 4.1).

The proportion of clinical symptoms reported in the two surveys are presented in Figure 4.2. Generally, the two study modes gained a very similar contribution of clinical symptoms in all defined groups in this study. While diarrhea was the most common adverse symptom reported in the general study population and in the self-reported food allergy group, hives was the dominant symptom in doctor-diagnosed food allergy and IgE-mediated food allergy in both survey modes.

In terms of triggering food items, no significant difference was seen in the contribution of food items in the surveys in regard to clinical symptoms. Seafood including fish, crustacean and shellfish stood out as the major triggering food items for food-induced adverse symptoms as well as doctor-diagnosed food allergy and IgE-mediated food allergy in both survey modes (Figure 4.3). Minor differences were seen for other food groups, where there were more cases reported in the WBS than in the PBS.

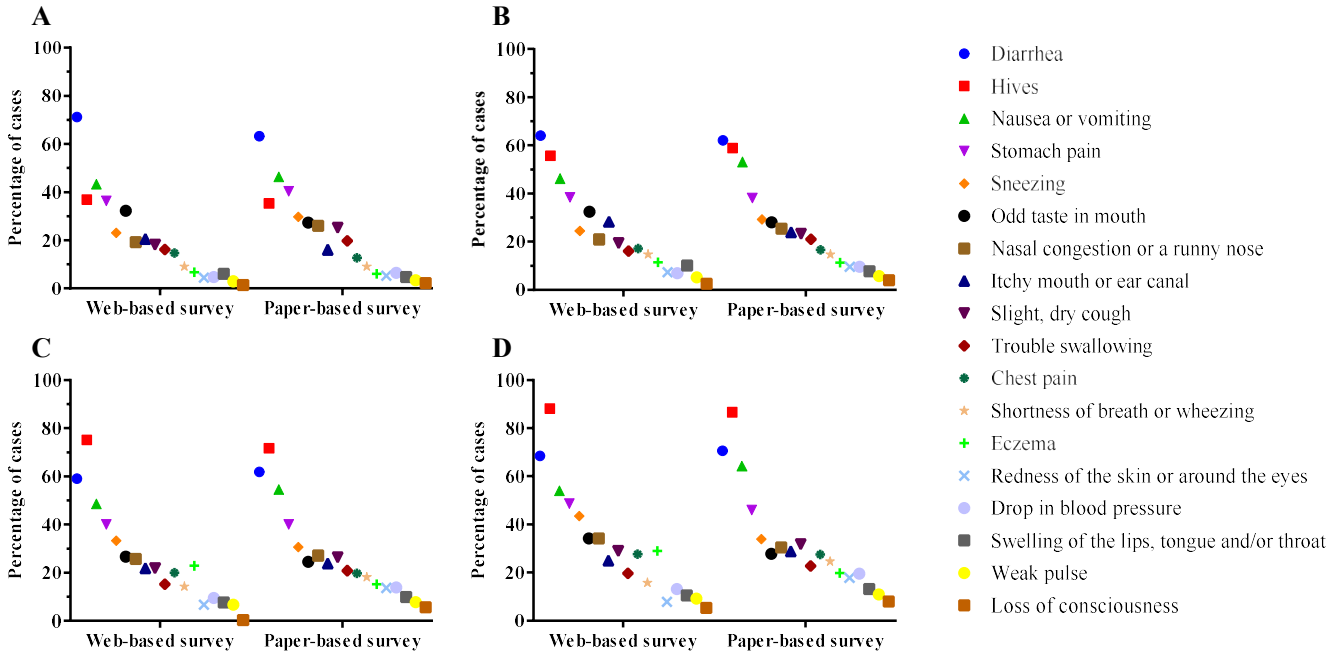

Figure 4.2 Proportion of clinical symptoms reported in two population-based survey modes.

(A) Reported adverse reactions caused by food consumption in the web-based survey (*n* = 1,595) and paper-based survey (*n* = 6,563). (B) Reported adverse reactions in self-reported FA participant in the web-based survey (*n* = 515) and paper-based survey (*n* = 1,629). (C) Reported adverse reactions in doctor-diagnosed FA participants in the web-based survey (*n* = 105) and paper-based survey (*n* = 527). (D) Reported adverse reactions in the IgE-mediated FA group in the web-based survey (*n* = 91) and paper-based survey (*n* = 433). The criteria to define IgE-mediated FA include: anaphylaxis reactions (i.e. drop in blood pressure, loss of consciousness, chest pain and weak pulse) or hives/urticaria or angioedema or anaphylaxis reactions after food intake. FA, food allergy.

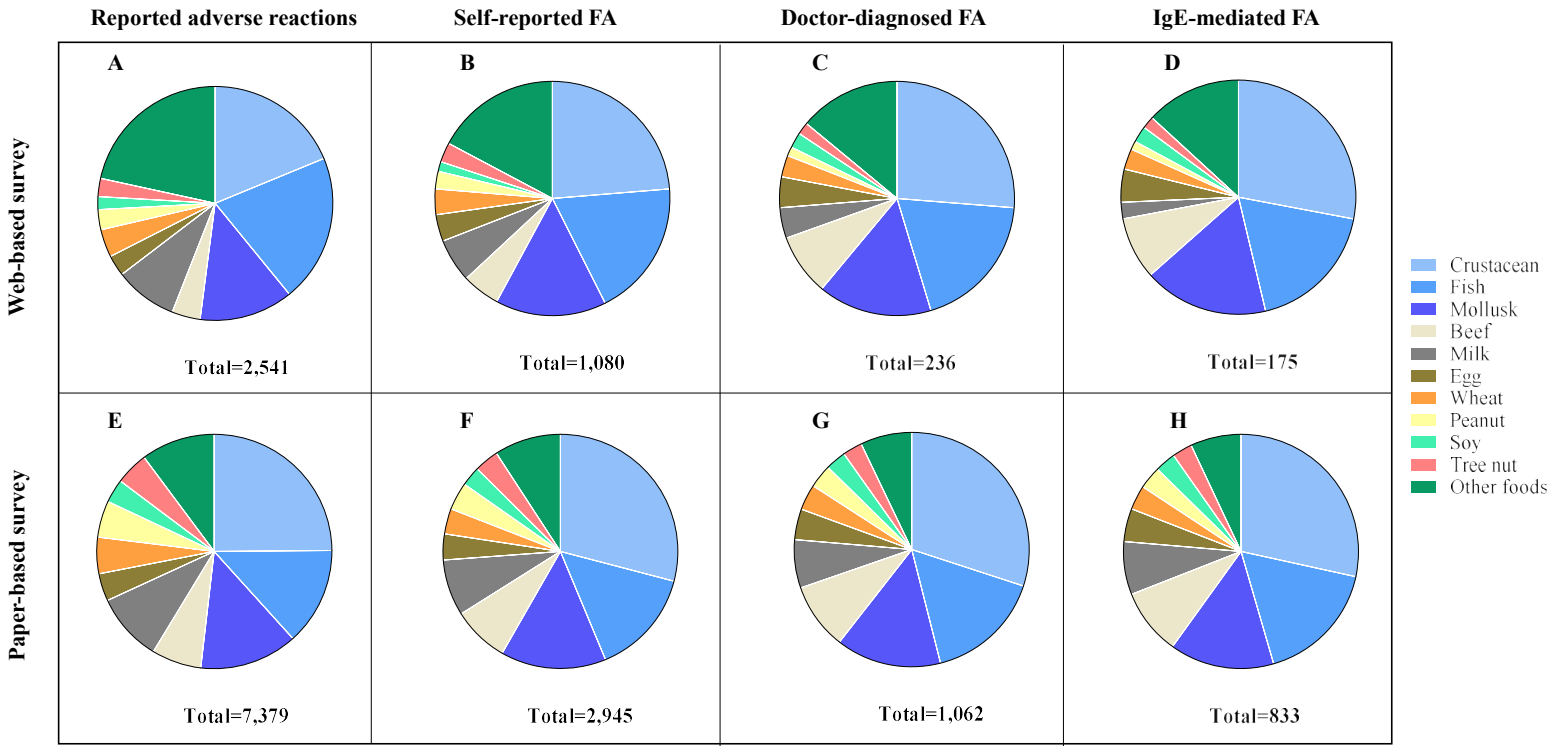

Figure 4.3 Comparison of the distribution of reported food items eliciting clinical adverse reactions in two survey modes.

In the web-based survey: (A) Reported food-induced adverse reactions (number of participants *n* = 1,595); (B) Self-reported FA (*n* = 515); (C) Doctor-diagnosed FA (*n* = 105) and (D) IgE-mediated FA (*n* = 91). In the paper-based survey: (E) Reported food-induced adverse reactions (*n* = 6,563); (F) Self-reported FA (*n* = 1,629); (G) Doctor-diagnosed FA (*n* = 527) and (H) IgE-mediated FA (*n* = 433). FA, food allergy.

4.4.3 Comparing the prevalence of food allergy between the two survey modes

The prevalence of self-reported food allergy, doctor-diagnosed food allergy and IgE-mediated food allergy were calculated based on the defined criteria of the study (see material & method section). The prevalence rates were generated from crude data and the difference of these proportions was analyzed by two-tailed *z*-test between the two independent populations (Table 4.2)

In the self-reported food allergy group, the two survey modes gained statistically different prevalence for most food items ($P < .001$), except in the cases of beef, peanut, soy and tree nut. However, in the doctor-diagnosed food allergy and IgE-mediated food allergy groups, the differences were seen in the prevalence of food allergy to other foods (doctor-diagnosed food allergy) ($P < .001$), as well as beef and tree nut allergy (IgE-mediated food allergy) ($P < .01$). There was no statistical evidence for the differences in food allergy prevalence between the two survey modes, with accepted of a type II error of 0.05. Additionally, when considering the 95% CIs of the prevalence from each variable, there was no difference in the prevalence of food allergy between WBS and PBS. In summary, regardless of the survey modes and the different response rates, the WBS and PBS reported very similar prevalence rates of most of food allergy types in this study.

Table 4.2 Prevalence of food allergy in Vietnam.

	Self-reported FA			Doctor-diagnosed FA			IgE-mediated FA		
	Web-based survey	**Paper-based survey**	**Difference, *P***	**Web-based survey**	**Paper-based survey**	**Difference, *P***	**Web-based survey**	**Paper-based survey**	**Difference, *P***
Any food	27.8 (25.7-29.8)	18.0 (17.2-18.8)	0.0000	5.7 (4.6-6.7)	5.8 (5.4-6.3)	0.7795	4.1 (3.2-5.0)	4.1 (3.7-4.6)	0.9590
Crustacean	13.8 (12.2-15.4)	9.5 (8.9-10.1)	0.0000	3.3 (2.5-4.2)	3.5 (3.2-3.9)	0.6928	2.6 (1.9-3.4	2.6 (2.3-3.0)	0.6277
Fish	11.0 (9.6-12.4)	4.8 (4.3-5.2)	0.0000	2.4 (1.7-3.1)	1.9 (1.6-2.2)	0.1233	1.7 (1.1-2.3)	1.6 (1.3-1.8)	0.3281
Mollusk	8.9 (7.6-10.2)	4.7 (4.3-5.2)	0.0000	2.0 (1.4-2.6)	1.7 (1.4-2.0)	0.3829	1.6 (1.0-2.2)	1.3 (1.1-1.6)	0.8912
Beef	3.0 (2.2-3.8)	2.5 (2.2-2.9)	0.2314	1.1 (0.6-1.6)	1.1 (0.9-1.3)	0.9829	0.8 (0.4-1.2)	0.1 (0.0-0.3)	0.0194
Milk	3.5 (2.7-4.3)	2.5 (2.2-2.9)	0.0186	0.5 (0.2-0.9)	0.8 (0.6-1.0)	0.2612	0.2 (0.0-0.4)	0.7 (0.5-0.8)	0.9465
Egg	2.2 (1.5-2.8)	1.2 (0.9-1.4)	0.0007	0.5 (0.2-0.9)	0.5 (0.4-0.6)	0.8182	0.4 (0.1-0.7)	0.4 (0.5-0.8)	0.7748
Wheat	2.1 (1.4-2.7)	1.2 (0.9-1.4)	0.0019	0.4 (0.1-0.7)	0.4 (0.3-0.6)	0.7933	0.3 (0.0-0.5)	0.3 (0.2-0.4)	0.1638
Peanut	1.4 (0.9-1.9)	1.2 (1.0-1.5)	0.5668	0.2 (0.0-0.3)	0.4 (0.3-0.5)	0.1485	0.1 (0.0-0.3)	0.3 (0.2-0.4)	0.6999
Soy	0.8 (0.4-1.2)	0.9 (0.7-1.1)	0.7485	0.3 (0.0-0.5)	0.3 (0.2-0.5)	0.6664	0.2 (0.0-0.4)	0.3 (0.2-0.4)	0.4565
Tree nut	1.6 (1.0-2.1)	1.1 (0.9-1.3)	0.0879	0.2 (0.0-0.4)	0.3 (0.2-0.4)	0.4533	0.2 (0.0-0.3)	0.3 (0.2-0.4)	0.0063
Other foods	10.0 (8.7-11.4)	3.0 (2.7-3.4)	0.0000	1.8 (1.2-2.4)	0.8 (0.6-1.0)	0.0002	1.2 (0.7-1.7)	0.6 (0.5-0.8)	0.9397

Values reported as % (95% CI).

FA, food allergy

4.4.4 The association of demographic factors with food allergy between the two survey modes

Multivariable logistic regression models were performed to analyze the association of demographic factors with food allergy (Table 4.3). The predictor variables were gender, family history and co-existence of other allergic diseases and outcome variable was doctor-diagnosed food allergy. The two-tailed t-test was used to compare the odds ratios of risk factors between WBS and PBS. The family history of food allergy was the strongest predictor of doctor-diagnosed food allergy ($P < .001$) regardless of survey modes. There is no statistical evidence for the difference of ORs of family history as a risk factor between the two survey modes ($\beta = .05$). Gender and atopy conditions showed no effects on doctor-diagnosed food allergy in both survey modes.

Table 4.3 Multivariable logistic regression analysis of demographic factors on doctor-diagnosed food allergy.

Risk factor, (95%CI)	**Web-based survey**	**Paper-based survey**
Sex *(Female/Male)*	1.4 (0.9-2.1)	1.18 (0.97 - 1.44)
Family history of food allergy *(Yes/No)*	**4.0 (2.5-6.5) ***	**7.26 (5.72 - 9.22) ***
Co-existing other allergic diseases *(Yes/No)*	1.1 (0.7-1.7)	1.09 (0.87 - 1.37)

'*' statistically significant ($P < .05$)

Data on the residential locations of the survey respondents were grouped into four different geographical regions: the South Central Coast, the Central Highlands, the South East and the Mekong Delta. Participants from the South East, including Ho Chi Minh City, the biggest metropolitan area of Vietnam, were considered as living in urban areas. Participants living in other parts of the country were considered to live in rural areas. A comparison was made to evaluate the impact of geographical location on food allergy incidence. First, we observed a higher number of doctor-diagnosed food allergy subjects in the South East compared to other parts of the country in both survey

types (Figure 4.4A-B). However, there were no statistical evidences for the difference in prevalence of doctor-diagnosed food allergy among these regions between the two survey modes (β = .05). Only in the South East, we reported a statistically significant difference of the overall prevalence of doctor-diagnosed food allergy resulted from different survey modes ($P < .001$) (Figure 4.4C).

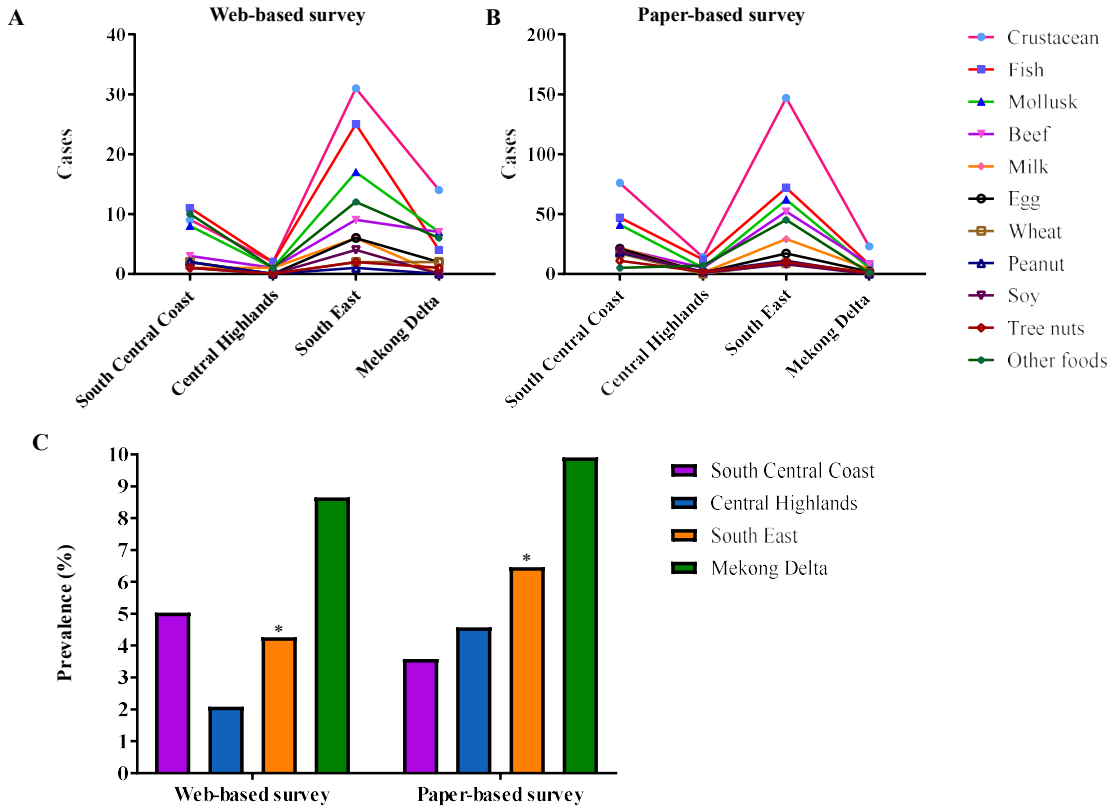

Figure 4.4 Distribution of doctor-diagnosed FAs by geographical regions of Vietnam in this study.

(A) Number of doctor-diagnosed FA (*n* = 94) by triggering food items in four geographical regions in the web-based survey. (B) Number of doctor-diagnosed FA (*n* = 401) by triggering food items in four geographical regions in the paper-based survey. (C) Prevalence of FA in four geographical regions (the South Central Coast, the Central Highlands, the South East and the Mekong Delta. Asterisk '*' denote significant difference in the prevalence in the South East between survey modes ($P < .001$). This is the biggest metropolitan area in Vietnam. FA, food allergy.

4.5 Discussion

This is the first study to validate data from two survey modes, WBS and traditional PBS, using the same questionnaire in an identical population. In general, the data from this WBS were comparable to the PBS conducted at the same point of time in two independent sample populations, especially with respect to the prevalence rates of food allergy, food allergy patterns and the distribution of clinical presentation.

However, we also observed substantial variations in self-reported food allergy prevalence between WBS (27.8%) and PBS (18.0%). This more or less reflects the current understanding of Vietnamese participants about food allergy definition and its clinical manifestations. In reality, the prevalence of self-reported food allergy might vary from 3% to 35% when comparing different epidemiological studies in the US (17), Europe (18) and Asia (8). However, the overall prevalence of doctor-diagnosed food allergy and IgE-mediated food allergy across the two survey modes were similar and comparable to previous studies in adults in Taiwan (19), US and Canada (20). Furthermore, both surveys demonstrated beyond doubt that seafood allergy was the predominant food allergy in this population. Seafood accounts for more than half of the reported food-induced allergic reactions in this study, and this observation was reported previously from population-based questionnaire surveys in children in Thailand, the Philippines and Singapore (10, 21). Additionally, very low rates of peanut, tree nut and wheat allergy were established, closely correlated to other studies performed in Asian countries (10, 22).

Food allergy can often be confused with other non-allergic food hypersensitivities due to its wide spectrum of clinical symptoms (23). In spite of using different survey types, we observed a very similar pattern of reported clinical symptoms among defined food allergy groups. Although there were more self-reported food allergy participants reporting gastrointestinal symptoms (diarrhea, nausea or vomiting or stomach pain) in the WBS than in the PBS, we found no significant effect of survey modes to the outcomes of clinical manifestations. Hives was the most frequent adverse symptom for food adverse immune responses, followed by diarrhea in doctor-diagnosed food allergy and IgE-mediated food allergy.

The multivariable logistic regression analysis of demographic factors to food allergy in the two survey modes strengthens the validation of WBS with respect to PBS. Family history of food allergy was the strongest indicator for food allergy in WBS and PBS ($P<.05$), whilst gender and atopy condition did not have any effects. With respect to the association of geographical region to food allergy incidence in the two survey modes, people living in rural areas showed a lower prevalence of food allergy than those in urban areas. A difference in prevalence of food allergy between survey types was only observed in the South East. In other regions, no statistical evidence was found to support a different incidence of food allergy between geographical regions.

As with all epidemiologic studies, there are several pitfalls that need to be considered prior to interpreting the results of a food allergy survey. In the case of a WBS, limitations include recall bias, response bias, participation bias and selection bias. In this study, our target population was young Vietnamese adults attending universities. Participants from the two survey modes have very similar ages (WBS: 21.6±3.4 years and PBS: 19.8±2.5 years) and educational level. Thus, the recall bias would be considered equal between the two survey types.

In terms of response bias, WBS showed a lower response rate (30.9%) than PBS (62.3%). Low response rate has previously been encountered in several paper-based food allergy surveys. For instance, in a food allergy survey in the UK, the authors reported a response rate of 36% (24) whilst in a nationwide Canadian study on food allergy, a participation rate of 34.6% was reported (25). In an epidemiological study, response rate is associated with study bias. Normally, investigators need to collect information from the non-response group to adjust for the final prevalence rate (20, 26). In our PBS, we assumed that people did not answer the questionnaire merely because of their non-interest in the topic. However, this ignorance might be a result of an absence of health problems arising from food ingestion. In this case, it is essential to have proper investigation on non-response bias to generate more accurate prevalence of food allergy in this population. In the WBS, there are a number of potential reasons that could explain the low response rate: the survey email did not reach participants; participants did not check their email frequently; the survey email was automatically placed into the participant's spam mailbox; the participants were not

interested in the survey or the participants had no food-related complaints. Overall, in spite of variations in sample size and response rate, key findings on food allergy in Vietnamese adults are consistent between the two survey modes. This is corroborated by a recent study on food allergy in the US in which the authors revealed that non-respondents posed no effects on demographics and other key variables after conducting a non-response bias analysis (17).

With respect to participation bias, we observed a higher proportion of female participants compared to males in both WBS and PBS, while Vietnam has an equal ratio of male and female adults aged below 50 years as well as of male and female students (1:1). The tendency that a certain gender prefers a specific mode of epidemiological survey was also seen in other population-based studies (27). Thus, an appropriate adjustment needs to be made to generate the final prevalence rate.

A major limitation of WBS is the selection bias. WBS seem to be more feasible for young population with access to the internet than other groups in the general population (i.e. older people, workers) (28). In case of Vietnam, people under the age of 35 years account for 60.5% of the population (29). Furthermore, this country has a high proportion of internet users (52.1%) compared to the average internet penetration in Asia with 45.2% (30). Most universities provide work-domain email addresses to their students and email is the major official channel for information exchange in educational institutes in Vietnam. University students were selected as the target population for this food allergy survey as they represent the young population of Vietnam and there is no foreseen bias between educational levels and food allergy incidence. Besides, this population is better educated overall and represents frequent internet users who are more likely to check their email inbox at regular intervals and enter the survey. Selection bias can be adjusted in combination with other surveys tailoring for other age groups (i.e. children) and people with occupational allergy to obtain a more accurate prevalence of food allergy in a community. Apart from that, no difference in the bias between the paper-based survey and internet survey could be demonstrated (13, 31).

To increase the response rate, incentives could be considered (32). However, the decision to use incentives and the type of incentives are dependent on the available financial capacity of the research project as well as the culture of each community where the study will be implemented. Suggestions on using incentives were mentioned elsewhere (33). In this study, we decided not to use incentives to limit the chance that participants might enter the survey more than once and thus might be a potential thread for participation bias.

In summary, we demonstrated that WBS could provide very comparable results to the traditional PBS. The economic efficiency of WBS was confirmed (27), as this study was conducted in Vietnam, a reflection of a typical developing economy in Asia. Before this study, there was no information available about food allergy incidence nor national clinical guidelines on food allergy in Vietnam. The consistence of key outcome values from WBS compared to PBS indicated the potential application of online surveys in epidemiological studies in other populations with limited capital and resources. Moreover, there are numerous available survey algorithms available, including free software that are accessible to all internet users. In our opinion, this online survey could combine with national campaigns on food allergy to increase awareness and understanding of food allergy in the general population. With the continuing rise of internet penetration in the general population, this method can be applied widely in schools and in offices. However, appropriate considerations need to be given to ensure the privacy of the respondents, the study design and the questionnaires need to be reviewed by relevant Human Research Ethics Committees.

In conclusion, the comparable results of the WBS to PBS were validated in this study. Taking into consideration all possible biases against advantages of WBS, we suggest the application of WBS as a low-cost, time-saving, labor-efficient and convenient platform to conduct surveys on food allergy on a population-based scale, particular in low income countries.

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4.7 Chapter 4 summary

From this chapter, the research aim was met as below:

- Compare the food allergy survey outcomes from two population-based survey modes: web-based survey and paper-based survey.

- By conducting the same food allergy survey in a consistent target population with two different survey modes allowed us to validate the survey outcomes. In general, web-based survey on food allergy provided comparable data as in the traditional paper-based survey in terms of the prevalence rate, the food allergen pattern and the food allergy determinants. Taking into consideration of the advantages of web-based survey, we suggest to apply this survey mode in future population-based survey

CHAPTER 5 CLINICAL PRESENTATIONS AND IMMUNOLOGICAL PROFILES OF SEAFOOD ALLERGY IN VIETNAM

Manuscript in Preparation:

Thu T.K. Le, Khanh V. Bui, Yen, T.H. Phan, Thimo Reuthers, Aya C. Taki, Sandip D. Kamath, Hieu C. Chu and Andreas L. Lopata. Clinical presentations and serum IgE reactivity patterns to local seafood species among shellfish allergic adults from Vietnam. Clinical & Experimental Allergy.

5.1 Introduction

Seafood allergy is the most common food allergic disorder reported in the adult population worldwide (1). Shellfish allergy was reported among adults in the US (2), Canada (3), Iceland and in southern Europe (4). In the Asia Pacific, shellfish is the most predominant cause of food allergy (5), with prawns and crabs being the leading cause of allergic reactions in Singaporean and Filipino children (6), Taiwanese adults (7), South Korean adults (8), Hong Kong children (9) and Chinese preschool children of Guangdong Province (10). Prawns are also the primary trigger of food-induced anaphylaxis in Singapore (11). The highest rate of self-reported allergy to fish is in the Philippines, reported at 3.84%, followed by 0.6% in Singapore and 0.39% in Thailand (12).

From the population-based surveys in Chapter 2 and Chapter 3 of this thesis, the findings highlighted the predominance of shellfish and fish allergy in the Vietnamese population. The prevalence of doctor-diagnosed crustacean, fish, and mollusk allergy among Vietnamese children was 3.83%, 1.24% and 1.03% respectively (13). Similarly among Vietnamese adults the rates were 2.95%, 1.58% and 1.27%, respectively (14). Besides the fact that seafood is an essential food commodity in Vietnam and the prevalence of seafood allergy seems to be as comparably high as other neighbouring countries in the Asia Pacific (15), there is limited information about the etiology and pathophysiology of seafood allergy in this country.

In tropical regions, other factors may contribute to the higher incidence of seafood allergy. For instance, a close correlation between HDM sensitization and shellfish allergy has been observed in South-East Asia (16). The local children born in Singapore and the Philippines showed a higher rate of shellfish allergy than expatriate children (mostly Caucasian) currently residing in the same area (6). Thus, it is crucial to address the impacts of putative triggers on the development of food allergy for more effective management of this chronic condition in Asia.

The current gold standard for food allergy diagnosis in a clinical setting is the application of a double-blind placebo-controlled food challenge (17). However, oral food challenges require elaborate preparations, trained health professionals, and high readiness of the emergency procedures (18). The application of oral food challenges

in diagnosing a food allergy is very limited in Vietnam. To the best of the author's knowledge, the currently available food allergy diagnostic tests are skin tests and allergen-specific immunoglobulin E (IgE) measurements using commercial immunological assays, with a review of patients' clinical history. There is no study assessing the effectiveness of current diagnostic tests in diagnosing food allergy in this population.

This study aims to investigate the clinical presentations and immunological profiles of seafood allergic subjects in Vietnam. The skin prick test and specific IgE measurements against a comprehensive panel of fish and shellfish species frequently consumed in Vietnam will be utilized to reveal the sensitization patterns among seafood allergic participants. Furthermore, the association between HDM, cockroach, and mealworm sensitization with sensitization to shellfish will be investigated to assess the likelihood of cross-reactivity. This study seeks to determine the pattern of typical seafood allergy symptoms, identify the allergy-triggering seafood components, assess the contribution of possible environmental factors (exposure to indoor allergens) to the development of seafood allergy, and evaluate the effectiveness of the current seafood allergy diagnostic tests applied in Vietnam. The data generated from this study will be invaluable to improving the current seafood allergy diagnostics and allergy management strategies in Vietnam, and possibly the Asia Pacific region.

5.2 Aims

The aims of this chapter are detailed below:

- To document the clinical presentations of seafood allergy in the participants to outline the typical symptoms and the onset of seafood allergic manifestations in this population.
- To investigate the sensitization profiles of the seafood allergic subjects by using different diagnostic tests, including the skin prick test and allergen-specific IgE determination.
- To examine the allergenicity of major seafood allergens and identify the most allergenic seafood commodities in Vietnam.
- To evaluate the likelihood of cross-reactivity across seafood species to estimate their contributing risk of triggering an allergic response in sensitized individuals.
- To evaluate the likelihood of cross-reactivity between seafood allergens and indoor allergens in order to assess the contribution of these environmental factors to the seafood allergy pathogenesis.
- To examine the correlation between the clinical presentation and the sensitization profile to evaluate the effectiveness of the current seafood allergy diagnostic tests.

5.3 Materials and Methods

5.3.1 Patient recruitment

Participants with a history of seafood allergy were recruited from outpatients at the Centre for Allergology and Immunology, Bach Mai Hospital, Vietnam. Participants were informed about the objectives and procedures of the study. This study was approved by the Research Ethics Committee at Bach Mai Hospital (2919/QD-BM) and the Human Research Ethics Committee at James Cook University (Ethics Approval #H7233, Appendix A2). Five participants with no history of food allergy were recruited as healthy controls. Written informed consent was obtained from each participant. The participant recruitment procedure for the study is presented in Figure 5.1.

5.3.2 Patient interviews

Participants were invited to complete a pre-designed questionnaire via a face-to-face interview with an experienced food allergy clinician. The questionnaire consisted of 16 different questions and was modified from previously published food allergy studies (6, 13, 19). Details of the questionnaire can be found in Appendix B3. The participants' clinical history of food allergic presentations were also collected.

5.3.3 Skin prick testing

After completing the interview, participants with probable seafood allergy were invited to undergo a skin prick test (SPT). The SPT was performed directly following the interview or at an alternate suitable time. The study selection criteria included: a) no antihistamine medication used within the last seven days, and b) the participant was in a good physical condition for the test. The 16 food allergens and outdoor allergens used for SPT were tuna (f040), cod (f003), shrimp (f024), sardine (f308), mussel (f037), crab (f023), squid (f258), beef (f027), egg (f245), octopus (f059), six grass mix (mg01), moulds (p902), German cockroach (i901), house dust mite (m608), positive control (k200), negative control (k100), as provided by Inmunotek (Inmunotek, Madrid, Spain). The procedures for the SPT followed the guidelines of the European Academy of Allergy, Asthma and Clinical Immunology (20). The test was conducted on the

participant's volar aspect of the forearm. Histamine (K200) and glycerol-saline (K100) were used as the positive and negative controls, respectively (Inmunotek, Spain). The 16-holes Prick-Film® (Inmunotek, Spain) was used to record the weal forming. The resulting weals were measured 15 to 20 minutes following the skin prick. A weal with a mean diameter greater than or equal to 3 mm was considered a positive response.

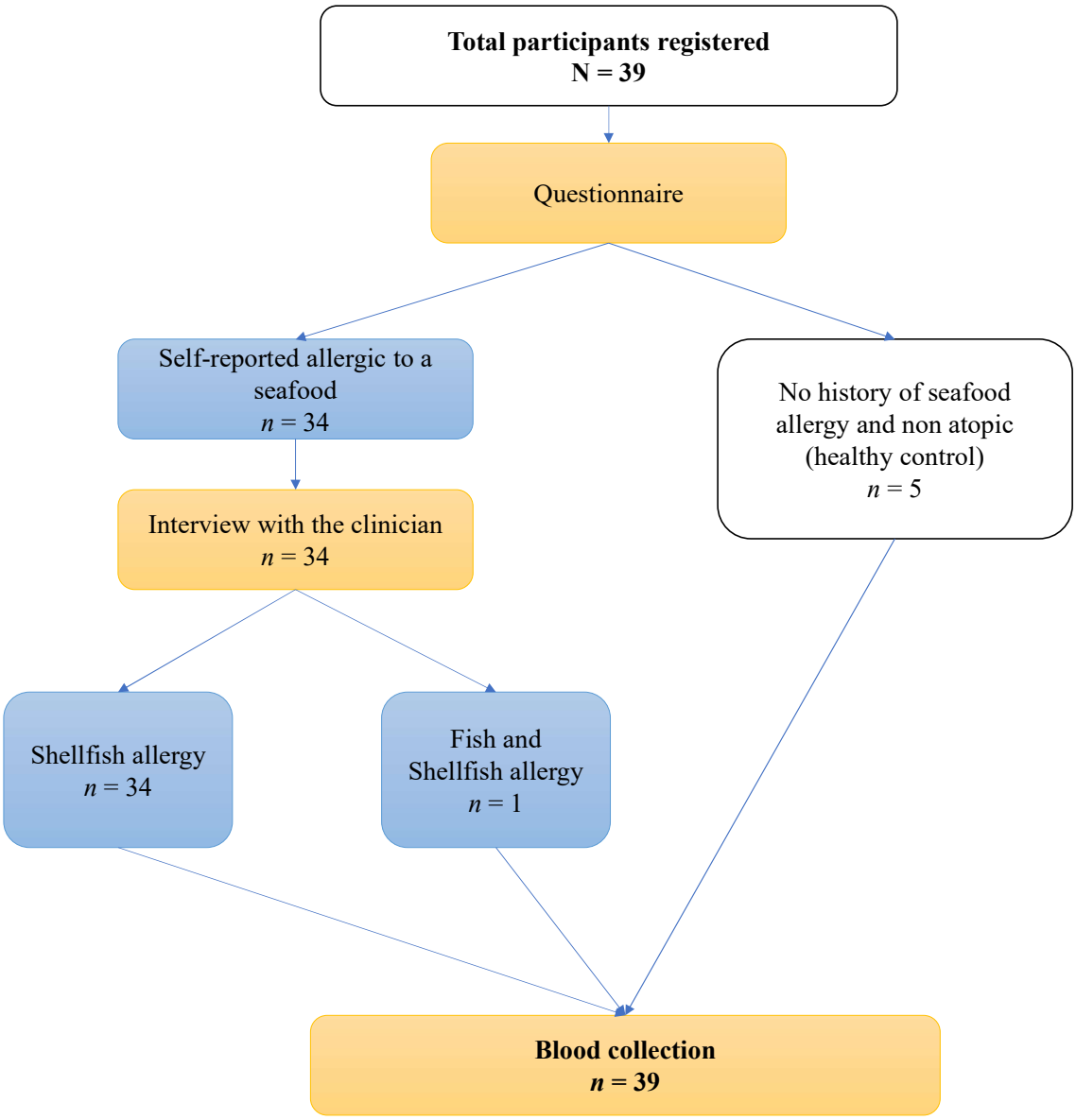

Figure 5.1 The flowchart presents the participant recruitment procedure and the outcomes of the seafood allergy study in Vietnam.

Shellfish allergy is a group of participants who reported a history of allergic reactions towards crustacean and mollusk consumption. Participants with the non-atopic condition are the group of participants that have no history of any of the following conditions: allergic rhinitis, allergic conjunctivitis, asthma, hay fever, eczema, food allergies.

5.3.4 Serum collection

Blood samples were collected from the participants with perceived seafood allergy, as well as the healthy control group, up to a volume of 10 mL, to be used for further *in vitro* analysis. Blood specimens were collected in sterile tubes and labeled with the participants' name, their date of birth and the laboratory identification number. Sera were separated from whole blood and aliquoted into 1 mL tubes. The collected sera were stored at -20^{o}C during transportation to the laboratory at JCU. At the laboratory at JCU, serum samples were kept at -80^{o}C until further use. All serum samples were collected with informed consent.

5.3.5 Seafood protein extraction

Fresh and frozen specimens were collected from local markets and distributors in Townsville and Melbourne, Australia and the correct species determined. All species used are also commonly consumed species in Vietnam. Specimens were kept on ice and frozen at -20^{o}C during transportation to the laboratory and stored at -30^{o}C in the laboratory for further use. Proteins were extracted based on the protocol developed by Kamath et al. (21) with minor modifications.

5.3.5.1 Preparation of raw protein extracts

An amount of 25 g of seafood muscle was placed in a glass bottle and then homogenized in 50 mL PBS (PBS, 10mM, pH 7.4) in a fume hood. After homogenization, samples were kept overnight at 4^{o}C with gentle agitation to maximize the extraction of water-soluble proteins from the specimen into the buffer. The next day, samples were centrifuged at 22,000 x*g* for 30 min at 4^{o}C to separate the supernatant containing water-soluble proteins. The supernatant was taken and centrifuged again at the same speed then filtered through glass fiber sheets. The supernatant was filtered again through a 0.45 µm cellulose acetate filter membrane to collect the final soluble proteins (Millipore, Billerica, MA, USA). The supernatant was aliquoted and stored at -80^{o}C until further use.

5.3.5.2 Preparation of heated protein extracts

The edible parts of shellfish specimens were heated in phosphate-buffered saline (PBS, pH7.1, 2x volume of PBS per weight) at 100°C for 15 minutes to mimic actual cooking practices. Samples were left to cool down to room temperature before proceeding to homogenization. The final slurry was kept at 4°C overnight with gentle agitation to maximize the extraction of water-soluble proteins into the buffer. The heated soluble protein extracts were collected by subsequent centrifugations (22,000 x*g*, 30 min) and filtrations (glass-fiber filter and 0.45µm membrane filter). Samples were aliquoted and kept at -80°C until further use. The protein concentration of each extract was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Uppsala, Sweden).

5.3.6 Purification of natural tropomyosin

Tropomyosin from different species was purified from heated extracts using ammonium sulfate precipitation with subsequent dialysis against 100 mmol/L ammonium bicarbonate, and Biologic LP fast protein liquid chromatography system with a CHT™ Ceramic Hydroxyapatite column (Bio-Rad, Hercules, California, USA). In brief, samples were dialysed into a buffer of 25 mM Tris, 5 mM $NaPO_4$, 150 mM NaCl, pH6.8) overnight before loading onto a Bio-scale Mini CHT™ Ceramic Hydroxyapatite column. TM was eluted by increasing the concentration of phosphate (500 mM $NaPO_4$) and then collected by pooling the purest of the TM-containing fractions. Purified TMs were dialysed overnight against PBS and presence confirmed by SDS-PAGE and mass spectrometry. All samples were freeze-dried and kept at -80°C for later use.

5.3.7 Other allergens used in immunoassays

Tropomyosin from Anisakis and house dust mite were supplied by the Molecular Allergy Research Laboratory (MARL) at James Cook University, Australia. Recombinant proteins analyzed during this study, include myosin light chain (rMLC), hemocyanin (rHC) and sarcoplasmic calcium-binding proteins (rSCP) from vannamei

prawn (*Litopeaneaus vannamei*), and tropomyosin from cockroach (rTM) (MARL). Additional protein extracts utilised include cockroach and mealworm extracts (MARL), as well as European house dust mite extract (HDM) supplied by DST (Diagnostische Systeme & Technologien GmbH, Schwerin, Germany). The complete list of all protein extracts and allergen components, including scientific names, used in this chapter is detailed in Table D5.1 (Appendix D).

5.3.8 Protein profiling by SDS-PAGE

The protein profile of all extracts and allergens were determined by performing Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a Dual Double-Wide Mini-Vertical Electrophoresis system (CBS Scientific, California, USA). Five µg of protein extract was heated to 95^{o}C in 5x sampling buffer containing Dithiothreitol (DTT) for 5 min. A volume of 10 µL sample was loaded into each well (12-16% acrylamide, 1 mm thick gel) and 2.5 µL Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA) was used as the protein marker. The proteins were separated at 100V for 20 min, then 220V until the dye front reached the bottom line of the cassette. The separated proteins were stained with Coomassie Brilliant Blue R-250 CBB staining (Bio-Rad, USA) and visualized using the Odyssey® CLx Imager (Li-cor, NE, USA) in the 700 nm channel.

5.3.9 Immunoassays

5.3.9.1 Grid-immunoblotting

A grid-immunoblotting technique (modified from Reese et al. (2001) was used to screen for the presence of specific IgE in serum, to determine sensitization patterns to multiple allergens and protein extracts (22). The assay utilized a surf blot apparatus (Idea Scientific, MN, USA) which was assembled according to the manufacturer's instructions. Either five µg of protein extract or one µg of purified allergen in 200 µl PBS was pipetted into each channel and immobilized on to the nitrocellulose membrane (Bio-Rad, USA) for 1 h at room temperature with end-to-end rocking. To analyze the IgE-binding patterns to the panel of proteins/allergens, the membrane was

blocked with 1x Casein (Casein Blocking Buffer 10x, Sigma, MO, USA) in PBS with 0.05% Tween-20 (PBS-T) (Bio-Rad, USA) and subsequently incubated with 1:20 diluted patient sera in 0.5x Casein in PBS-T for 3 hours (the membrane was rotated 90^{o} with respect to the apparatus to create the grids). IgE binding was detected using polyclonal rabbit anti-human IgE antibody (Dako, Glostrup Denmark) diluted 1:20,000 in 0.5x Casein in PBS-T. For the detection of rabbit antibodies, 1:20,000 diluted goat anti-rabbit IgG antibody (Dylight™ 800, Thermo, IL, USA) in 0.5x Casein in PBS-T was used. Binding was visualized using the Odyssey CLx Imager (Li-cor, NE, USA) and data was imported into the Image Studio™ software to analyze the binding intensity.

A panel consisting of 19 slots was employed with 18 slots for protein extract/allergens and 1 slot containing PBS only (blank). Similarly, 18 sera and 1 blank containing 0.5x Casein in PBS-T were pipetted into the slots vertically. Serum from a healthy control and a shellfish allergic patient were used as negative and positive controls, respectively. HDM extract was used for all membranes as an internal control. An example of the grid-immunoblotting design is presented in Figure 5.2 below.

For the analysis of immunoblotting for IgE-protein reactivity, the fluorescence signal of each band was digitized using an analog to digital converter that converts the analog signal to a digital scale expressed by an arbitrary fluorescence unit. The final intensity values were subtracted for the local background and exported as comma-delimited text files into Microsoft Excel. The imported data was analyzed in Microsoft Excel and GraphPad Prism (version 8.2) was used to plot the IgE binding intensity against each extract/allergen component.

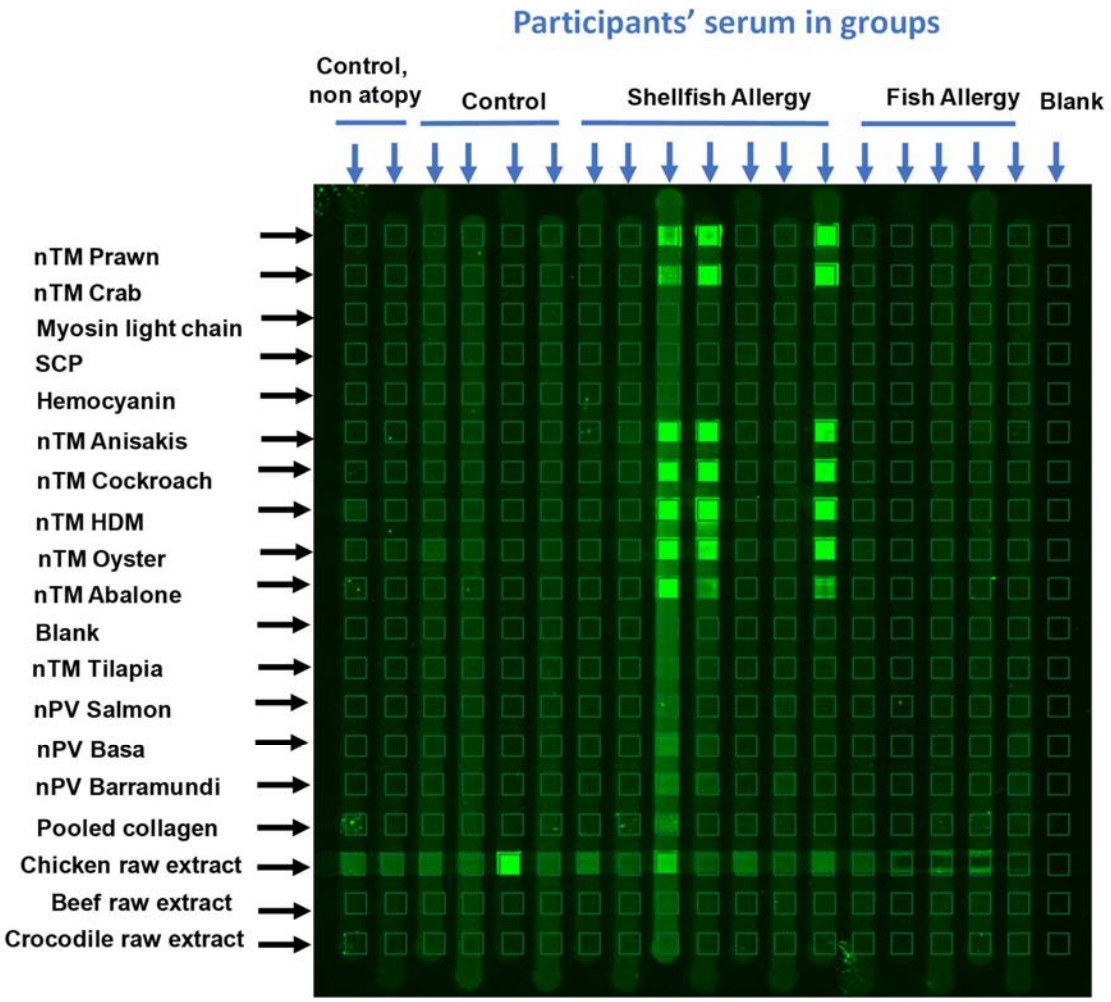

Figure 5.2 Grid-immunoblotting design to analyze the IgE reactivity against investigated extracts/allergens. Allergens are applied in the horizontal direction, while patient sera are applied afterwards in the vertical direction.

5.3.9.2 Immunoblotting

SDS-PAGE-separated proteins were transferred onto nitrocellulose membrane (16V, 30 min) (Bio-Rad, USA) and blocked with 1x Casein in PBS-T. The membrane was assembled in a surf blot apparatus (Idea Scientific, MN, USA) and incubated with 1:20 diluted patient serum in 0.5xCasein in PBS-T. IgE binding was detected using the same primary and secondary antibodies as used in the grid-immunoblotting.

5.3.10 Statistical analysis

SPSS Statistics version 25.0 for Windows and GraphPad Prism version 8.2 were used to perform statistical analysis and generate plots. The demographic and clinical features of the participants were tabulated for comparison. Continuous variables were presented as mean ± SD or number of cases and percent where appropriate. For the grid blot data, IgE reactivity signals were exported into Microsoft Excel files. Raw fluorescent signals were subtracted for the local background relevant to each protein extract/allergen to gain the blank-corrected readouts. The relative IgE binding intensity was estimated by transforming these readouts into $\log_{10}$ of the blank-corrected data. Replicates of positive and negative controls were averaged to generate the reference values of positive and negative responses to the analyzed proteins/allergens. The negative values were used as the threshold data to define positive IgE reactivity. The Friedman test or one-way ANOVA, followed by Dunn's test was used to compare the IgE reactivity of participants to different tested proteins/allergens where appropriate. The Wilcoxon matched-paired signed-rank test was used to compare the IgE reactivity between two tested proteins/allergens. The Pearson correlation was used to examine the correlation of the IgE reactivity between the seafood protein extracts and the purified allergens (i.e., tropomyosin and parvalbumin). The Spearman's rank-order correlation was used to evaluate the association between SPT performance and the relevant IgE reactivity (r_s and *p*-value), and non-linear regression was used to analyze the independence between the two variables. A *p*-value of less than .05 was considered to be significant in all tests.

5.4 Results

5.4.1 Demographics

Thirty-four participants (50.0% female) with a history of developing adverse reactions to either crustacean or mollusk or fish were recruited for this study (Table D5.2, Appendix D). The average age of the participants was 30.9 ± 11.9 years. All participants reported allergic symptoms via the ingestion pathway. Prawns (46.2%) and crabs (40.4%) were the most frequently reported allergy-causing seafood in this cohort, with some patients reporting several different types of offending shellfish (Table 5.1).

Table 5.1 The implicated seafood species that trigger adverse reactions among 34 participants.

Seafood item	Responses	
	N	Percent
Prawn	24	46.2%
Crab	21	40.4%
Clam	2	3.8%
Sea snail	2	3.8%
Lobster	1	1.9%
Squid	1	1.9%
Oyster	1	1.9%
Total	**52**	**100.0%**

Regarding symptom onset, 73.5 % of the participants manifested adverse responses within one hour of food ingestion. Two participants (5.9%) reported delayed adverse symptoms occurring within four hours after food consumption. Four participants (11.8%) failed to recall the symptom onset (Table 5.2).

Table 5.2 Reported onset of clinical symptoms among 34 participants.

Symptom onset	Responses	
	N	Percent
10-30 min	12	35.3%
30 min - 1 h	7	20.6%
Less than 10 min	6	17.6%
1- 2 h	3	8.8%
2-12 h	2	5.9%
Do not know	4	11.8%
Total	**34**	**100.0%**

Most of the participants (82.4%) experienced skin problems such as hives, redness of skin or skin itching. Twenty-two participants (64.7%) reported oral allergy symptoms involving lips, mouth, tongue, and throat. Respiratory problems were reported in 17 participants (50.0%). Thirteen participants (38.2%) reported gastrointestinal symptoms. Severe symptoms were reported by five participants (14.7%) (Table 5.3). Generally, participants presented with multiple clinical symptoms (an average of 4.5 episodes per subject) during the allergic response.

Table 5.3 The distribution of reported clinical symptoms of participants with a history of seafood allergy (*n* = 34).

Symptom	Responses	
	N	Percent
Itching	27	17.8%
Hives/urticaria	24	15.8%
Swelling of lips or face	19	12.5%
Itchy throat or mouth	11	7.2%
Abdominal pain	11	7.2%
Nausea/vomiting	10	6.6%
Wheezing	9	5.9%
Lip or tongue tingling	8	5.3%
Tight chest/chest pain	6	3.9%
Congested or running nose	6	3.9%
Diarrhea	5	3.3%
Redness of the skin	5	3.3%
Tight throat	3	2.0%
Faint/dizzy	3	2.0%
Shock	2	1.3%
Drop in blood pressure	2	1.3%
Swelling elsewhere	1	0.7%
Total	**152**	**100.0%**

In the most severe episodes, most of the participants (63.3%) were taken to hospital, while others took no action (23.3%) or used an antihistamine (10.0%) (Table 5.4). None of the participants possess or carry emergency tool kits such as an EpiPen®.

Regarding current atopic conditions, twelve participants (35.3%) have other concurrent allergic conditions, mostly allergic rhinitis; five participants (14.7%) reported having childhood eczema, and three participants (8.9 %) currently suffer from other food allergies additional to seafood allergy (Table D5.2, Appendix D).

Table 5.4 Participants' action during the most severe food-triggering episodes (*n* = 34).

Action during the most severe episodes	Responses	
	N	Percent
Go to hospital	19	63.3%
Take no action	7	23.3%
Use antihistamine	3	10.0%
Go to the pharmacy for medication	1	3.3%
Total	**30**	**100.0%**

Since the most severe episodes, twenty-two participants (64.7%) actively avoid the suspected seafood. The remaining group still consume the seafood regularly, with 14.7% continuing to experience adverse reactions, and 5.9% of them reacting only occasionally (Table 5.5). Most of the participants in this study (76.5%) currently live in urban areas; eighteen participants (52.9%) reported having at least one immediate family member with a food allergy, and none of them have any pets.

Table 5.5 The current food allergy status of the participants (*n* = 34).

Current food allergy status	Responses	
	N	Percent
Avoiding the suspected food	22	64.7%
Still have reactions	5	14.7%
Do not know	5	14.7%
Only react sometimes	2	5.9%
Total	**34**	**100.0%**

5.4.2 Protein profiles of seafood extracts and allergens

SDS-PAGE analysis was performed to determine the protein profile of all extracts and allergens used in the immunoassays. The protein marker ranges from 10 to 250 kDa. The gels were stained with Coomassie Brilliant Blue and visualized using the Odyssey CLX Imager at 700 nm. The output images were imported into pdf files and presented in Figure D5.1, Appendix D.

5.4.3 IgE reactivity against crustacean heated protein extracts

Figure 5.3 displays the relative IgE binding intensity of thirty-four participants to different crustacean heated protein extracts. Subjects displayed the strongest IgE reactivity to the heated protein extract of BTP, whereas, vannamei prawn had the lowest recognition ($p < .01$). Among three crab species, mud crab demonstrated the highest IgE binding intensity, followed by blue swimmer crab ($p = .0293$). Softshell crab is the least allergenic crab of all ($p < .05$).

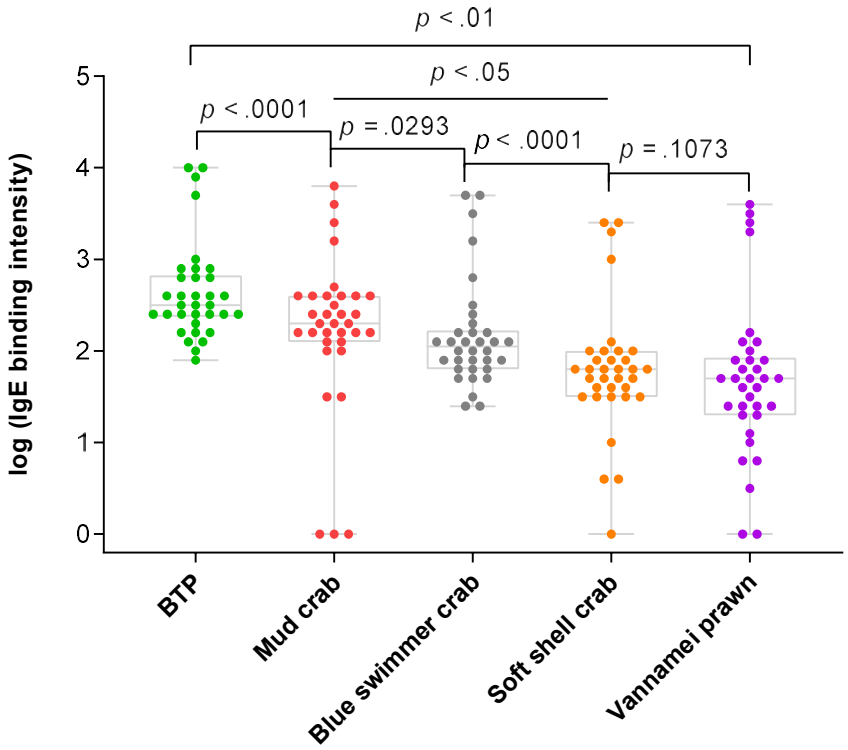

Figure 5.3 IgE reactivity of shellfish allergic patients (n = 34) against five crustacean species. BTP: back tiger prawn. The Wilcoxon matched-paired signed-rank test was performed to compare the IgE reactivity to each pair of extracts.

5.4.4 IgE reactivity against mollusk heated protein extracts

The heated extracts of eight mollusk species were used to determine IgE reactivity. Patients showed the highest level of IgE binding to white clam extract (Figure 5.4). Oyster appeared to be less reactive than other bivalves (p < .0001). Within the gastropoda class, participants showed a significantly higher IgE reactivity to abalone than snail extract (p < .0001). Within the Cephalopoda, no difference in IgE sensitization was seen between octopus and cuttlefish extracts, but a much lower reactivity to squid extract was exhibited (p < .0001).

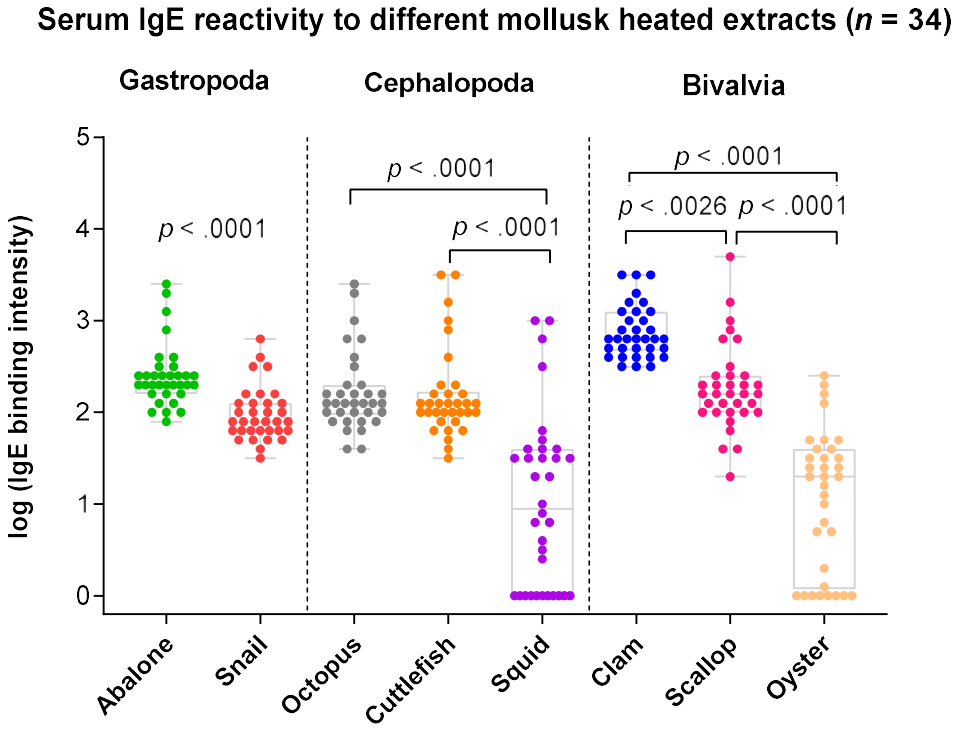

Figure 5.4 Serum IgE reactivity of shellfish allergic patients (n = 34) against eight **mollusk species**. The Friedman test was used to compare the means of the difference between groups (Friedman statistic = 198.1, p < .0001). The IgE reactivity of each pair of extracts was compared by the Dunn's multiple comparisons test (Table E5.1, Appendix E).

5.4.5 IgE reactivity against different allergens from vannamei prawn

Figure 5.5 shows IgE reactivity of individual patients to different allergens from vannamei prawn. The heated protein extract resulted in the highest amount of positive tests (73.5%). Although nTM and rHC have the same positive ratio, the positive IgE reactivities are often demonstrated in different patients. Four patients (#7, #8, #10, #15) showed very strong IgE binding to nTM but not to rMLC, rHC or rSCP, while patients #18, #19 and #21 showed IgE reactivity to only rHC. There were five patients (#14, #16, #20, #22 and #23) with only a weak signal or no binding to any of the analyzed allergens from vannamei prawn.

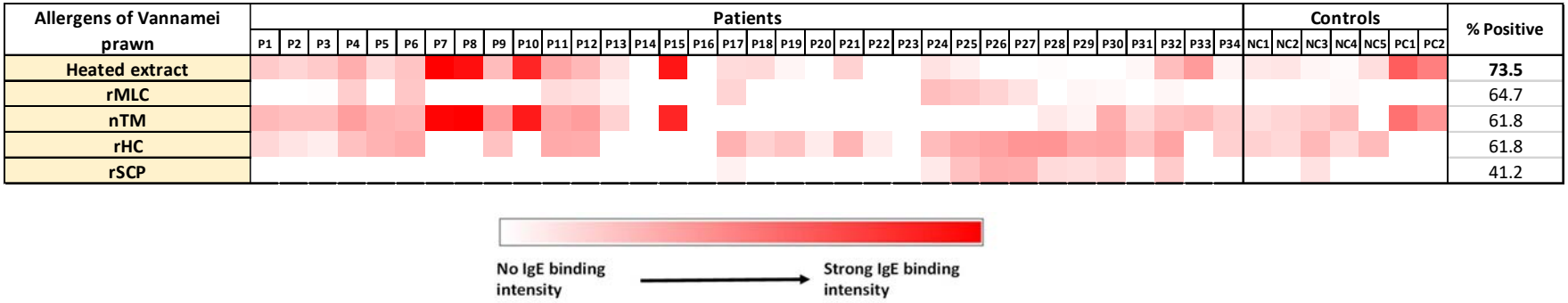

Figure 5.5 A heat-map displaying the specific IgE reactivity to **heated protein extract** and **purified allergens of Vannamei prawn** among seafood allergic patients (*n* = 34) and control groups. The average binding intensity of the five negative controls (labeled NC1 to NC5) was used to define the cutoff value for each tested allergen. The patients demonstrating an IgE binding signal greater than the cutoff value is defined as positive. The percentage of positive tests is determined by the ratio between the number of positive patients and the total number of patients. The binding intensity is expressed in color from white (no binding) to red (strong binding). PC1 and PC2: positive control #1 and #2. rMLC: recombinant myosin light chain; nTM: natural tropomyosin; rHC: recombinant hemocyanin; rSCP: recombinant sasco-plasmic calcium-binding protein.

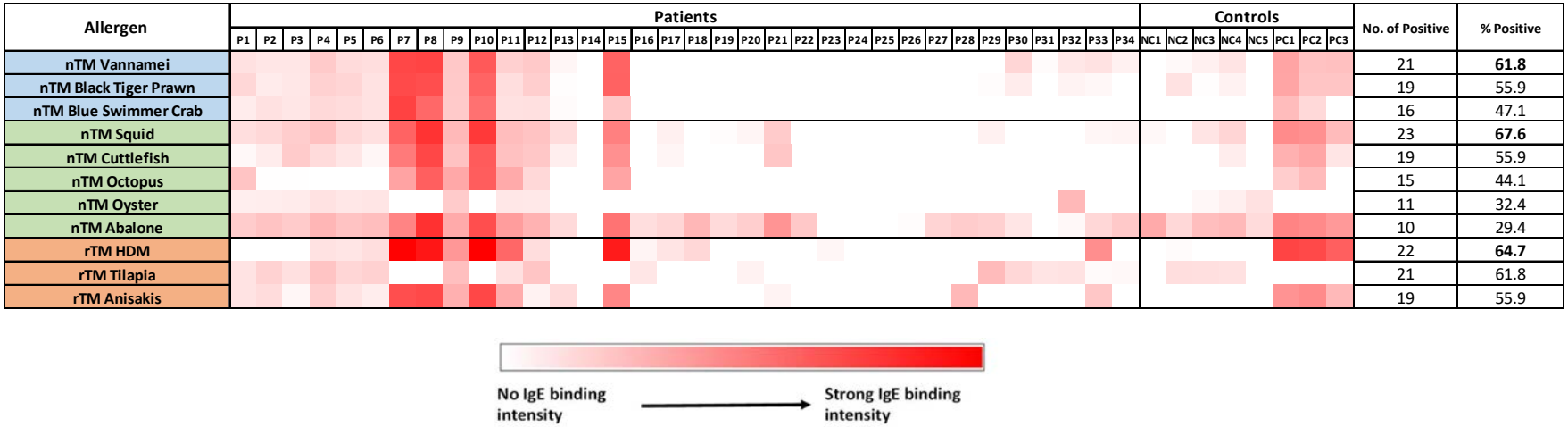

Figure 5.6 A heatmap displaying the specific IgE reactivity to **tropomyosin** from food and non-food sources among seafood allergic patients (*n* = 34) and the control groups. The average binding intensity of the five negative controls (labeled NC1 to NC5) was used to define the cutoff value for each tested allergen. A positive test is defined as having an IgE binding signal greater than the cutoff value. The percentage of positive tests is determined by the ratio between the number of positive patients and the total number of patients. The binding intensity is expressed in color from white (no binding) to red (strong binding). PC1 and PC2: positive control #1 and #2. nTM: natural tropomyosin. HDM: house dust mite.

5.4.6 IgE reactivity against the tropomyosin allergen

The IgE reactivity to different natural and recombinant tropomyosins are presented in Figure 5.6. In general, seafood allergic patients showed diverse IgE reactivity patterns to different tropomyosins. There were four patients (#7, #8, #10, #15) showing extreme IgE binding intensity to nearly all analyzed allergens, excluding the natural tropomyosin of oyster and tilapia. Two patients (#9 and #11) showed IgE reactivity to all analyzed tropomyosin allergens. The most reactive allergen was the natural tropomyosin of squid (67.6% positive) and those of HDM (64.7% positive). The natural tropomyosin from abalone showed the least reactivity (29.4% positive). In the crustacean group, most patients who demonstrated IgE reactivity to vannamei prawn tropomyosin also showed IgE reactivity to the same allergen of black tiger prawn (90.5%) and blue swimmer crab (76.2%), except for the case of patient #30, #32 and #33 who showed species-specific sensitization.

5.4.7 Correlation between the IgE reactivity against seafood protein extracts and their purified tropomyosins

Tropomyosin is widely known as the major allergen in crustacean and mollusk however, new allergen components from crustacean and mollusk have recently been identified and characterized. This analysis investigated the contribution of tropomyosin sensitization to the overall reactivity to crustacean and mollusk extract in 34 seafood allergic patients, by comparing the sIgE level to heated extracts to that of its respective purified tropomyosin (Figure 5.7, Figure 5.8 and Figure 5.9). Generally, patients demonstrated a strong positive correlation between their sIgE levels to the heated extract and the respective natural tropomyosin, in seven out of eight investigated species ($p < .0001$). Only oyster showed a weak and non-significant correlation ($r = .3069$, $p = .0775$).

Among crustacean extracts (Figure 5.7), the correlation between the IgE recognition to nTM and the heated extract descended from vannamei ($r = .8239$) to crab ($r = .7165$) and BTP ($r = .7073$), whereas more participants reacted to the heated extract but were negative to crab nTM (52.9%) than BTP nTM (47.1%) and vannamei nTM (32.4%). These participants may react to other heat-stable allergens in the crustacean extracts other than TM.

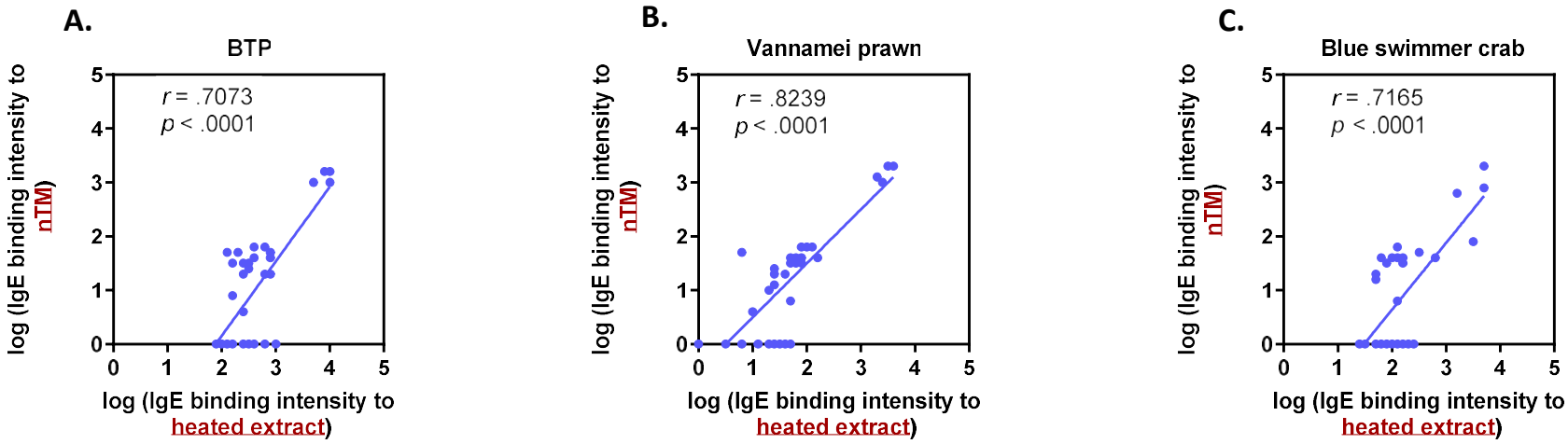

Figure 5.7 The relationship between patient IgE reactivity (n = 34) to **crustacean** heated protein extracts and the relevant purified **tropomyosin**. A) BTP; B) Vannamei prawn; C) Blue swimmer crab. The IgE binding intensity was transformed into logarithm with a base of 10. The Pearson correlation coefficient was computed, and the linear regression line plotted using Graphpad Prism to visualize the correlation.

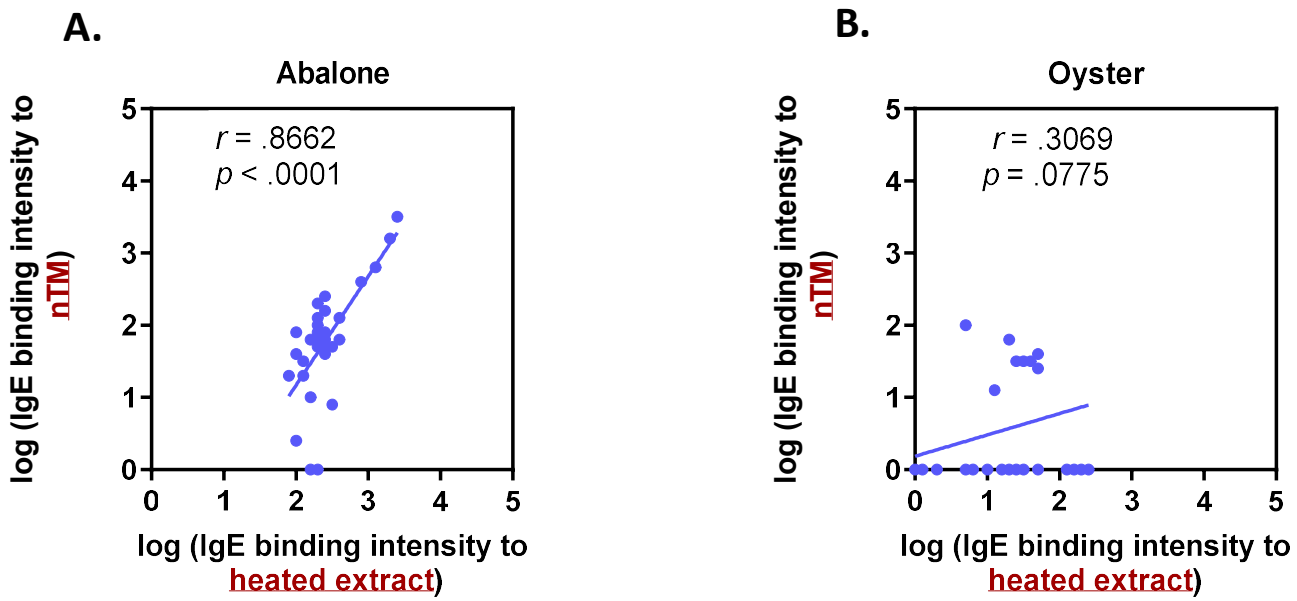

Figure 5.8 : The relationship between patient IgE reactivity (*n* = 34) to **mollusk heated protein** extract and the relevant purified **tropomyosin** of mollusks. A) Abalone and B) Oyster; The IgE binding intensity was transformed into logarithm with a base of 10. The Pearson correlation coefficient was computed, and the linear regression line plotted using Graphpad Prism to visualize the correlation.

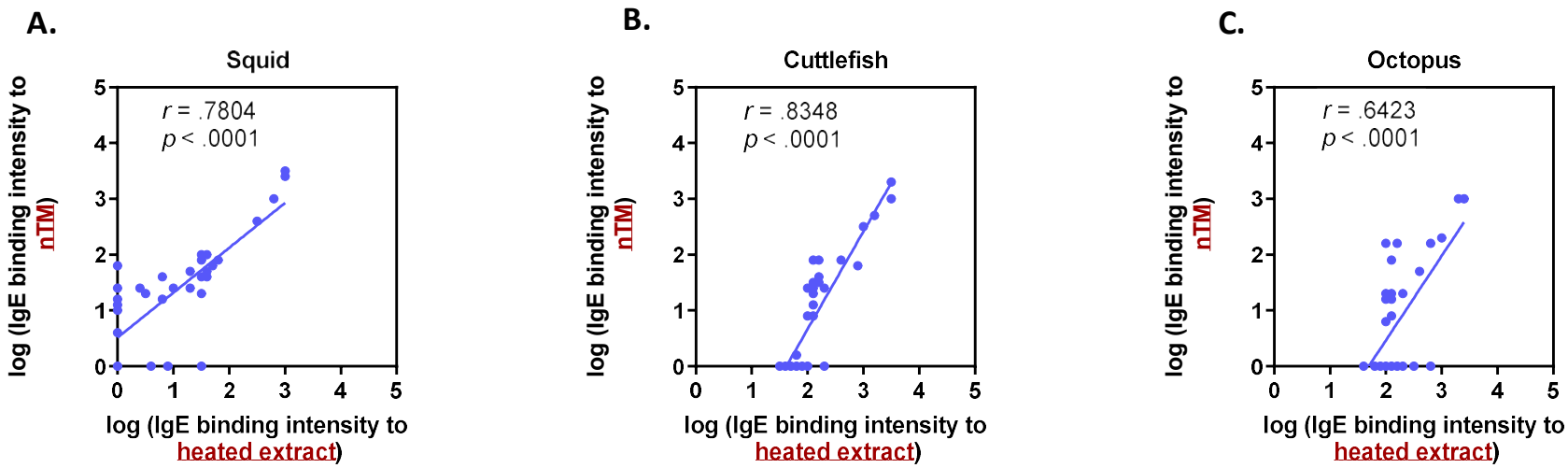

Figure 5.9 The relationship between patient IgE reactivity (n = 34) to **Cephalopoda** heated protein extract and the relevant purified **tropomyosin.** A) Squid; B) Cuttlefish and C) Octopus. The IgE binding intensity was transformed into logarithm with a base of 10. The Pearson correlation coefficient was computed, and the linear regression line plotted using Graphpad Prism to visualize the correlation.

5.4.8 IgE reactivity to protein extracts and allergens of house dust mite, cockroach and mealworm

Sera from shellfish allergic patients were analyzed for their IgE reactivity against non-food allergen sources (Figure 5.10). 97.1% of patients had positive IgE antibody responses to HDM extract (mean of relative IgE binding intensity = 3.38) and 61.8% of these patients showed IgE reactivity to HDM purified tropomyosin, but with a lower IgE binding intensity (mean of relative IgE binding intensity = 1.27, $p < .0001$). Patients demonstrated a higher IgE binding intensity to HDM extract than to cockroach extract ($p < .0001$) and mealworm raw extract ($p < .0001$). However, cockroach recombinant tropomyosin demonstrated a higher IgE binding intensity (mean of relative IgE binding intensity = 1.05) than the cockroach extract (mean of relative IgE binding intensity = .72), but no statistical difference was seen ($p > .9999$).

Mealworm is an edible insect that is frequently consumed in Vietnam. In this cohort, there are eight patients reported clinical history to silkworm pupae consumption. The IgE reactivity of seafood allergic patients against mealworm proteins was investigated. Twenty-five individuals presented a positive response to mealworm raw extract and 28 subjects reacted to mealworm heated extract. Also, the patients showed much stronger IgE reactivity to mealworm heated extract than the raw extract ($p = .0002$). Among eight participants who reported clinical response to silkworm pupae, seven out of eight have a positive response to mealworm raw extract, and all of them showed a positive response to mealworm heated extract.

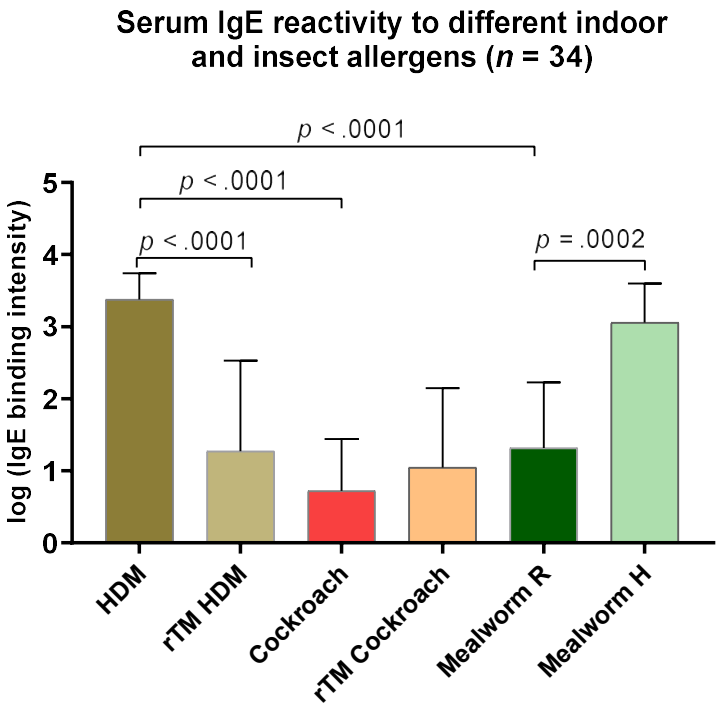

Figure 5.10 Patient IgE reactivity against **HDM, cockroach and mealworm** proteins. Proteins included HDM extract and purified TM, cockroach extract and purified TM, and mealworm raw and heated extracts. The Friedman test was used to compare the means of the difference between groups (Friedman statistic = 154.9, p<.0001). The IgE reactivity of each pair of extracts was compared using the Dunn's multiple comparisons test (Table E5.2, Appendix E).

5.4.9 Correlation between the serum IgE reactivity against house dust mite and cockroach extracts and their tropomyosins

Tropomyosin is implicated as the major allergen corresponding to the cross-reactivity between shellfish and HDM and/or cockroach allergy (23). The amino acid sequence similarity is in general very high between different arthropod groups. However, only a weak positive correlation between the IgE recognition to HDM natural tropomyosin and its extract was observed (Figure 5.11 A). Eleven patients (32.4%) showed IgE reactivity to HDM extract but not to its respective tropomyosin. In an experiment with cockroach proteins, a negative correlation was seen between cockroach extracts and the recombinant tropomyosin (Figure 5.11 B). 13/34 (38.2%) patients did not

demonstrate any IgE binding to cockroach tropomyosin but displayed binding to cockroach extract. Conversely, 14/34 (41.2%) subjects demonstrated IgE binding to the purified tropomyosin but were negative to the extract. There were three subjects that did not react to either the extract or the purified tropomyosin.

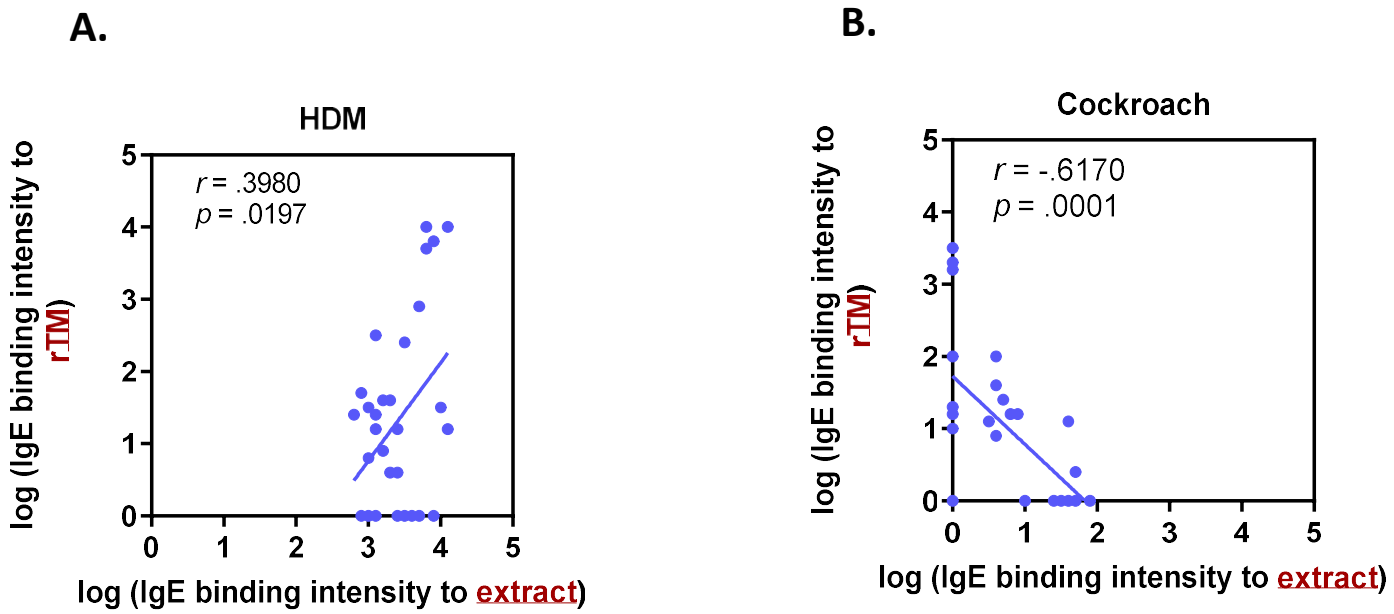

Figure 5.11 Correlation between serum IgE reactivity to **HDM** extract (A) and **cockroach** extract (B) and their **tropomyosin** (*n* = 34). The IgE binding intensity was transformed into logarithm with a base of 10. The Pearson correlation coefficients were computed, and the linear regression line was plotted in Graphpad Prism to visualize the correlation.

5.4.10 The effect of heat treatment on patient IgE reactivity against oyster allergens

Oyster is one type of seafood that is often consumed raw; thus, investigating the IgE reactivity of seafood allergic patients to oyster under different preparation/cooking forms could demonstrate how its allergenicity alters through different cooking practices. The IgE reactivity of participants to raw and heated extracts, as well as purified natural tropomysin, was investigated. As shown in Figure 5.12, compared to the IgE reactivity to the raw extract, the IgE reactivity decreased in the heated extract ($p < .0001$) and the purified oyster tromomyosin ($p < .0001$). Although no statistical difference was seen in the IgE response between the heated extract and tropomyosin ($p = .2067$), only 11/34 (32.4%) subjects had positive IgE responses to oyster tropomyosin. Overall, heat treatment reduced the allergenicity of oyster. Patients displayed selective IgE binding to oyster tropomyosin, however some patients may exhibit specific IgE to alternate oyster allergens in the extracts.

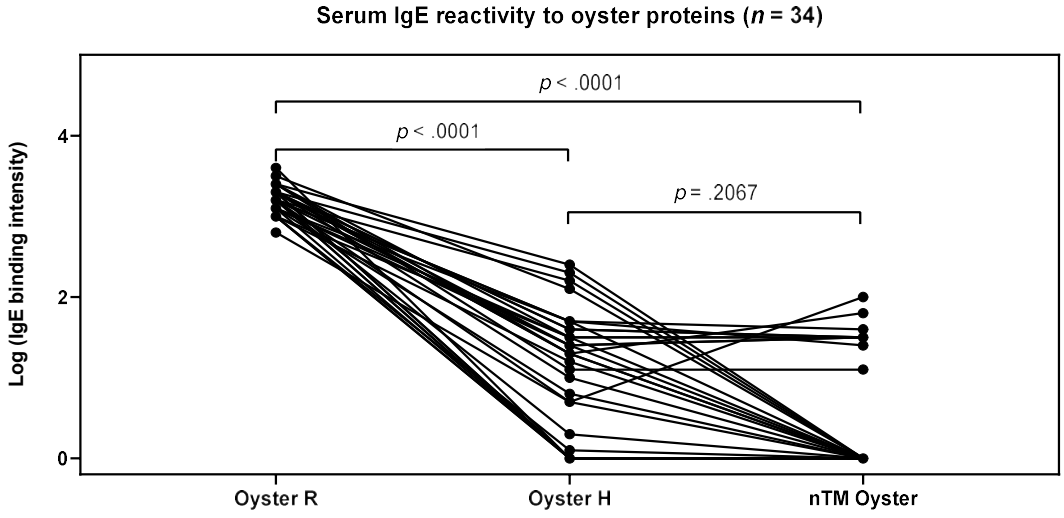

Figure 5.12 IgE reactivity to different **oyster** preparations.

The IgE binding intensity was transformed into logarithm with a base of 10. R: raw extract, H: heated extract, nTM: natural tropomyosin. The IgE reactivity of each pair of extracts was compared using the Dunn's multiple comparisons test.

5.4.11 IgE reactivity against the fish heated protein extracts

Of all thirty-four participants, only one individual (#23) reported adverse reactions to fish. The serum IgE reactivity of all patients to thirteen commonly consumed fish species in Vietnam is shown in Figure 5.13. Basa fish, goby fish, Atlantic salmon, tilapia and yellowtail scad are the top five fish species demonstrating the IgE recognition. The least IgE reactive fish species were walking catfish and blue mackerel.

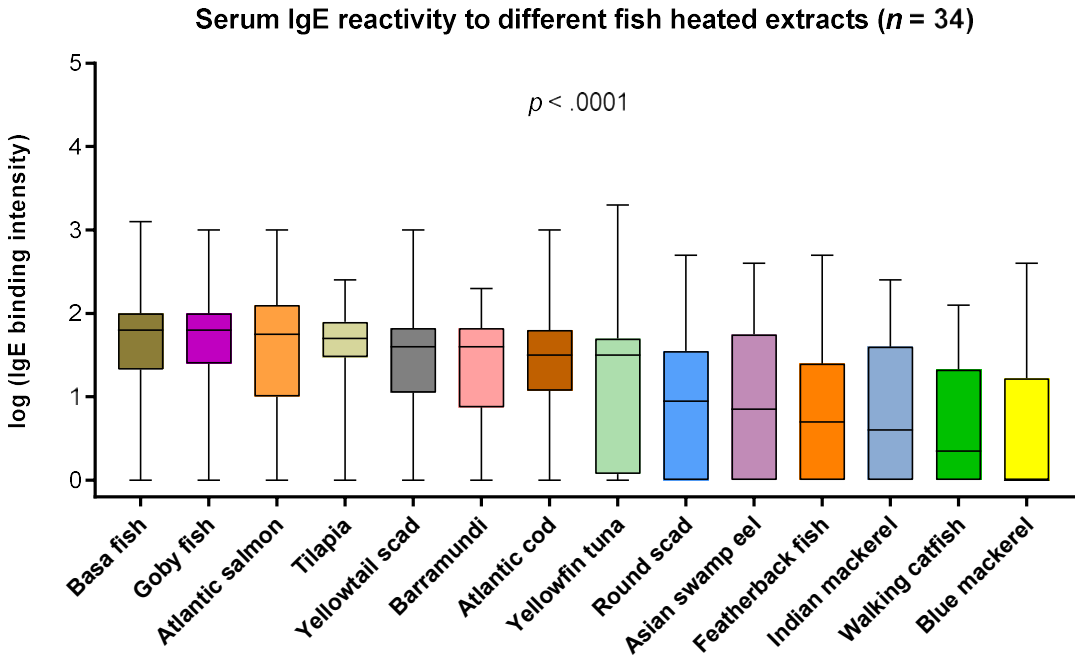

Figure 5.13 Comparison of IgE reactivity to different **fish species** among participants (n = 34). The IgE binding intensity was transformed into logarithm with a base of 10. The Friedman test was used to compare the means of the difference between groups (Friedman statistic = 172.6, p < .0001). The IgE reactivity of each pair of extracts was compared by the Dunn's multiple comparisons test (Table E5.3, Appendix E).

The pattern of IgE reactivity to the heated fish extract is presented in Figure 5.14. Among the investigated fish panel, tilapia and basa fish had the highest positive rate of 91.2% and 82.4%, respectively. Atlantic cod (82.4%), yellowtail scad (79.4%), barramundi (79.4%), Goby fish (76.5%) and yellowfin tuna (70.6%) had high positive

reactivity rates. Fish species with the lowest numbers of positive tests in this cohort include round scad, Asian swamp eel, featherback fish, Indian mackerel, walking catfish and blue mackerel. Participants demonstrated a diverse pattern of serum IgE reactivity to different heated proteins from fish. Two subjects (#26 and #29) showed no IgE reactivity to any fish extracts.

Fish species	Patients																																No. of positive	% Positive
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34		
Tilapia																																			31	**91.2**
Basa fish																																			28	82.4
Atlantic cod																																			28	82.4
Barramundi																																			27	79.4
Yellowtail scad																																			27	79.4
Goby fish																																			26	76.5
Atlantic salmon																																			24	70.6
Yellowfin tuna																																			24	70.6
Indian mackerel																																			19	55.9
Featherback fish																																			19	55.9
Asian swamp eel																																			18	52.9
Round scad																																			18	52.9
Walking catfish																																			17	50.0
Blue mackerel																																			12	35.3

Figure 5.14 A heat-map displaying the specific IgE reactivity to **heated fish** extracts among seafood allergic patients (*n* = 34).

The average binding intensity of the five negative controls was used to define the cutoff value for each tested allergen. Patients showing an IgE binding intensity greater than the cutoff value is defined as a positive test. The percentage of positive tests is determined by the ratio between the number of positive patients and the total number of patients. The binding intensity is expressed in color from white (no binding) to dark blue (strong binding).

Parvalbumin is a major allergen found in fish, therefore parvalbumin from four different fish species: barramundi, Atlantic cod, basa fish and Atlantic salmon, were used to examine the IgE reactivity among the 34 patients (Figure 5.15). The IgE reactivity to the natural tropomyosin of barramundi differed to those of salmon (p = .0387). No difference in the IgE reactivity was seen among other fish parvalbumins.

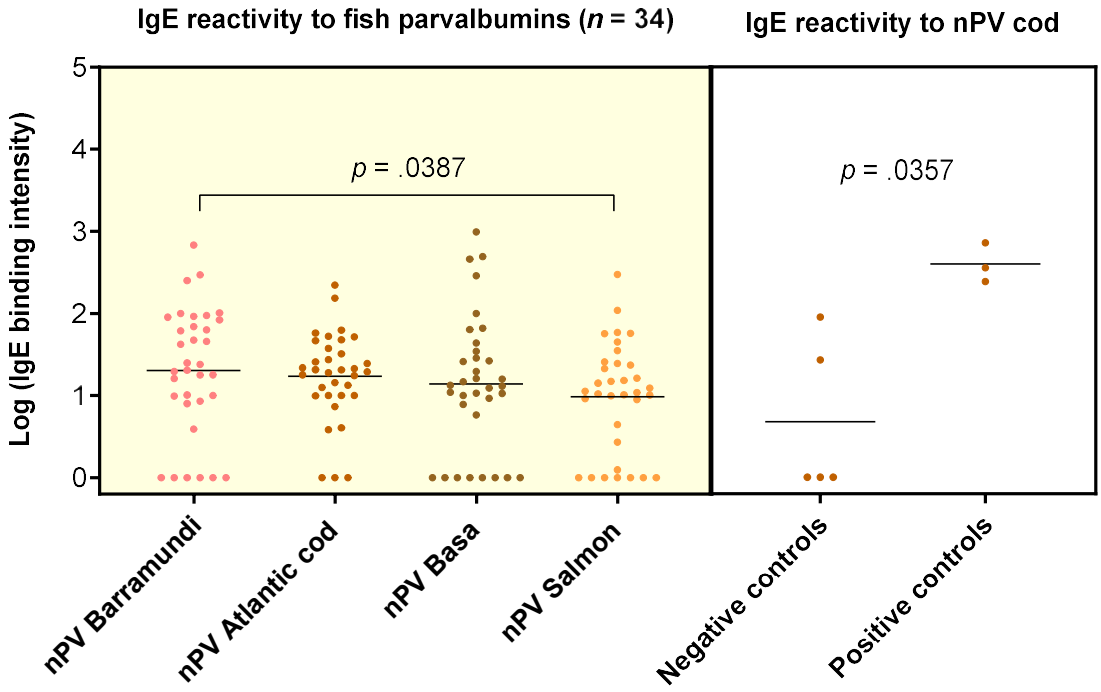

Figure 5.15 Serum IgE reactivity to different fish parvalbumins (**nPV)** among 34 patients and eight controls. The IgE binding intensity was transformed into logarithm with a base of 10. The Friedman test was used to compare the means of the difference between groups (Friedman statistic = 7.962, p = .0468). The IgE reactivity of each pair of fish allergens was compared by the Dunn's multiple comparisons test (Table E5.4, Appendix E).

5.4.12 Correlation between SPT results and serum IgE reactivity against seafood protein extracts and allergens

The SPT was performed with nineteen patients to identify their clinical sensitization to prawn, crab, mussel, squid, octopus, cod, tuna, salmon, cockroach, HDM, beef, egg, molds and mix grass by commercial SPT reagents. The correlation of the SPT results

(weal diameter in mm) and the IgE reactivity (logarithm of IgE binding intensity) was determined by the non-parameter Spearman correlation test to generate the correlation coefficient *r*. A weal diameter ≥ 3mm was considered as a positive result. Patient IgE reactivity ≥ the mean of the IgE reactivity of five healthy controls was considered as a positive IgE antibody result to the relevant investigated allergens/extracts.

Of all 19 patients, 13 patients (68.4%) have positive results to prawn by SPT (Figure 5.16A). Within this subpopulation, 12/13 patients had positive IgE binding to BTP and vannamei prawn *in vitro*. A moderate positive correlation was observed between the SPT result and prawn extracts (BTP: $r = .5511$, $p = .0145$; vannamei prawn: $r = .5345$, $p = .0184$).

Looking at the results of SPT with crab , 11/19 patients (57.9%) had positive results (Figure 5.16B), of which, 7/11 patients showed positive IgE binding to mud crab extract, 9/11 patients were positive to soft shell crab and 10/11 patients were positive to blue swimmer crab. In general, SPT crab outcome and the IgE reactivity to three crab species indicated a very weak correlation ($r = .2814 \sim .399$, $p > .05$).

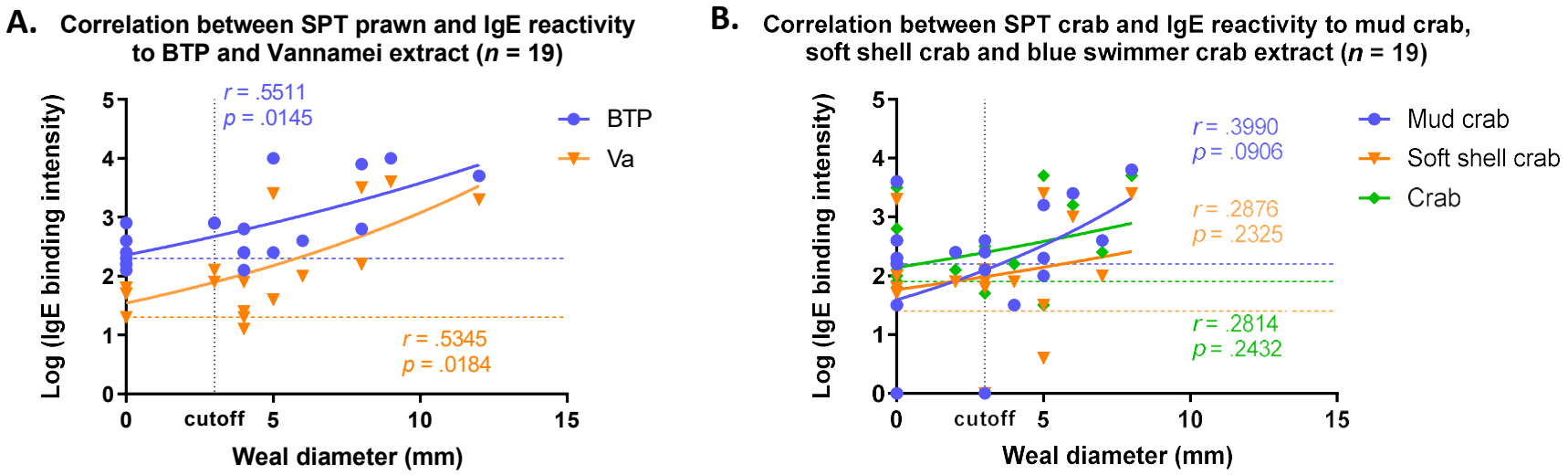

Figure 5.16 Correlation between **SPT results** and serum IgE reactivity to **crustacean** among 19 shellfish allergic patients. A) Serum IgE reactivity to BTP and vannamei prawn; B) Serum IgE reactivity to mud crab, soft shell crab and blue swimmer crab. The IgE binding intensity was transformed into logarithm with a base of 10. The cutoff values were determined by averaging the IgE binding intensity of five healthy controls from the same extracts/allergens. The colored dotted lines indicate the threshold lines for the relevant extracts. The Spearman's rank correlation coefficients were computed, and the regression line was plotted in GraphPad Prism to visualize the correlation.

In this cohort, four patients reported a clinical history to a mollusk. SPT with mussel was performed to confirm the clinical sensitization and identify possible cross-reactivity among 19 shellfish-allergic patients. However, all four patients with clinical history to oyster and mollusk (#3, #19, #29, #31) did not display a positive result to SPT with mussel. In contrast 7/19 patients (36.8%) showed positive SPT results to mussel extract (Figure 5.17). There was no correlation between SPT results and serum IgE reactivity to the investigated mollusk species.

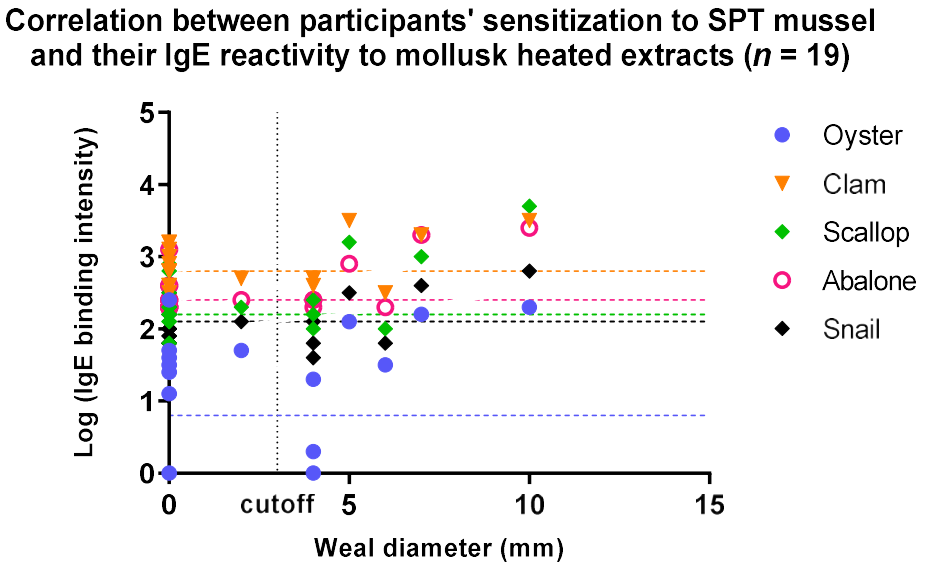

Figure 5.17 Correlation between **mussel SPT** results and serum IgE reactivity to various **mollusk heated extracts**. The IgE binding intensity was transformed into logarithm with a base of 10. The colored dotted lines indicate the threshold lines for the relevant extracts. The cutoff values were determined by averaging the IgE binding intensity of five healthy controls from the same extracts/allergens.

SPT to salmon, tuna, and cod was performed among the 19 patients (Figure 5.18). More subjects demonstrated a clinical sensitization to tuna (6 patients) than salmon (4 patients) or cod (3 patients) by SPT. For tuna patients did show a strong correlation between the SPT result and serum IgE reactivity (r = .6897, p = .0011), but this correlation was not seen for the salmon and cod extracts, or for purified cod parvalbumin (p > .05).

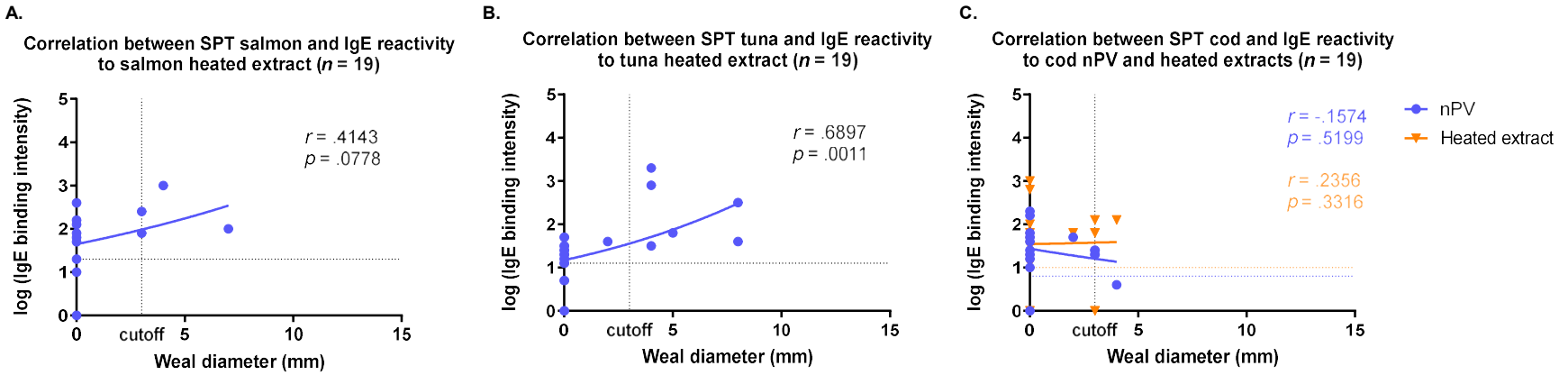

Figure 5.18 Correlation between **SPT results** and serum IgE reactivity using **fish extracts**. A. Salmon SPT and IgE to heated extract. B. Tuna SPT and IgE to heated extract. C. Cod SPT and IgE heated extracts and purified cod nPV.

The IgE binding intensity was transformed into logarithm with a base of 10. The colored dotted lines indicate the threshold lines for the relevant extracts. The cutoff values were determined by averaging the IgE binding intensity of five healthy controls from the same extracts/allergens. The Spearman's rank correlation coefficients were computed, and the regression line was plotted in GraphPad Prism to visualize the correlation.

The cross-reactivity of shellfish allergic patients with indoor allergens was investigated (Figure 5.19). For the nineteen patients, SPTs were performed to HDM and cockroach using available commercial extracts. Results indicate that 17/19 patients (89.5%) have positive SPT results to HDM and 13/19 patients (68.4%) react positively to cockroach SPT. In comparison to *in vitro* IgE binding tests, a weak correlation was seen between SPT positive results and serum IgE reactivity to these indoor allergens. One patient presented a positive response to the positive control reagent (histamine) but not to any SPT reagent.

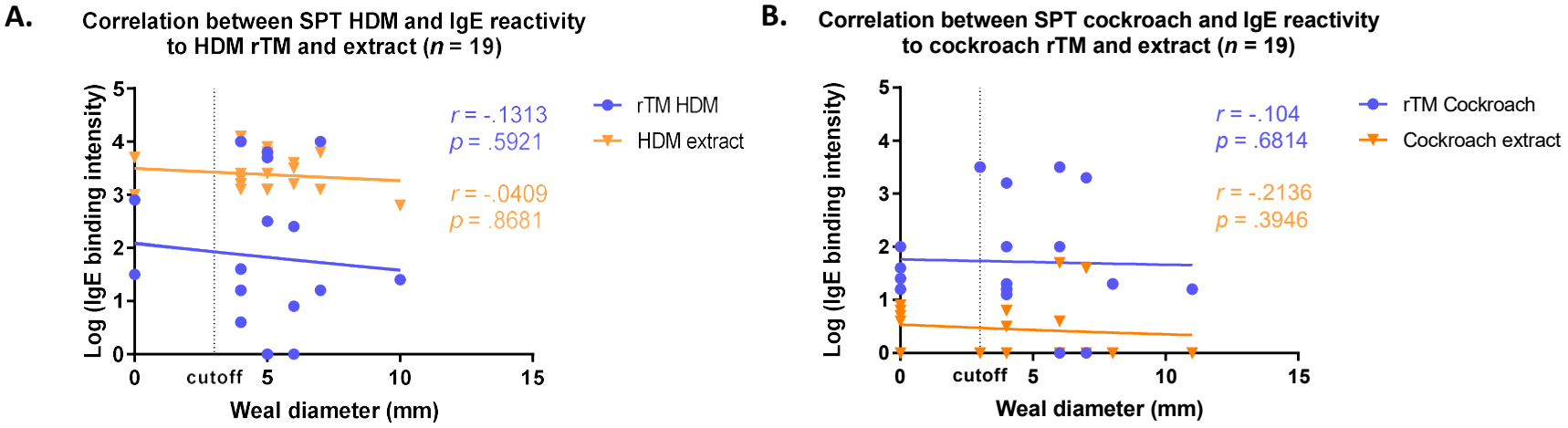

Figure 5.19 Correlation between **SPT outcomes** and IgE reactivity to **indoor allergens** (*n* = 19).

A) SPT using purified tropomyosin and extract from HDM; B) SPT using purified tropomyosin and raw extract from cockroach. The IgE binding intensity was transformed into logarithm with a base of 10. The colored dotted lines indicate the threshold lines for the relevant extracts. The cutoff values were determined by averaging the IgE binding intensity of five healthy controls from the same extracts/allergens. The Spearman's rank correlation coefficients were computed, and the regression line was plotted in GraphPad Prism to visualize the correlation.

5.5 Discussion

From the population-based surveys in Chapter 3 and Chapter 4, crustacean, mollusk and fish were the dominant allergy-inducing food groups among Vietnamese children and adults. This study sought to identify the seafood allergic patients' sIgE reactivity against crustacean, mollusk and fish species; to investigate the possible cross-reactivity between seafood allergens themselves and with other non-food allergen sources. The correlation between sIgE level and the SPT outcomes of the relevant allergen components were determined. To the author's knowledge, this is the first comprehensive study providing immunological and serological profiles of a seafood-sensitized subpopulation in Vietnam.

In line with the previous studies in Asia, prawn and crab were the leading allergy-inducing food groups among thirty-four seafood allergic participants. The dominance of shellfish allergy in this region may link to the high shellfish consumption in the general Vietnamese population (24). Most of the patients presented with an acute onset after seafood ingestion, implying the dominance of an IgE-mediated food allergy response. Skin problems including hives/urticarial or redness of the skin were the most frequently reported symptoms. This information is in accordance with the findings from the previous published population-based survey among the doctor-diagnosed food allergy populations in Vietnam (13, 14) (see Chapter 3 and Chapter 4). Noticeably, many patients with seafood allergy in this study appeared to develop their food allergic condition in adulthood with some patients recalling tolerating seafood when they were younger. Seafood has been reported as the leading food allergy type in adults (14, 25). Besides the long-lasting nature of this food allergy, the prolonged exposure to indoor allergens such as tropomyosin from HDM was implicated as the primary sensitizer leading to the later development of seafood allergy (26).

Tropomyosin (MW: 34-38 kDa) is a double-stranded α-helical coiled-coil actin-binding protein found in cell cytoskeletons and contractile muscle systems. Great efforts have been made to identify and molecularly characterize this water-soluble, heat-stable allergen from different edible crustacean and mollusk species. In food allergy, tropomyosin is the primary allergen among shellfish allergic populations (27-29). This

protein is, thus, considered a marker for sensitization to crustacean, mollusk or even fish (30). Generally, an allergen that incurs a positive response in >50% of the investigated population is regarded as a major allergen (31). In this study, tropomyosin purified from three crustacean species and five mollusk species was used to identify serum sIgE sensitization among thirty-four patients and five healthy controls. Despite the highly conserved amino acid sequence of arthropod tropomyosins (32), species-specific IgE reactivity to tropomyosin was indicated in our cohort. Patients demonstrated a positive response rate ranging from 29.4% - 67.6% against the investigated natural tropomyosins. With an average of 49.3% of patients reacting positively to a tropomyosin protein from either crustacean or mollusk, tropomyosin is at the borderline to be considered a major trigger among seafood allergic patients in this population. In practice, several allergens have been applied as biomarkers for seafood allergy diagnosis. A study by Gámez et al. (33) specified recombinant tropomyosin as a good predictor of shrimp allergy with a positive predictive value of 0.72 and a negative predictive value of 0.91. This finding, in Spanish shrimp allergic patients, was in line with studies of shrimp allergy in Brazil (34) and the US (35) where the authors revealed a strong correlation between sIgE response to shrimp tropomyosin and the clinical manifestations among confirmed prawn allergic patients. Interestingly, recent studies in South China (36), Japan (37) Singapore (38) and Italy (39) highlighted that tropomyosin was just a minor allergen among patients with shrimp allergy. Hence, it is crucial to investigate the involvement of other allergens in shellfish that could trigger an allergic response in the sensitized population.

In addition to tropomyosin, the proteins myosin light chain, sarcoplasmic calcium-binding protein and hemocyanin have been shown to be involved in clinical hyperactivity to shrimp, among shrimp-sensitized populations (40-42). For instance, sensitization to SCP and TM is associated with a positive challenge to shrimp among shrimp-allergic patients in the US (43). In our study, the analysis of sIgE to heated extract, recombinant myosin light chain, sarcoplasmic calcium-binding protein, and hemocyanin, and natural tropomyosin from vannamei prawn were performed and IgE reactivity compared (Fig. 5.5). As expected, there were more patients showing sIgE responses to heated extract (73.5%) than solely purified/recombinant allergen (p < .0001). Both recombinant hemocyanin and natural tropomyosin demonstrated a positive response rate of 61.8%. Mono-sensitization to vannamei prawn hemocyanin

was observed in patients #20, #22, #24 and #35. This clearly indicates the involvement of hemocyanin in sensitization to prawn in this population.

Each seafood species itself might contain different allergen concentrations, with different levels of allergenicity (44). The quantification of allergens and associated sIgE across species could support the prediction of clinically relevant sensitization, and to a certain extent, aid in the design of improved specific *in vivo* tests. In this investigation, the heated extract of BTP demonstrated higher levels of IgE recognition than those of other crab species (mud crab, soft shell crab and blue swimmer crab) and vannamei prawn ($p < .0001$) (Fig. 5.3). Among the mollusk group, the white clam was the most allergenic species compared to others (scallop and oyster) in the bivalve group ($p < .0001$). Octopus and cuttlefish are more allergenic than squid ($p < .0001$). Similarly, among the two investigated gastropods, the abalone bound more sIgE than snail ($p < .0001$).

Measurement of sIgE is of importance in predicting clinical reactivity, but it is also essential to examine the clinical evidence of sensitization through *in vivo* testing. In the current study, SPT to five commercially available shellfish reagents was performed among 19/34 patients. SPT with prawn extracts induced the highest clinical response (68.4%), followed by SPT with crab (57.9%), SPT with mussel (36.8%), SPT with octopus (31.6%) and SPT with squid (21.1%). A moderately positive correlation was observed between the SPT result and sIgE tests using prawn extracts (BTP: $r = .5511$, $p = .0145$; vannamei prawn: $r = .5345$, $p = .0184$) but not for other investigated species. For five subjects with simultaneous clinical history to prawns, crabs and mollusks, all SPT results to prawn, crab and mollusk were negative. However, four of them had a positive response to a SPT with HDM extract. This may be implicated in the cross-reactivity between the indoor allergen and seafood allergen in this subpopulation. However, it should be noted that most of the commercial SPT reagents that are produced in Europe or America (this study used the SPT reagents provided by the Immunotek from Spain) might not be specific for the local patients in Asia, not to mention the variability of the allergen components in each of the commercial extracts, as has been documented in the literature (45).

Immunological and clinical cross-reactivity between tropomyosin from dust mites and shellfish has been well documented (32). Prolonged exposure to tropomyosin from the living environments via the inhalant pathway is assumed to be the primary sensitizer for later sensitization to shellfish (46). Lam et al. (47) reported a high sensitization rate to storage mites in the population in the North of Vietnam. In the current investigation, many shellfish allergic patients (91.2%) reveled IgE binding to HDM, and 17/19 (89.5%) had a positive result to a HDM SPT. In the Asia Pacific region, cross-reactivity between shellfish and mites has previously been described in Singapore (38) and Australia (48). With a predominance of mites in regions of temperate and warm climates (49), mite sensitization remains a hidden risk for the later development of shellfish allergy in these populations.

However it is not solely tropomyosin attributing to shrimp-mite cross-reactivity; other important mite allergens have also been identified and characterized (50, 51). For example, a mite and crustacean allergen with a molecular weight of 20 kDa (52), and some higher molecular weight proteins from invertebrates (39). Ubiquitin and α-actinin are two new allergens that were identified among mite-shrimp allergic subjects in Spain (53). In the current study, a weak correlation between IgE binding to HDM tropomyosin and HDM raw extract was seen among the 34 patients (r = .2283, p = .1941), with 61.8% of patients' IgE recognizing HDM tropomyosin compared to 97.1% recognizing the HDM raw extract (p < .0001). There is a need for further investigations to identify other possible cross-reactive components implicated in mite and shellfish sensitization.

Cockroach is the second most important allergen source that is known to cross-react with shellfish allergens. Cockroach sensitization is common due to the widespread occurrence of this indoor insect (54, 55). Cockroach sensitization could occur early in childhood and signify an important trigger for the development of many allergic conditions later in life (55, 56). Several allergens from cockroach have been identified and characterized (57, 58), along with their cross-reactivity to shellfish allergen (36, 59, 60). German cockroach (*Blattella germanica*) was selected for the examination due to its abundance in the Asia regions (61). Higher rates of IgE recognition to cockroach recombinant tropomyosin, as compared to cockroach extract, was noted, and likely to contribute to the cross-reactivity of cockroach to other invertebrate allergens, including crustacean. 68.4% of the analyzed patients showed a positive response to the SPT

reagent. However, no correlation between sIgE measurements and the relevant clinical manifestation was observed (r = -.02136, p = .3946). The disagreement in the test outcomes of the serological and immunological diagnostic methods for cockroach sensitization was addressed in a recent study (62). The variation of cockroach allergen distribution and/or their concentration in commercial SPT extracts and extracts used for the immunoassay was demonstrated (63). Thus, when exploiting concurrent multiple *in vitro* and *in vivo* tests to assess cockroach sensitization, it is important to ensure the consistency of cockroach allergens to support a compelling outcome.

Mealworm (*Tenebrio molitor L.)* is a group of edible insects that is frequently consumed in Asia (64); it contains allergens that have also shown to cross-react with shellfish allergens (65, 66). The potential cross-reactive allergens include arginine kinase, tropomyosin, α-tubulin, β-tubulin, actin, fructose-biphosphate aldolase, myosin light chain and troponin-T (66). In a study from the Netherlands, up to 87% of shrimp-allergic patients cross-reacted to mealworm allergens (65). In this study, 73.5% and 82.4% of patients showed sIgE binding to mealworm raw and heated extracts, respectively. Additionally, sIgE recognition to mealworm heated extract was much stronger than to raw extract (p < .0001). This phenomenon might be attributed to the stability of mealworm allergens during heat treatment (67) as well as the increased allergenicity of a major heat-stable 27 kDa glycoprotein in mealworm (68). 8/34 patients in this study reported adverse reactions to silkworm (*Bombyx mori*) pupae, confirming the clinical relevance of arthropod cross-reactivity. Anaphylaxis due to silkworm consumption has also been reported in China (69) and the US (70). Although the present study could not confirm the patients' sensitization to silkworm allergens to match with their clinical history, it can be seen that the majority of shellfish allergic patients in this population cross-reacted to mealworm allergens. Thus, consumption of this edible insect might put seafood allergic patients at risk of developing allergic reactions.

Among the investigated mollusk species, oyster is often consumed raw, thus, it is crucial to understand the allergenicity of oyster in raw and cooked preparations. Heat treatment has been known to change the allergenicity of food allergens (71-73). However, whether thermal treatment increases or decreases the allergenicity of a food antigen varies with its structure or composition, and the extent of the heat treatment. When heat is applied such as boiling, frying or roasting, it could denature heat-labile

proteins in food. In addition, high temperatures could lead to the rearrangement of protein structure, thus altering the availableIgE binding epitopes; or it could expose previously hidden epitipopes. Heat treatment was found to increase the IgE reactivity of many mollusk extracts, including Sydney rock oyster (*Saccostrea glomerata*), blue mussel (*Mytilus edulis*), saucer scallop (*Amusium balloti*), and southern calamari (*Sepioteuthis australis*) (72), as well as tropomyosin of the Pacific oyster (*Crassostrea gigas*) (74). However, the opposite tendency was seen in the tropical oyster (*Crassostrea belcheri*) (75). In the current study, a stronger patient IgE reactivity was observed to raw Pacific oyster extract than to heated extract ($p < .0001$) and purified nTM ($p < .0001$), confirming the loss of allergenicity due to heat treatment. Furthermore, with 11/22 subjects reacting to heated extract but not to the nTM implicates a likely participation of other heat-stable allergens residing in the oyster heated extract. Currently, tropomyosin from only two oyster specifies are characterized and registered in the IUIS database (Cra g 1 and Sac g 1). However, with the application of biochemical and computational tools, Nugraha et al. (2018) reported 23 unrecognized allergens in the Pacific oyster on top of the well-known TM (76). Thus, it is essential to identify the putative allergens present in raw and heated oyster extracts that may be important in provoking allergic reactions among this population.

In this cohort, only one patient reported clinical reactivity to fish. Other participants either avoided consuming fish as a consequence of having shellfish allergy, or only consumed this food occasionally. We sought to identify the risk of cross-reactivity among shellfish allergic patients to fourteen commonly consumed fish species in Vietnam. Generally, patients demonstrated diverse patterns of sIgE reactivity to heated extracts. In this study all patients reacted positively to at least one fish species. Patients with strong IgE responses to crustacean and mollusk also showed strong IgE reactivity to investigated fish species. The strongest IgE response was, in descending order: basa fish, goby fish, Atlantic salmon, tilapia, yellow scad, barramundi, Atlantic cod, and yellowfin tuna. There was no significant difference in IgE binding to purified parvalbumin of barramundi, cod, basa, or salmon ($p = .2843$), but 61.8% of participants reacted positively to fish tropomyosin from Tilapia. When confirmed with fish SPT reagents, the positive clinical reactivity decreased to 31.6% for the tuna SPT, 21.1% for the salmon SPT and 15.8% for the cod SPT. So, shellfish allergic patients demonstrated some clinical cross-reactivity to fish species, but it is not known whether

TM or any other allergens in fish were responsible for the cross-reactivity among the population in this study. Due to the unavailablity of SPT extracts for basa fish, we were unable to confirm the clinical reactivity to this allergen in this population. Given the fact that basa fish is one of the common fish species in Vietnam, and it appeared to be implicated in reactions in the greatest numbers of patients in this study, it is crucial to conduct further investigation of this fish to identify its allergen profile.

Vietnam is one of the highest seafood consuming nations in the tropics (24) and is the biggest seafood production and processing hub in the world market (77). Similar to other developing economies, Vietnam has been suffering from the burdens of the allergic epidemic due to the rapid urbanisation and substantial changes in lifestyle (78, 79). In the field of food allergy, it is still in its infancy, with limited specialists and medical facilities. Food allergy diagnosis in Vietnam focuses heavily on two allergy units in Hanoi and Ho Chi Minh City. From our population-based surveys that revealed a comparably high seafood allergy rate in both children and adults, we followed-up this investigation in an effort to provide more insight on the clinical presentation and the immunological profiles of seafood allergy sufferers in Vietnam. The author is aware that this study has a number of limitations that need to be addressed.

Seafood allergy is prevalent in both Vietnamese children and adults. However, in the serological studies, the investigator was unable to recruit enough pediatric participants to confirm the immunological profiles of seafood allergy in this age group and thus, missed the opportunity to compare the manifestation of this health condition between children and adults. The picture of the seafood allergy in Vietnam is therefore still incomplete.

In this study, sera from five healthy controls were used in the immunoassays to establish the threshold for the sIgE levels to investigated proteins/allergens. Even though these participants have no previous clinical reaction towards seafood consumption, these subjects might likely have a certain amount of sIgE antibodies to investigated allergens/extracts. Hence, the cutoff value used to define a positive or negative response in this study remains relative to the controls utilized. Furthermore, the author understands that the comparison between sIgE measurement and SPT

result might be irrelevant due to the inconsistency between the seafood species in the commercial SPT reagent and the ones used in the immunoassays in this investigation.

It is well known that traditional tests for food allergy have imitations; SPT and sIgE measurement are the approved tools for detecting sensitization to foods, but are not necessarily predictive of reaction severity. In our cohort, four participants (#7, #8, #10 and #15) presented with an exceptionally high IgE reactivity to most of the tested allergens, about 10 times higher than the positive control. These participants are in good agreement with their SPT results (largest weal of 12 mm) and clinical history of shellfish allergy. Of these four participants, three presented with severe reactions including wheezing, faint, and shock after consuming seafood. Thus, it is suggested that SPT weal size and IgE levels can correlate with the likelihood of a reaction to the suspected trigger. Numerous international studies were conducted in the attempt to identify the cut-off value of the weal size to the clinical relevance. For example, a study among Australian infants with egg, peanut and sesame allergy set up the 95% predictive value for allergic reactions for egg (SPT weal >=4 mm), peanut (SPT weal >=8 mm) and sesame (SPT weal >=8 mm) (80). However, in this investigation, we were unable to confirm the food allergy status using oral food challenge. To the author's best knowledge, performing oral food challenges are very limited in Vietnam due to technical and personnel constraints. Furthermore, the SPT reagents used in this study were produced in Europe and missed some seafood species which cause allergic reactions in Vietnam. We were unable to perform SPT to some of the seafood species implicated by some patients.

Despite these limitations, this study is the first step towards discovering and understanding seafood allergy in the Vietnamese population. This is the first population-based study on FA in Vietnam; it provided useful information about the current FA situation for the Vietnamese population, healthcare professionals and public health policymakers. The survey was thought to increase local people's awareness about FA, particularly contributing to a better understanding of the disease and its typical clinical symptoms.

The author would like to suggest some course of actions to better manage FA in Vietnam:

- Developing a national guideline on FA and food allergens.
- Developing a guideline/cheat sheet on food anaphylaxis.
- Reviewing and validating the effectiveness of the current diagnostic tests for FA in Vietnam.
- Educating the general public about this health condition and increasing their awareness.
- Taking food allergens into account in the current food labelling policies and regulations.

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5.7 Chapter 5 summary

- Thirty-four seafood allergic patients and five healthy controls were recruited. Their clinical history of seafood allergy was collected and analyzed. Seafood allergic subjects reported the acute onset of clinical symptoms, of which skin problems and oral allergy syndrome are the most frequent reactions after consuming seafood.

- Skin prick tests were performed in 19 participants. IgE reactivity to the investigated seafood panel was performed in all participants to explore their sensitization profiles.

- IgE reactivity to a comprehensive panel of the most frequently consumed crustacean, mollusk and fish species weas presented and analyzed. Prawns and crabs are the main allergy-triggering food items. The variation of IgE sensitization to different prawn allergens was addressed. Prawn hemocyanin and tropomyosin seem to be the most frequently implicated allergenic proteins. Black tiger prawn, abalone, octopus and clam are are associated with the most seafood allergy sensitizations among the investigated seafood panel. Subjects with shellfish allergy also showed IgE sensitization to heated fish extract. The most implicated fish species was basa and the least was blue mackerel. Participants also showed IgE reactivity to purified parvalbumin, and a significant difference of IgE recognition was seen between parvalbumin from barramundi and salmon only. Heat treatment was shown to alter the allergenicity of the Pacific oyster extracts.

- Participants showed species-specific IgE reactivity to natural tropomyosin from different crustacean and mollusk species. The strongest IgE recognition was seen to natural tropomyosin from vannamei prawn and squid.

- Tropomyosin is the primary trigger for the cross-reactivity between shellfish and house dust mite and cockroach. However, tropomyosin is not the major allergen for dust mite and cockroach sensitization in this population.

- A weak correlation between SPT outcomes and IgE reactivity was seen for most of the seafood species, excluding vannamei prawn which demonstrated a moderate correlation between the SPT and IgE reactivity tests.

CHAPTER 6 CLINICAL PRESENTATIONS AND IMMUNOLOGICAL PROFILES OF SEAFOOD ALLERGY IN AUSTRALIA

Manuscript in preparation:

Thu T.K. Le, Aya C. Taki, Sandip D. Kamath, Elecia Johnston, Anthony Leicht, Kenji Doma, Darlene Wallace, Dianne E. Campbell and Andreas L. Lopata. Serum IgE reactivity to commonly consumed seafood products among Australian adults with seafood allergy. Clinical & Experimental Allergy.

6.1 Introduction

Food allergy is an immunological disorder resulting from the immune system reacting to a harmless component in food. Although the risk of fatality is rare (1), food allergy strongly affects the quality of life of its sufferers and imposes financial burdens. It is estimated that one out of ten Australian infants and up to 8% of children have a food allergy (2). Australia also has the highest rate of challenge-proven food allergy in the world (3).

The most frequent allergy-inducing food items in Australia are cow's milk (8.3%), followed by peanut (6.9%), shellfish (5.9%), wheat (5.6%), fruit (5.3%), egg (3.4%), vegetables (2.7%), fish (2.5%), tree nuts (2.2%), soy (1.7%) and other foods (6.3%) (4). However, the prevalence of food allergy and the offending food allergen sources seem to differ among age groups in this continent. According to the Australian Health Survey in 2014, the leading food allergy types in children (2-18 years) were peanut (2.9%), tree nuts (1.6%), fish (0.5%) and prawn (0.5%). In adults (19-30 years) the food allergy pattern and frequency were starkly different with prawn being the highest (2.3%), followed by peanut (1.3%), fish (1.1%) and tree nuts (0.5%) (5). It should be noted that peanut, tree nuts and seafood are persistent food allergies that most children do not outgrow in adulthood. Thus, the finding that prawn and fish allergy are also the predominant food allergy types in adulthood in this population is interesting, and worth further investigation.

Shellfish and fish allergy have been reported to be the most common food allergies in the adult population in several countries. In the US, from a population-based survey among 40,443 adults, shellfish allergy was reported at 2.9%, followed by milk allergy (1.9%), peanut allergy (1.8%), tree nuts (1.2%) and fish allergy (0.9%) (6). Similarly, in Europe, the highest shellfish and fish allergy rates were 6% and 2% among Italian adults, respectively (7). In the Asia Pacific, a population-based survey among 30,018 Taiwan adults revealed the frequency of shellfish allergy to be 7.05% and fish allergy 1.17% (8). Our recently published population-based survey in Vietnam demonstrated the predominance of shellfish and fish allergy among Vietnamese adults with doctor-diagnosed crustacean, fish and mollusk allergy at 3.5%, 1.9%, and 1.7%, respectively. In Australia, fish was the second highest culprit in food-induced anaphylaxis (1). However, seafood allergy studies among adults in Australia are far outnumbered by

pediatric food allergy investigations. There is a lack of information about the epidemics and etiology of seafood allergy in Australian adults.

Tropomyosin is one of the major seafood allergens, accounting for sensitization in about 80% of crustacean allergy cases (9). This heat-stable protein (MW: 34-38 kDa) can also be found in non-food sources such as house dust mite and cockroach (10, 11). Due to the high homology of arthropod tropomyosin, the cross-reactivity to tropomyosin from food and non-food sources has been highlighted (9, 12). Sensitization to tropomyosin from indoor allergen sources has been indicated as the primary sensitizer for the later development of shellfish allergy (13). An investigation among Spanish shrimp allergic patients demonstrated IgE reactivity to shrimp tropomyosin correlated well with clinical shrimp allergy and tropomyosin was suggested as a biomarker for shrimp allergy diagnosis (14). However, it is unknown how shellfish tropomyosins and other allergen components contribute to the pathophysiology of shellfish allergy among the sensitized population in Australia. Whether or not prolonged exposure to an indoor allergen is a trigger for elevated shellfish allergy incidence in adulthood in this population is not known.

The current study aims to investigate the clinical presentations and immunological patterns of seafood allergy among adults in North Queensland, Australia. The IgE reactivity of seafood allergic subjects against a comprehensive panel of crustacean, mollusk and fish extracts will be investigated to examine the putative allergenicity of the investigated seafood products. The IgE reactivity to purified tropomyosins from seafood and indoor allergen sources will be examined to assess the likelihood of cross-reactivity among arthropod tropomyosins. The attribution of individual allergen components to the IgE response will be compared and discussed. This study seeks to provide insights into the seafood allergy pathogenesis in Australia. The findings will provide scientific evidence for improving seafood allergy diagnosis in Australia and in the Asia Pacific.

6.2 Aims

The aims of this chapter are as below:

- To collect clinical data of seafood allergic participants and investigate the clinical manifestations of seafood allergy in Australian adults.
- To investigate the IgE reactivity of seafood allergic participants to the most commonly consumed crustacean, mollusk, and fish species in Australia to examine the allergenicity of seafood species in this population.
- To examine the attribution of the major allergens tropomyosin and parvalbumin in sensitization to seafood.
- To investigate the correlation between reported clinical symptoms and the presence of specific IgE in seafood allergic participants.
- To compare the specific IgE levels determined by lab-based immunoblotting techniques and by ImmunoCAP (the commercial test kit currently available in Australia).

6.3 Materials and methods

6.3.1 Participant recruitment:

A seafood allergy study campaign was promoted in North Queensland, Australia. Participants were recruited via two intakes in November 2017 and May 2018. A total of 69 participants were interviewed via a detailed questionnaire (Figure 6.1). Forty-one subjects with a clinical history of developing adverse symptoms upon seafood consumption were invited to an interview with the study food allergist. Twenty-eight subjects with no clinical history to seafood consumption were invited as healthy controls. All participants gave their written informed consent to the study and the relevant scientific reports and publications.

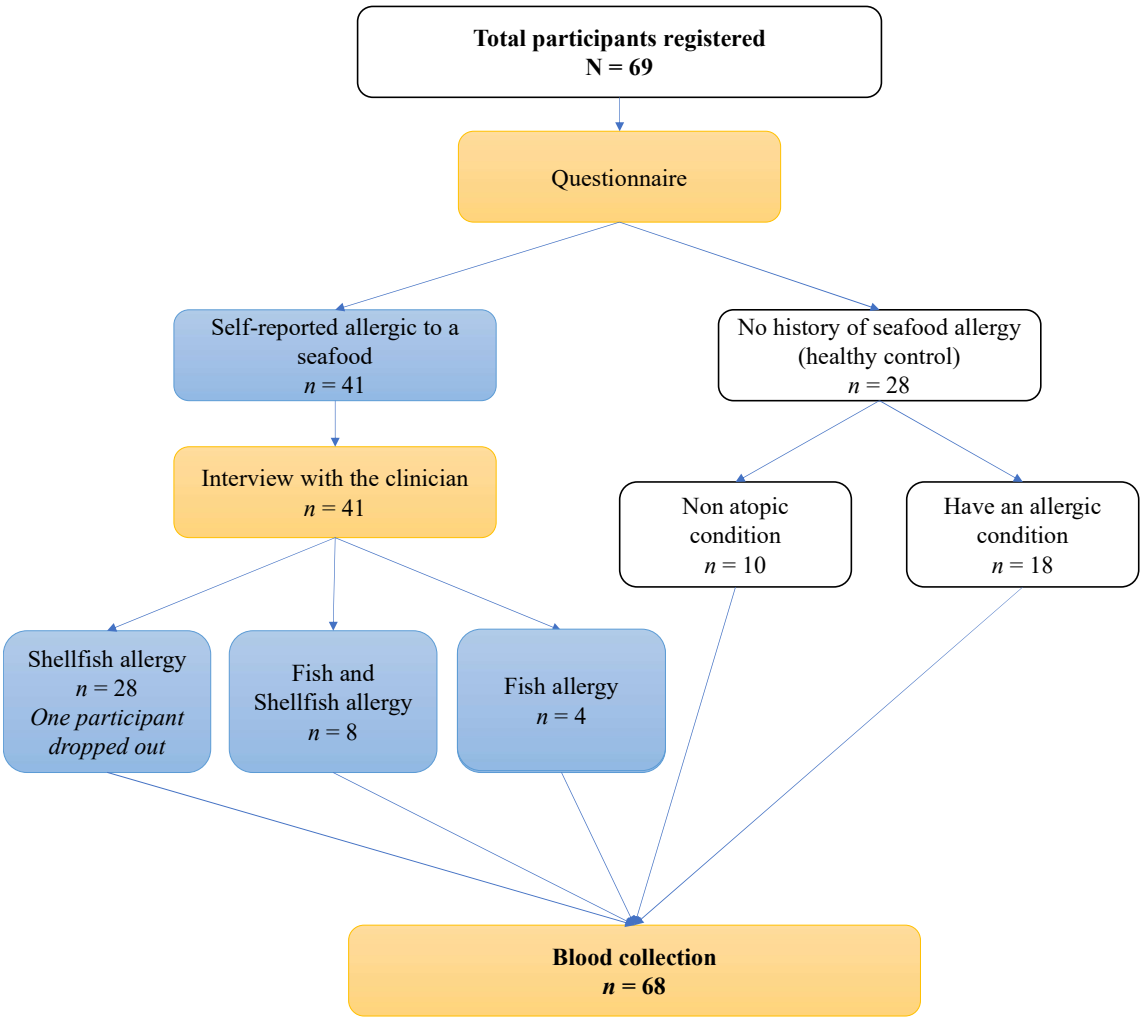

Figure 6.1 Study design and flow chart.

6.3.2 Participant interview

Participants with self-reported clinical history to fish, crustacean, or mollusk were invited to an interview with the food allergist. A pre-designed questionnaire (Appendix B6) was used to collect demographic information and details of the clinical manifestations. Participants were classified into three groups according to their clinical history: shellfish allergy, fish allergy, and seafood allergy. The shellfish allergy group consisted of individuals with a clinical history to crustacean or mollusk. The fish allergy group included subjects that reported allergic reactions to fish. The seafood allergy group were participants with clinical history to both shellfish and fish consumption.

6.3.3 Serum collection

Both the seafood allergic participants and the healthy controls were invited to donate blood samples. A volume of up to 10 mL blood was collected into a BD vacutainer® blood collection tubes containing clot activator. Blood samples were kept at room temperature to clot prior to the centrifugation (1000 x *g*/ 10 min). Sera were pipetted into a clean vial and labeled with the participant's name, their date of birth and the laboratory identification number. Serum aliquots were kept at -80^{o}C until further use.

6.3.4 Allergen panel

A comprehensive panel of fish, crustacean and mollusk species which are commonly consumed in the region was used for IgE analysis. Raw and heated seafood protein extracts and single purified allergens were prepared as previously described in section 5.3.5 of Chapter 5. Additional allergens used in the immunoassays were provided by the Molecular Allergy Research Laboratory at James Cook University, Australia and include: recombinant myosin light chain (rMLC), hemocyanin (rHC) and sarcoplasmic calcium-binding proteins (rSCP) from vannamei prawn (*Litopeaneaus vannamei*), as well as extract and rTM from cockroach. European house dust mite extract was supplied by DST (Diagnostische Systeme & Technologien GmbH, Schwerin, Germany). The list of all protein extracts and allergens used in this chapter is summarized in Table D6.2 (Appendix D).

6.3.5 IgE measurement by ImmunoCAP

Serum specific IgE to Pilchard (f61), Tuna (f40), Salmon (f41), Cod (f3), Squid (f258), Shrimp (f24), HDM (d1) was quantified using Phadia ImmunoCAP® test kits (Phadia-Thermofisher, Uppsala, Sweden). According to the manufacturer, the prawn reagent (f24) is a mixture of boiled, frozen Atlantic shrimp and raw, frozen prawns from the Indo-West-Pacific of four species *Pandalus borealis, Penaeus monodon, Metapenaeopsis barbata, Metapenaus joyneri* (http://www.phadia.com/en/Products/Allergy-testing-products/ImmunoCAP-Allergen-Information/Food-of-Animal-Origin/Shellfish/Shrimp/). The tests were outsourced to the Sullivan Nicolaides Pathologist (Brisbane, Australia). The IgE levels were ranked from negative to very high following the guidelines from the test provider (Sullivan Nicolaides Pathology, Brisbane, Australia): class 0 – negative: value of less than 0.1 kUA/L; class I – low: < 0.7 kUA/L; class II - moderate <3.5 kUA/L; class III – high: <17.5 kUA/L; class IV - very high <52.5 kUA/L and class VI – very high: <100 kUA/L.

6.3.6 Protein profiling by SDS-PAGE

Protein profile of all extracts and allergens was completed by SDS-PAGE, as described in Section 5.3.8 of Chapter 5.

6.3.7 Immunoassays

Grid blot and western blot were performed as described in Section 5.3.9 of Chapter 5.

6.3.8 Ethics approval

This study was approved by the James Cook University Human Research Ethics Committee. Approval ID: H6829 (Appendix A3).

6.3.9 Data analysis

The software SPSS Statistics version 25.0 for Windows (IBM Corp., Armonk, N.Y., USA) and the GraphPad Prism version 8.2 for Windows (GraphPad Software, La Jolla, California, USA) were used to perform statistical analysis and generate plots. The demographic and clinical features of the participants were tabulated for comparison. Continuous variables were presented as mean ± SD or as the number of cases and percentages where appropriate. For the grid blot immunoassay data, IgE reactivity signals were exported into Microsoft Excel files. Raw fluorescent signals were blank-corrected by subtracting the local background, relevant to each protein extract/allergen. The relative IgE binding intensities were estimated by transforming these readouts into $\log_{10}$ of the blank-corrected data. Replicates of positive and negative controls were averaged to generate the reference values of positive and negative responses to the tested proteins/allergens. The negative values were used as the threshold to define positive IgE reactivity. The Friedman test or one-way ANOVA, followed by Dunn's test, were used to compare the IgE reactivity of participants to different tested proteins/allergens where appropriate. The Wilcoxon matched-pairs signed-rank test was used to compare the IgE reactivity between two tested proteins/allergens. The Pearson correlation was used to examine the relationship of IgE reactivity between the seafood protein extracts and the purified allergens (i.e., tropomyosin and parvalbumin). The Spearman's rank-order correlation was used to evaluate the association between the ImmunoCAP test results (ranks) and the relevant IgE reactivity (r_s and *p*-value), and the linear regression was used to analyze the independence between the two variables. A *p*-value of less than .05 is considered to be significant in all tests.

6.4 Results

6.4.1 Demographics

Forty-one participants (63.4% female) who had a clinical history to fish, crustacean, or mollusk were interviewed (Table D6.1, Appendix D). The average age of the participants was 42.5 ± 15.8 years. In this cohort, the majority of participants are Caucasian (80.5%); Asian heritage accounts for 17.1%, and one participant is Latin American. Twenty-nine subjects (70.7%) self-reported allergic disorders to crustacean and mollusk; four subjects (9.8%) self-reported fish allergy and eight subjects (19.5%) perceived allergies to both fish and shellfish. There were five subjects reporting adverse symptoms due to skin contact, and the remaining subjects manifested allergic symptoms via the ingestion pathway. 87.8% and 31.7% of participants reported allergic symptoms to crustacean and mollusk, respectively. Prawns (37.5%) was the most implicated shellfish in this survey (Table 6.1). Among the different fish species, Atlantic salmon and tuna were frequently indicated as allergy-inducing foods.

In this cohort, 31/41 (75.6%) participants reported having seafood during early childhood (1-5 years) and 6/41 (14.6%) participants started consuming seafood at around 6-10 years. One participant strictly avoids seafood although there is no evidence or clinical history suggesting the case of seafood allergy. The majority of participants (78.0%) tolerated seafood for a certain period of time before reporting the first allergic reactions in their adolescence or adulthood.

Table 6.1 The implicated seafood species that trigger adverse reactions among 41 participants.

Items	Responses	
	N	Percent
Prawns	36	37.5%
Crabs	11	11.5%
Lobster	7	7.3%
Calamari	7	7.3%
Bugs	6	6.3%
Atlantic salmon	4	4.2%
Scallops	4	4.2%
Oysters	4	4.2%
Tuna	3	3.1%
Barramundi	2	2.1%
Mackerel	2	2.1%
Mussels	2	2.1%
Freshwater crayfish	2	2.1%
Whitings	1	1.0%
Breams	1	1.0%
Mullet	1	1.0%
Squid	1	1.0%
Octopus	1	1.0%
Clams	1	1.0%
Total	**96**	**100.0%**

The majority of the participants presented with acute allergic reactions (Figure 6.2). 75.6% of subjects had symptoms occur within thirty minutes after the food ingestion/contact. Up to 90.2% of participants recorded clinical disorders within two hours. Four participants (9.8%) reported a late allergic response with the symptom appearing later than 12 hours after food ingestion. The distribution of the reported clinical manifestations is presented in Table 6.2.

Most of the participants experienced a broad spectrum of clinical presentations, with an average of 4-5 symptoms per subject. Of these, skin problems including hives/urticaria, a flare of eczema, a redness of the skin or skin itching were predominant (73.2%). 68.3% of participants reported symptoms localized to the oral organs: itchy throat or mouth, lips or face swelling, lip or a tongue tingling or tight throat. Gastrointestinal symptoms (i.e., abdominal pain, nausea/vomitting or diarhea) occurred in 18/41 participants (43.9%), and 15/41 participants (36.6%) presented respiratory symptoms (tight chest/chest pain, coughing, congested or a running nose).

Six participants (14.5%) experienced anaphylaxis and were taken to the emergency room.

In the most severe episodes, many of the participants (39.2%) took no action, 12/41 (23.5%) participants used an antihistamine to relieve the symptoms, 10/41 (19.6%) participants visited doctors for their allergic symptoms. 8/41 (15.7%) participants currently carry an EpiPen®.

Forty subjects donated their blood for *in vitro* investigation; one participant dropped out of the study.

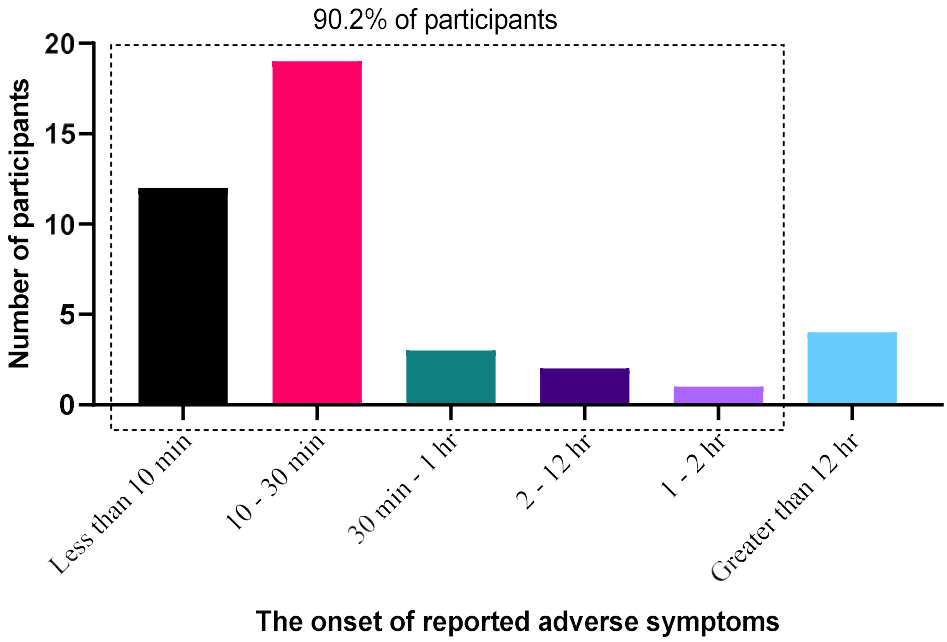

Figure 6.2 The reported onset of adverse symptoms due to seafood consumption/contact among 41 participants.

Table 6.2 The distribution of reported clinical symptoms of participants with a history of seafood allergy (*n* = 41).

Symptom	Responses	
	N	Percent
Itchy throat or mouth	22	12.6%
Redness of the skin	19	10.9%
Nausea/vomiting	18	10.3%
Itching	17	9.8%
swelling of lips or face	17	9.8%
Lip or tongue tinging	16	9.2%
Hives/urticaria	14	8.0%
Tight throat	10	5.7%
Tight chest/chest pain	10	5.7%
Abdominal pain	6	3.4%
Coughing	6	3.4%
Swelling elsewhere	5	2.9%
Diarrhea	4	2.3%
Wheezing	4	2.3%
Faint/dizzy	3	1.7%
Flare of eczema	1	0.6%
Congested or running nose	1	0.6%
Other symptoms	1	0.6%
Total	**174**	**100.0%**

There are many contributing factors to the current food allergy condition. 82.9% of participants indicated to have at least one other concurrent allergic conditions: twenty-five participants (61.0%) have allergic rhinitis, twenty-four participants (58.5%) have house dust mite allergy, eighteen participants (43.9%) have asthma, eleven participants (26.8%) experienced childhood eczema and ten participants (24.4%) concurrently suffers from other food allergies excluding seafood allergy. 58.5% of participants have an immediate family member with a food allergy or an allergic disease. Nearly half of the participants (48.8%) currently have pets, including cats, dogs or horses.

6.4.2 Clinical history and sensitization profile of the control group

Among 28 healthy controls, 18/28 (64.3%) of the subjects indicated they had an atopic condition, including childhood asthma, contact dermatitis, hay fever, allergic rhinitis, or food intolerance. sIgE to HDM and prawn was measured by ImmunoCAP® for 26/28 controls (Figure 6.3). Of these 6/26 and 10/26 subjects were sensitized to prawn and HDM, respectively, based on the ImmunoCAP results. The individuals who presented with no atopic condition and were negative to prawn and HDM by ImmunoCAP were used as the healthy controls in the immunoassays.

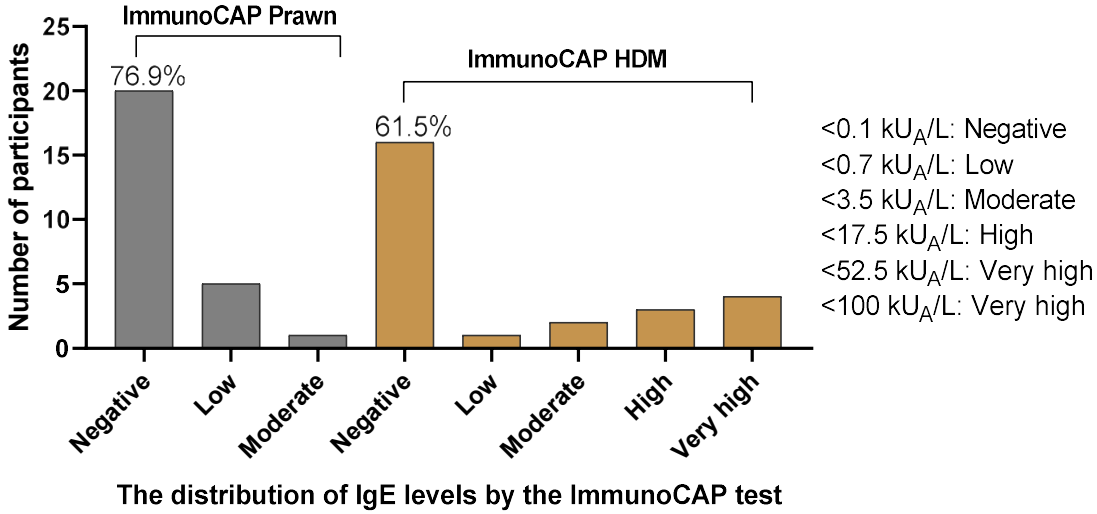

Figure 6.3 The distribution of sIgE levels to prawn and HDM as measured by ImmunoCAP, among 26 control participants.

6.4.3 IgE reactivity to crustacean, mollusk and indoor allergen sources among participants with perceived shellfish allergy

Sera from twenty-eight subjects diagnosed with IgE-mediated crustacean and/or mollusk allergy by the study clinician, based on clinical symptoms, were used to analyze for IgE reactivity to a panel of eight crustaceans, seven mollusks and two representative indoor allergen sources (HDM and cockroach). The negative control

group included six healthy participants. The positive control included two participants with confirmed shellfish allergy. Clinical data for these groups is included in Table D6.1, Appendix D. The IgE binding intensity of the negative control group was averaged and used as the cutoff to define the positive IgE reactivity test to the relevant investigated species. The percentage of positive tests was generated from the number of subjects showing positive IgE reactivity, over the total investigated population (n = 28). The IgE reactivity of twenty-eight participants against heated extracts from crustaceans and mollusks, and extracts from HDM and cockroach, are presented in Figure 6.4. The color scale is a grading of the IgE binding intensity, from no binding (white, lightest) to the strongest binding (red, darkest).

Out of the crustacean extracts tested, mud crab gained the highest positive IgE reactivity ratio (85.7%), followed by yabby and prawn. Prawn species share similar IgE reactivity ratio among participants, except BTP seems to be the least reactive species (50.0%). In the mollusk group, cuttlefish had the highest IgE recognition rate (82.1%), and this was double the amount of positive tests observed to Pacific oyster (39.3%). More than half of the cohort displayed positive IgE binding to abalone, squid, octopus and clam extracts. The lowest number of positive IgE tests was against scallop, with 17.9% positive IgE reactivity.

All subjects demonstrated positive IgE reactivity to at least one of the investigated protein extracts. Two subjects (#3, #20) showed the strongest and broadest range of IgE reactivity to all tested crustacean and shellfish species. Participants #1, #6 and #19 demonstrated IgE reactivity to crustacean but not to mollusk protein extracts. In general, most participants reacting to vannamei protein extract also reacted to other prawn extracts, excluding the case of participant #17 that showed IgE reactivity to vannamei prawn extract only. In the mollusk group, species-specific IgE binding was also demonstrated among subjects. For instance, participant #2 indicated a strong IgE response to cuttlefish, squid, and octopus (cephalopoda) but not to abalone (gastropoda). This participant also reacted to the clam extract but not to other bivalves (i.e. Pacific oyster, scallop).

Two representatives of indoor allergens employed in the screening were HDM and cockroach. About half of the subjects (53.6%) had a higher IgE binding intensity to HDM and cockroach extract compared to the negative control group, but each with a

distinct and separate set of implicated subjects. Generally, participants with a positive IgE response to HDM or cockroach had IgE reactivity against at least one other crustacean or mollusk extract. But in contrast, 14.6% of individuals displayed IgE binding to neither HDM nor cockroach extract, but demonstrated the positive reactivity to a shellfish.

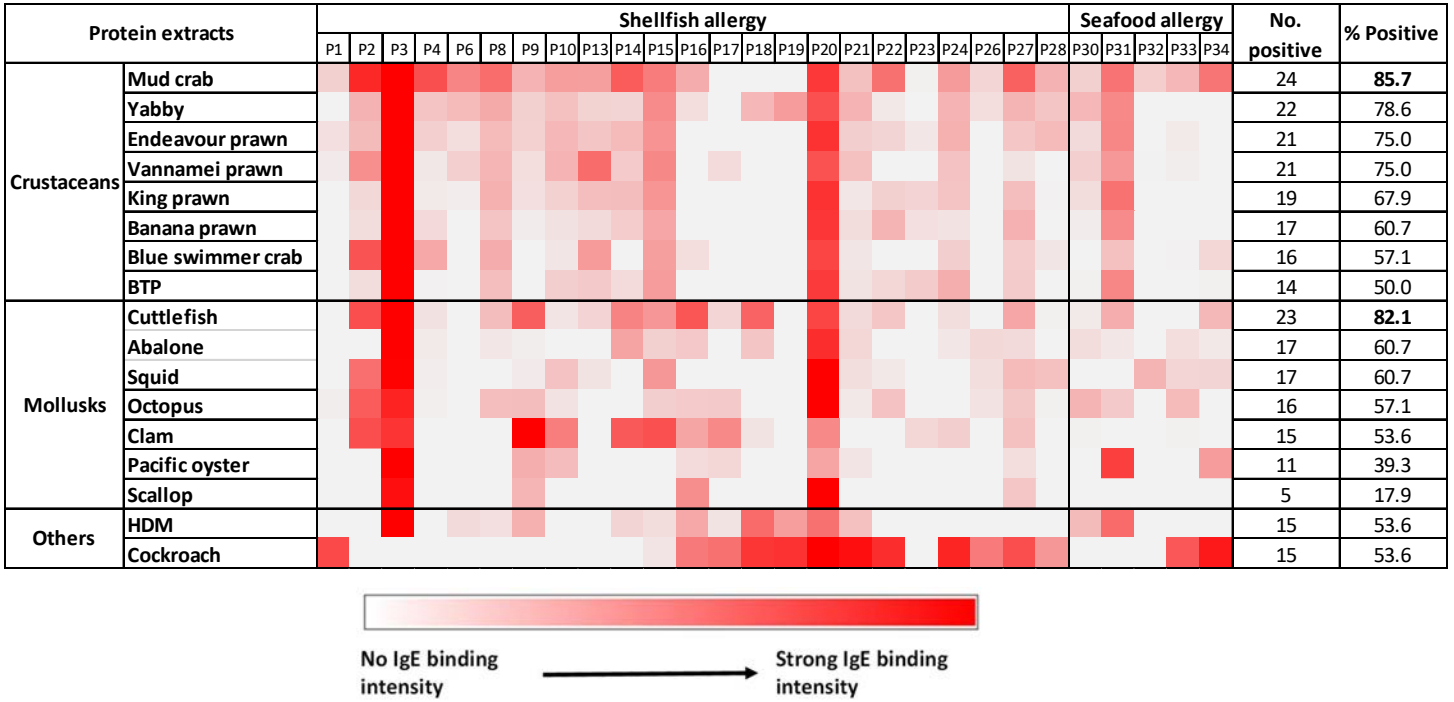

Figure 6.4 Heatmap displaying specific IgE reactivity to crustaceans, mollusks and indoor allergens among 28 participants.

Participants were divided into two groups: shellfish allergy, containing individuals with a clinical history to crustacean and mollusk only, and seafood allergy, containing individuals with a clinical history to crustacean, mollusk, and fish. The average binding intensity of five negative controls was used to define the cutoff value for a positive test, for each allergen tested. A participant showing an IgE binding intensity greater than the cutoff value is defined as a positive result. The percentage of positive tests is determined by the ratio between the number of positive participants and the total number of participants (n = 28). The IgE binding intensity is visualized using a color scale ranging from white (no binding) to dark red (strong binding).

The IgE binding intensity among participants against investigated crustacean and mollusk proteins was compared and presented as boxplots to compare overall binding intensities across different shellfish species (Figure 6.5). The Friedman test was applied to compare the IgE binding intensities between shellfish extracts and the paired t-test was used to compare the IgE binding intensities between HDM and cockroach. A statistical difference was observed in the serum IgE binding intensity within and across the crustacean and mollusk groups ($p < .0001$). Among the crustacean species, the highest IgE binding intensity was detected for yabby extract, whilst in the mollusk group, clam extract was the most reactive. Noticeably, subjects showed a much higher IgE binding intensity to HDM than cockroach ($p < .0001$).

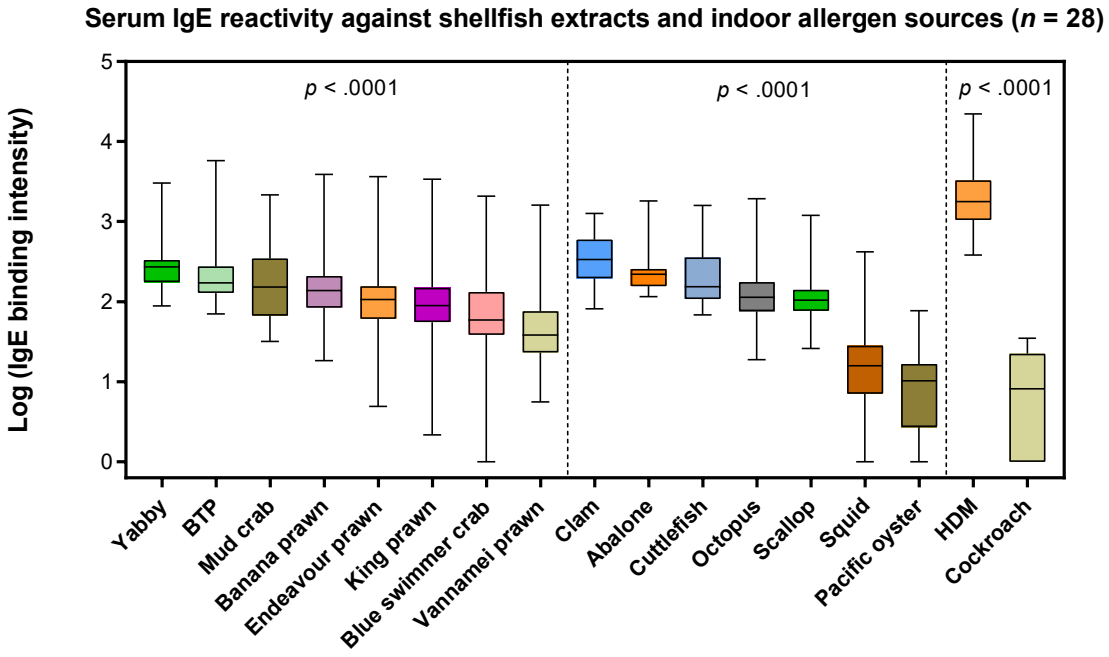

Figure 6.5 IgE binding intensity of the participants against **crustacean, mollusk, HDM** and **cockroach**. Boxplots present the median and interquartile range values of the IgE reactivity. The IgE binding intensity was transformed into logarithm with a base of 10. The Friedman test was used to compare the IgE binding intensity of the participants to crustacean extracts (Friedman statistic = 123.9, $p < .0001$), to mollusk extracts (Friedman statistic = 131.4, $p < .0001$). The IgE reactivity of each pair of extracts was compared by the Dunn's multiple comparisons test (Table E6.2, Appendix E). The Wilcoxon matched pairs signed rank test was used to compare the IgE reactivity between HDM and cockroach extract.

6.4.4 IgE reactivity to tropomyosin from food and non-food sources

Tropomyosins from six groups, including food (crustacean, mollusk and fish) and non-food (HDM, cockroach and Anisakis) sources, were used to analyze participants' IgE reactivity. The forty participants were divided into three groups: shellfish allergy, fish allergy, and seafood allergy according to their clinical history. A panel of twenty-seven healthy controls was used to generate the cutoff values to define positive tests to the relevant allergens. Positive controls included two patients with clinically confirmed shellfish allergy and known binding to prawn TM (Table D6.4, Appendix D).

The serum IgE reactivity against tropomyosin was presented in Figure 6.6. Both shellfish allergy and fish allergy groups showed distinctly positive IgE responses to tropomyosins. Three participants (#18, #19 and #37) showed no IgE binding signal to any of the tropomyosins. Similar to their IgE reactivity profiles to the protein extracts, participant #3 and #20 displayed strong IgE reactivity to all investigated tropomyosins. The remaining participants demonstrated reactivity to tropomyosins from food or non-food sources. Tropomyosin from HDM and blue swimmer crab demonstrated the highest rate of IgE recognition (63.4%), followed by cockroach (56.1%). The lowest IgE reactivity was to abalone tropomyosin (33.6%). Diverse patterns of species-specific IgE binding was demonstrated across all the participants.

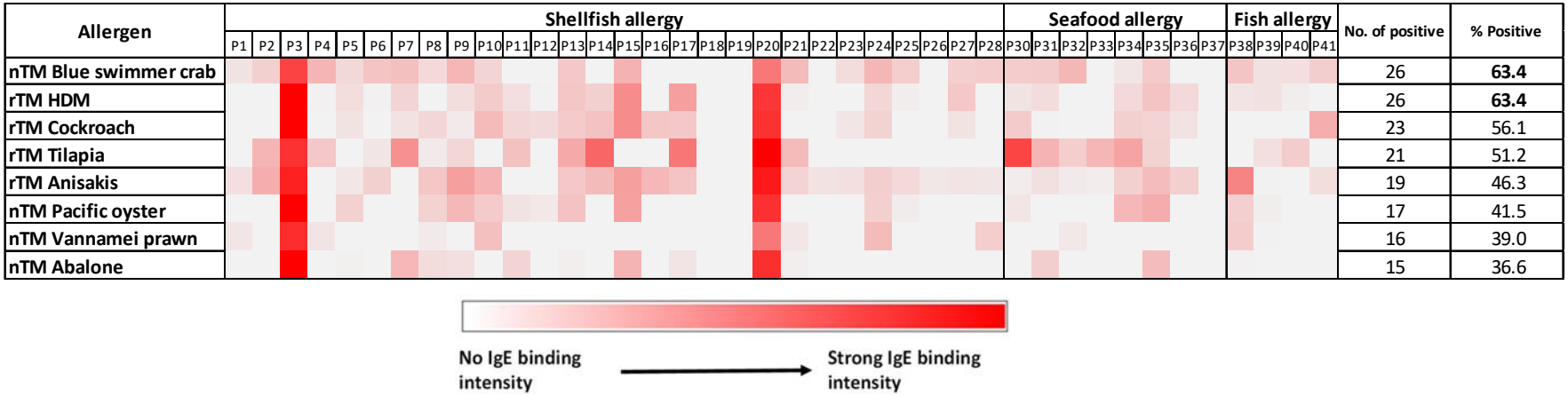

Figure 6.6 Heatmap displaying specific IgE reactivity to the **purified tropomyosins** from seafood and non-food sources among 40 participants. Participants were divided into three groups: the shellfish allergy group are individuals with clinical history to crustacean ormollusk; the seafood allergy group includes individuals with clinical history to crustacean/mollusk and fish; and the fish allergy group are individuals with clinical history to fish. The average IgE binding intensity of the five negative controls was used to define the cutoff value for each tested allergen. A participant showing an IgE binding intensity greater than the cutoff value is defined as a positive result. The percentage of positive participants is determined by the ratio between the number of positive participants and the total number of participants (n = 40). The IgE binding intensity is visualized using a color scale ranging from white (no binding) to dark red (strong binding). nTM: natural tropomyosin, rTM: recombinant tropomyosin. HDM: house dust mite.

6.4.5 IgE reactivity to allergen components in vannamei prawn

Tropomyosin is the major allergen in shellfish. However, many other proteins could also trigger an allergic response among seafood sensitized subjects. The investigation of serum IgE reactivity to different allergenic proteins from vannamei prawn is summarized in Figure 6.7. Scatter plots were employed to visualize the correlation between the participants' IgE reactivity against vannamei heated extract (y-axis) and the individual allergens from vannamei (x-axis): nTM, rMLC, rSCP, and the rHC. The nonparametric Spearman correlation test was computed to generate the Spearman correlation coefficient r_s and p-value for each pair of the allergen and the heated extract.

Of the four investigated allergens, TM, MLC and SCP are heat-stable while HC is known as a heat-sensitivity protein. Participants showed a moderate and significant correlation of IgE reactivity against heated prawn extract and nTM (r_s = .4018, p < .0340). A weak but insignificant correlation was recorded between the participants' IgE reactivity to the heated extract and the rMLC (r_s = .3379, p < .0787) and the rSCP (r_s = .3014, p < .1191). The IgE reactivity of the heated prawn extract and the rHC correlated negatively (r_s = -.0890, p < .0652).

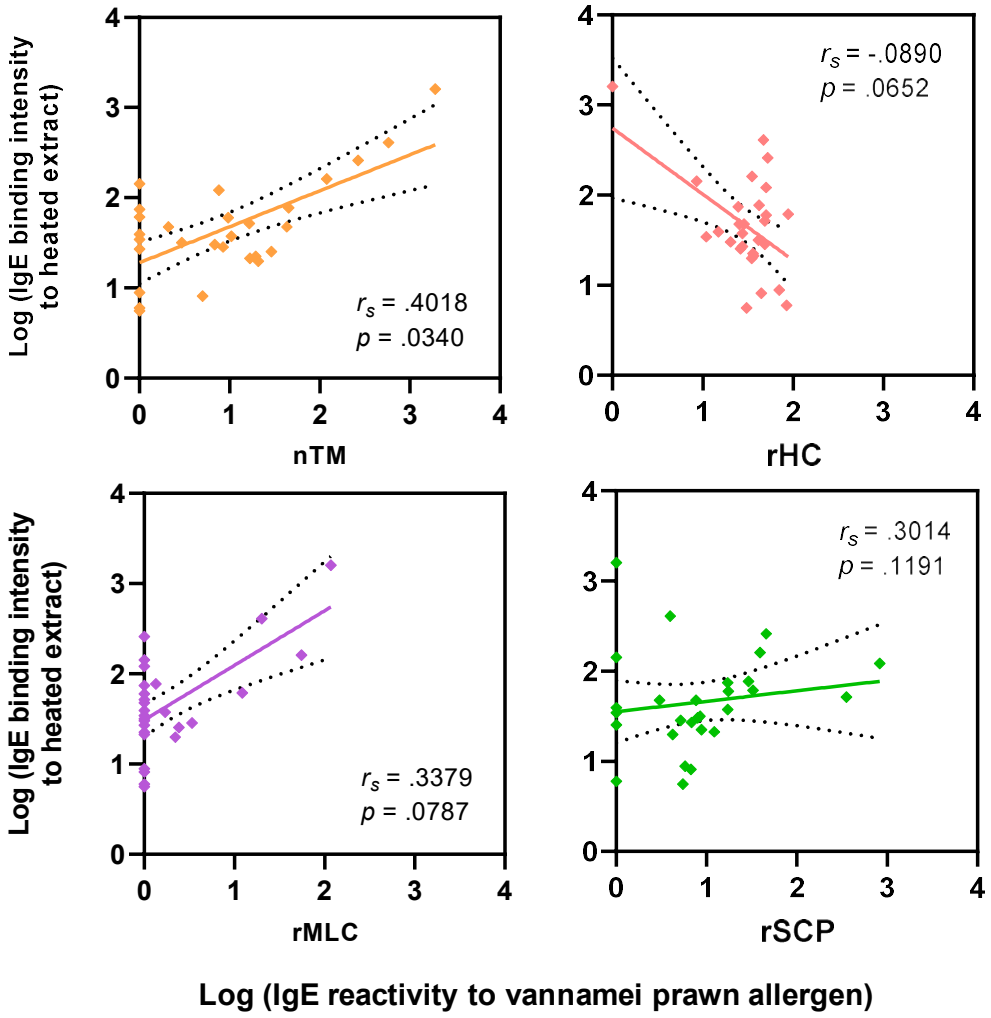

Figure 6.7 IgE reactivity to different allergens from **vannamei prawn** (n = 28). The nonparametric Spearman correlation test was computed to generate the Spearman correlation coefficient r_s and p-value for each pair of allergen and extract. The IgE binding intensity was logarithm transform to a base of 10. nTM: natural tropomyosin, rHC: recombinant hemocyanin, rMLC: recombinant myosin light chain, rSCP: recombinant sarcoplasmic calcium binding protein.

The IgE binding intensity of the participants against prawn allergen components was compared in Figure 6.8. All the participants showed IgE reactivity to the heated extract, with less participants binding to the individual allergens and therefore a reduced average IgE reactivity, in descending order: rHC, nTM, rSCP, and rMLC. The IgE reactivity against heated extract was significantly different compared to nTM (p =

.0014), rSCP (p = .0002) and rMLC (p < .0001). A significant difference was seen between the IgE response against rHC (the heat-sensitive allergen) and nTM (p = .0270), rHC and rSCP (p = .0053), and rHC and rMLC (p < .0001) but not between rHC and the heated extract (p > .9999).

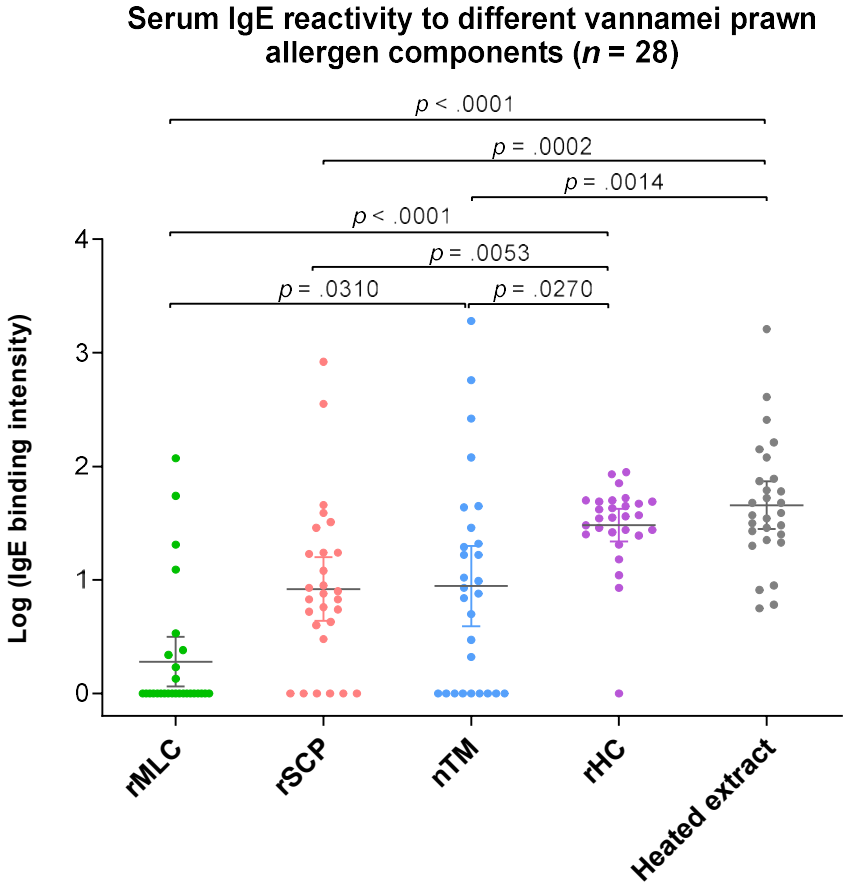

Figure 6.8 The comparison of sIgE reactivity to different **prawn allergen components** (n = 28). The IgE binding intensity was transformed into logarithm with a base of 10. Scatter dot plots present the mean IgE reactivity with 95% CI to each allergen component. The Friedman test was used to compare the IgE binding intensity of the participants to the prawn allergen components (Friedman statistic = 62.64, p < .0001). The Dunn's multiple comparisons test was computed to compare the IgE reactivity of each pair of allergens (Table E6.3, Appendix E).

6.4.6 Correlation between the IgE reactivity against prawn heated extract and the prawn ImmunoCAP results

The measurement of IgE by ImmunoCAP was outsourced to estimate the participants' IgE levels to prawn (CAP f24). The IgE level was ranked from negative to very high IgE concentration (kU_A/L), following the guidelines from the test provider (Sullivan Nicolaides Pathology, Brisbane, Australia). Among 36 participants with a shellfish allergic condition, 47.2% of them indicated a negative IgE reactivity to prawn, 25.0% had a low IgE reactivity, 8.3% had a moderate IgE reactivity, and 19.4% of the participants had a high and very high IgE concentration to prawn (Figure 6.9).

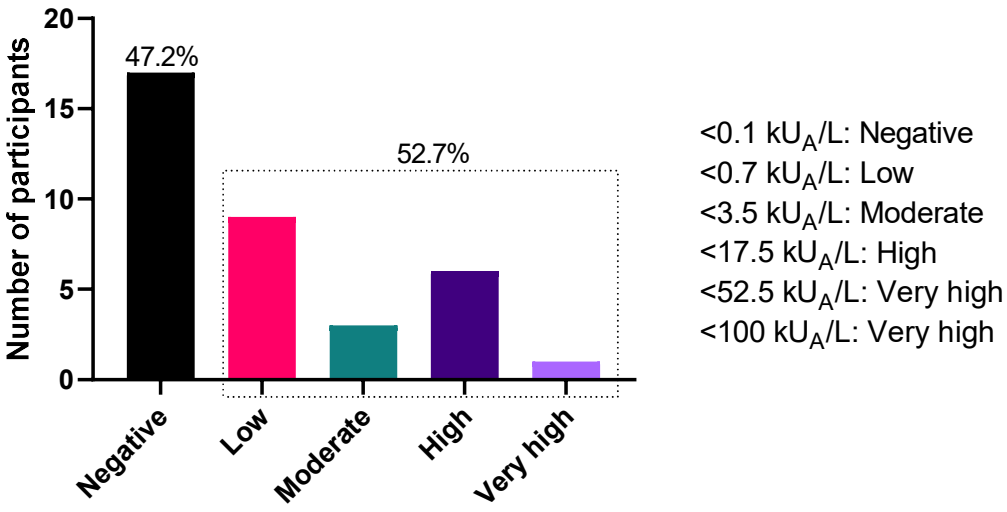

Figure 6.9 The distribution of ImmunoCAP testing results for the measurement of prawn-specific IgE among 36 shellfish allergic participants. The concentration of sIgE level was ranked from negative to very high following the guidelines from the test provider (Sullivan Nicolaides Pathology, Brisbane, Australia; CAP f24).

The correlation between IgE reactivity determined using the grid-immunoblotting assay and the commercial test outcomes was compared (Figure 6.10). Only participants demonstrating positive IgE reactivity by immunoblot (IgE binding intensity higher than the cutoff value as detailed in section 6.4.3) to the investigated extracts were selected

for the correlation test. There was a good correlation between the participants' IgE levels and their IgE reactivity to banana prawn (n = 17, r = .8516, p < .0001) and endeavor prawn (n = 21, r = .8606, p < .0001). Participants showed a moderate but significant correlation between the IgE levels to ImmunoCAP prawn and the IgE reactivity to BTP (n = 17, r = .6030, p < .0122) and vannamei heated extract by immunoblot (n = 20, r = .5077, p < .0223).

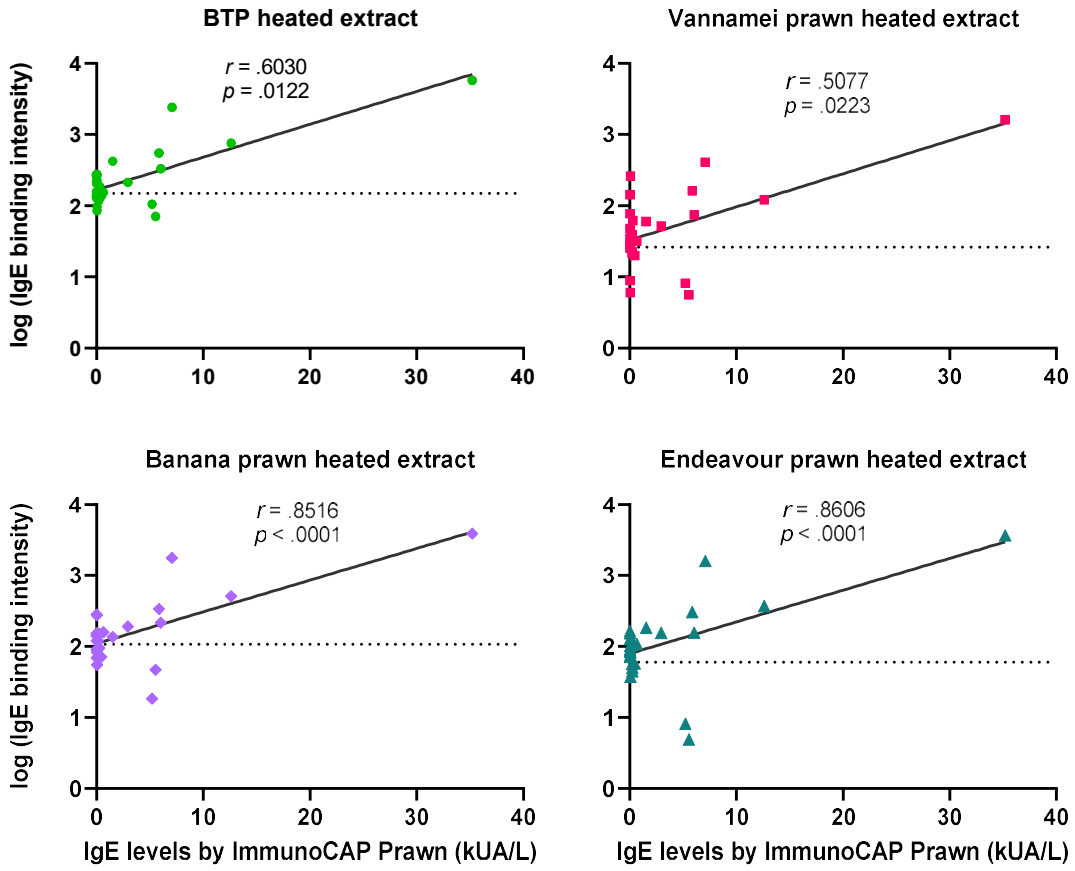

Figure 6.10 The correlation between the levels of prawn-specific IgE, determined by ImmunoCAP (*P. borealis, P. monodon, M. barbata, M. joyneri*) and the IgE binding intensity to heated protein extract of **BTP, vannamei, banana prawn and endeavour prawn** by grid-immunoblot. The IgE binding intensity was logarithm transformed. The dotted line indicates the cutoff value of each extract. Only participants with a positive IgE reactivity to the investigated extracts were selected for the correlation test. The Spearman correlation test was used to determine the correlation coefficient and *p*-value.

Among 17 participants who had less than 0.1 kUA/L specific IgE to prawn by ImmunoCAP, 9/17 (52.9%) demonstrated IgE reactivity to the heated vannamei prawn extract immunoblotting (Figure 6.11).

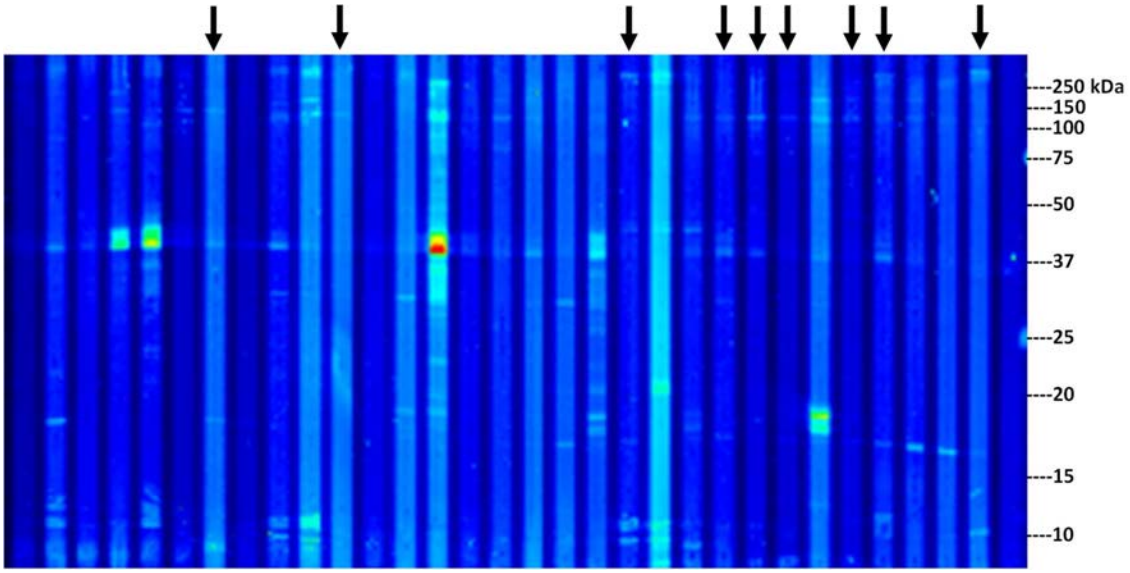

Figure 6.11 IgE reactivity of shellfish allergic participants against **heated vannamei** prawn extract by immunoblotting. The black arrow indicated participants that demonstrated an IgE reactivity to the heated extract but negative to ImmunoCAP prawn.

6.4.7 IgE reactivity to indoor allergen components

The IgE reactivity to indoor allergens among shellfish allergic participants was examined and presented in Figure 6.12. Subjects showed the strongest IgE reactivity to the HDM extract but not to the HDM tropomyosin ($p < .0001$). Evaluating IgE binding to cockroach allergens, the heated extract had significantly higher overall IgE intensities compared to the raw extract ($p < .0001$) and the cockroach recombinant tropomyosin ($p = .002$). Generally, participants demonstrated more IgE reactivity to relevant allergens of HDM than cockroach ($p < .0001$).

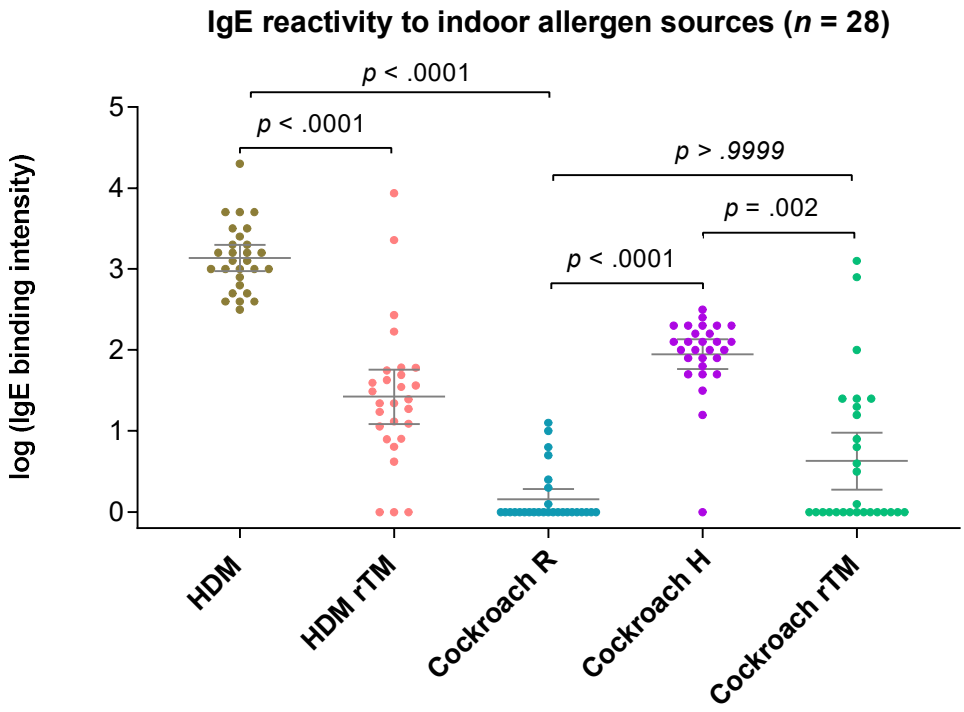

Figure 6.12 IgE reactivity against **cockroach** and **HDM allergens** (n = 28). Scatter dot plot presenting the mean IgE reactivity with 95% CI to each allergen component. The Friedman test was used to compare the IgE reactivity between groups (Friedman statistic = 92.31, p < .0001). The Dunn's multiple comparisons test was computed to compare the IgE reactivity of each pair of allergens (Table E6.4, Appendix E). HDM: house dust mite, R: raw extract, H: heated extract, rTM: recombinant tropomyosin.

6.4.8 IgE reactivity to fish protein extracts

In this cohort, there were only four subjects with self-reported allergic reactions to fish and eight subjects allergic to both fish and shellfish. Grid-immunoblotting was conducted to examine the IgE reactivity of participants against 13 fish species. Control groups included two healthy participants and two fish allergic patients (Table D6.3 and Table D6.4, Appendix D). Patterns of IgE reactivity to fish species were presented as a heatmap with the color scale ranging from white (no binding) to red (high binding) (Figure 6.13). Within the subpopulation of participants with clinical history to fish, 11/12 (91.7%) subjects showed positive IgE reactivity to at least one fish extract. Participant #34 had no positive IgE recognition to any investigated fish species. Among the shellfish allergy group, 24/28 (85.7%) subjects reacted to at least one fish protein

extract. Excluding participant #3 and #31 which displayed IgE binding to all fish extracts, most subjects showed a distinct IgE reactivity pattern to fish species. For instance, subject #1 showed strong IgE reactivity to proteins from salmon and basa fish but not to other fish species. Fish allergic participant #40 had positive IgE reactivity to proteins of coral trout and basa fish only. Participant #12 showed strong IgE reactivity to only heated proteins of coral trout.

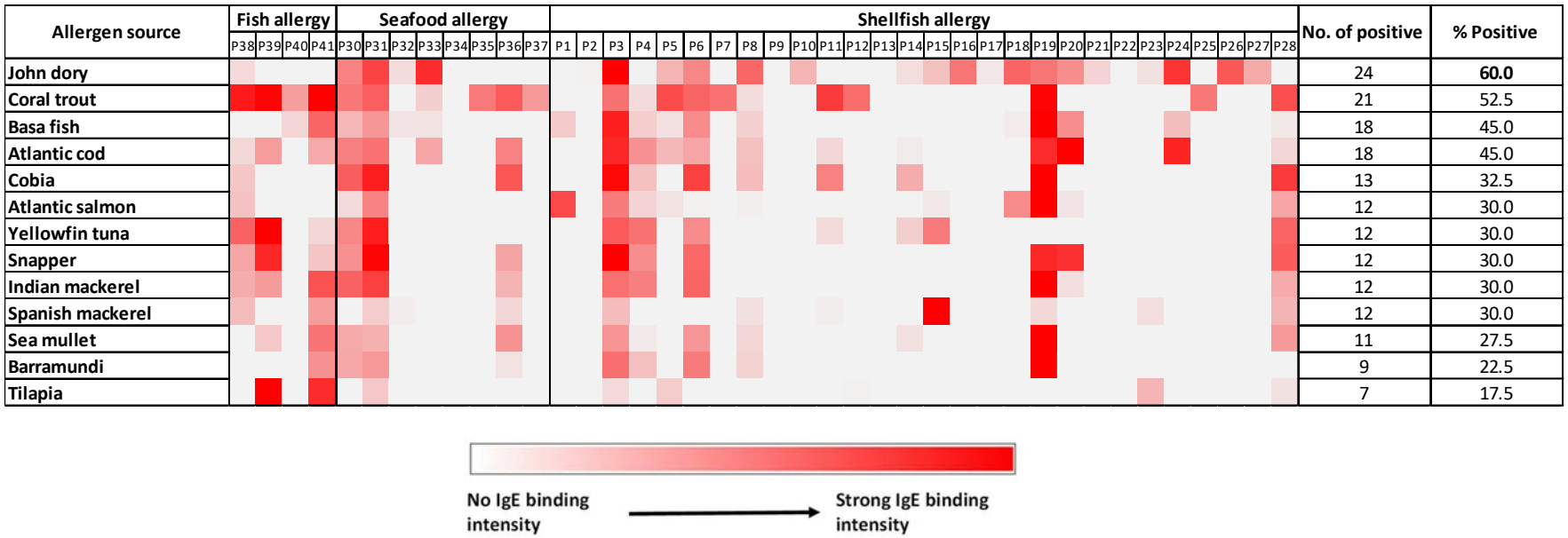

Figure 6.13 Heatmap displaying the specific IgE reactivity to **fish heated extracts** among 40 participants. Participants were divided into three groups: the fish allergy group contains individuals with clinical history to fish; the seafood allergy group includes individuals with clinical history to the crustacean, mollusk, and fish; the shellfish allergy group contains individuals with clinical history to crustacean and mollusk. The average IgE binding intensity of the five negative controls was used to define the cutoff value for each tested allergen. A participant with an IgE binding intensity greater than the cutoff value is defined as a positive result. The percentage of positive tests is determined by the ratio between the number of positive participants and the total number of participants (n = 40). The IgE binding intensity is visualized in a color scale ranging from white (no binding) to dark red (strong binding).

Overall, John dory gave the highest number of positive tests in this cohort, with 60.0% of participants displaying IgE binding to its heated proteins (Figure 6.13). Participants also showed the strongest IgE binding intensity to dory, compared to snapper (p < .001), barramundi (p < .0001), sea mullet (p < .0001), yellowfin tuna (p < .0001), cobia (p < .0001) and Indian mackerel (p < .0001) (Figure 6.14).

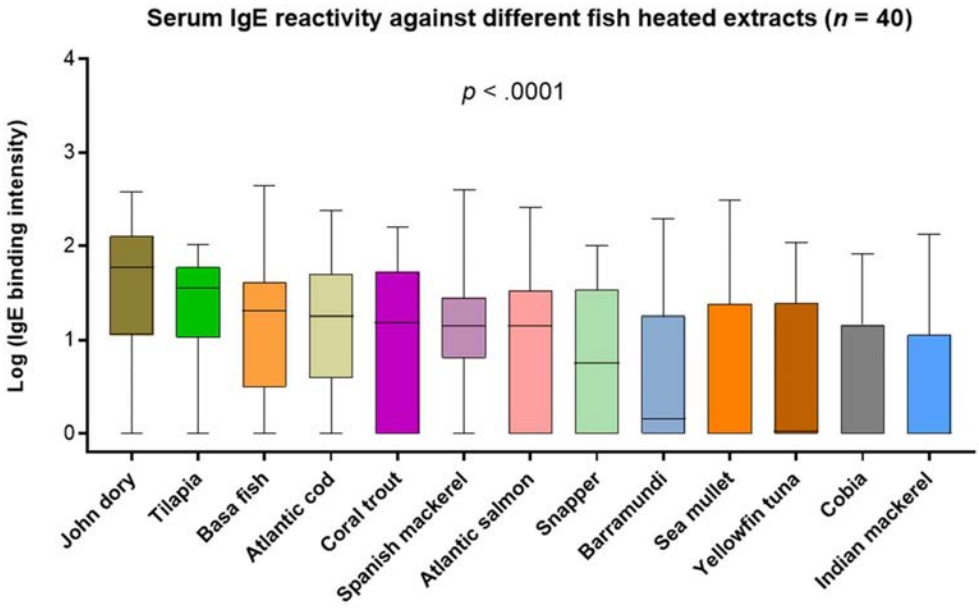

Figure 6.14 Comparison of patient IgE reactivity against **heated fish extracts** (n = 40). The IgE binding intensity was transformed into a logarithm with a base of 10. Boxplots present the mean of the IgE binding intensity and SD. The Friedman test was used to compare the IgE reactivity across all analyzed fish extracts (Friedman statistic = 161.3, p < .0001). The Dunn's multiple comparisons test was computed to compare the IgE reactivity of each pair of extracts and can be found at Table E6.5, Appendix E.

When stratifying the data into two subgroups according to the participants' clinical history, participants with a history of fish consumption (fish/seafood allergy) demonstrated higher IgE reactivity against 11/13 analyzed fish extracts than those with shellfish allergy but tolerate fish (Figure 6.15). However, a significant difference was only seen in the coral trout extract (p < .0338).

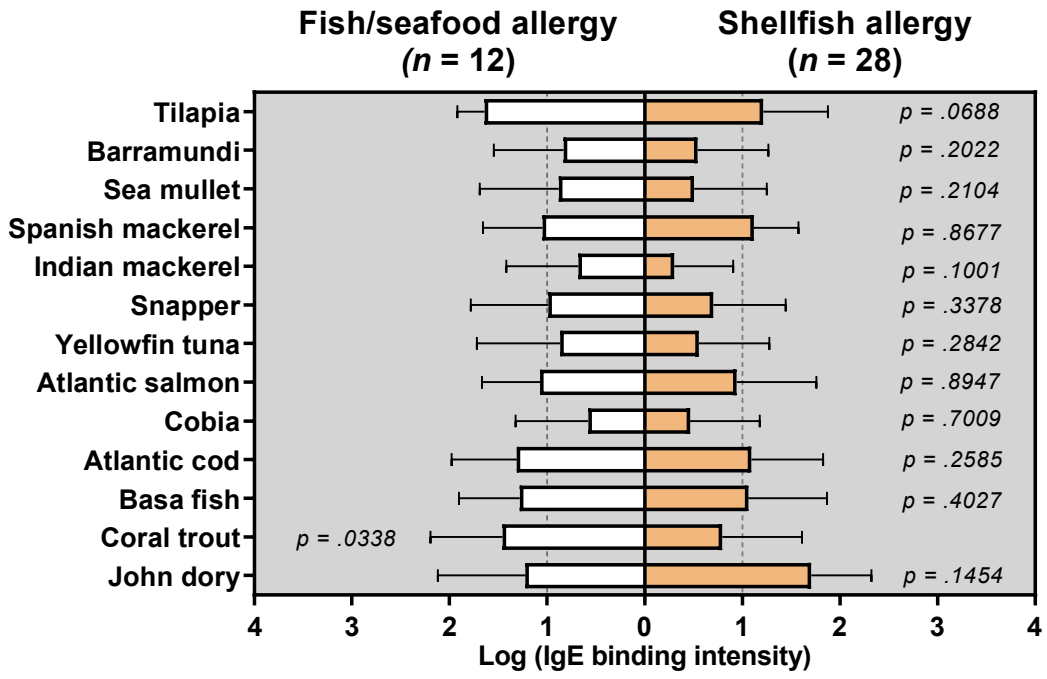

Figure 6.15 Comparison of IgE reactivity against **heated fish extracts** between two subgroups. The fish/seafood allergy group (consisting of participants with clinical history to fish, *n* = 12) and the shellfish allergy group (including participants allergic to shellfish but tolerate fish, *n* = 28). Each bar graph presents the mean and the standard deviation of the IgE reactivity signal. The IgE binding intensity against each extract between two studied groups was compared using the Mann-Whitney test.

6.4.9 IgE reactivity to the fish allergen parvalbumin

Parvalbumin is the major fish allergen. Purified natural parvalbumins from five fish species: Atlantic cod, basa fish, barramundi, Atlantic salmon and yellowfin tuna were used to investigate the IgE reactivity among the study population. Patterns of serum IgE reactivity against fish parvalbumin were visualized by a heatmap as shown in Figure 6.16. IgE binding intensity was expressed by a color scale ranging from white (no binding) to red (binding). The darker the color represents the stronger IgE reactivity. The purified parvalbumin from Atlantic cod demonstrated the highest positive rate (67.5%), followed by the parvalbumin from basa fish and barramundi (60.0%). The parvalbumin of Atlantic salmon (47.5%) and yellowfin tuna (40.0%) were the least reactive in this study population.

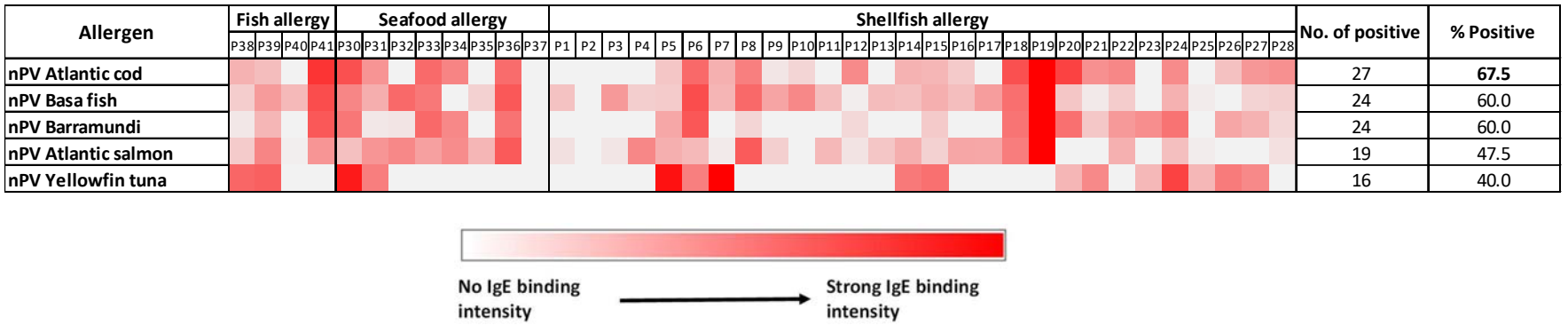

Figure 6.16 The heatmap displays the specific IgE reactivity to the purified natural **fish parvalbumin** among the 40 participants.

Participants were divided into three groups: fish allergy group are individuals with clinical history to fish; seafood allergy group includes individuals with clinical history to the crustacean, mollusk; and shellfish allergy group are individuals with clinical history to crustacean and mollusk. The average IgE binding intensity of the five negative controls was used to define the cutoff value for each tested allergen. A participant shows an IgE binding intensity greater than the cutoff value is defined as a positive response. The percentage of positive is determined by the ratio between the number of positive participants and the total number of participants (n = 40). The IgE binding intensity is visualized in color scale ranging from white (no binding) to dark red (strong binding). nPV: natural parvalbumin.

When investigating the IgE reactivity among participants with a clinical history to fish consumption, participant #37 had no IgE binding to any analyzed fish parvalbumins. Participant #30, #38 and #39 recognized parvalbumins of all fish species, but the remaining fish allergic subjects indicated species-specific IgE binding patterns. Overall, the fish/seafood allergy group demonstrated a higher IgE binding intensity than the shellfish allergy group but not statistically significant (Figure 6.17).

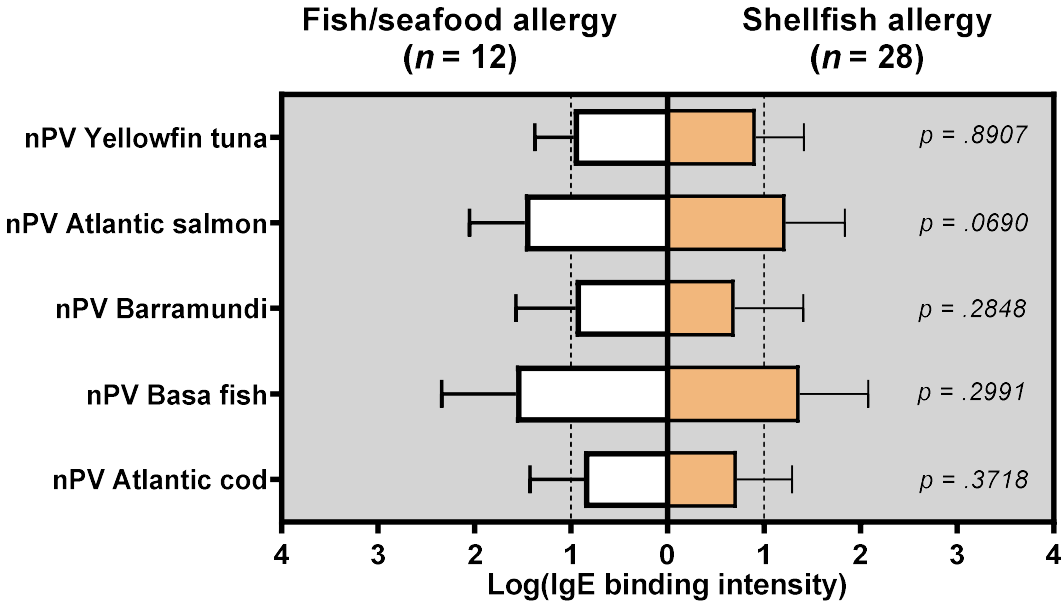

Figure 6.17 Comparison of IgE reactivity against **fish parvalbumins** between two subgroups. The fish/seafood allergy group (consisting of participants with clinical history to fish, *n* = 12) and the shellfish allergy group (including participants allergic to shellfish but tolerate fish, *n* = 28). Each bar graph presents the mean and the standard deviation of the IgE reactivity signal. The IgE binding intensity against each extract between two groups was compared using the Mann-Whitney test.

6.4.10 IgE reactivity to salmon raw and heated protein extracts and the correlation with salmon parvalbumin

Salmon is among the most frequently consumed fish species, and is also commonly consumed raw. The participants' IgE reactivity against raw and heated extracts from salmon were investigated and compared to the IgE recognition to purified salmon parvalbumin. Among 40 participants, no difference in the IgE reactivity to raw and

heated salmon extracts was seen (Wilcoxon matched-pairs signed-rank test, p = .3499). A low correlation of IgE reactivity was demonstrated between raw and heated fish proteins to the purified parvalbumin among two subgroups. The heated salmon extract appeared to correlate better to the purified parvalbumin than the raw extract, among the shellfish allergy group, but this was not statistically significant (r_s = .2488, p = .2017 and r_s = .0416, p = .8334, respectively) (Figure 6.18). In contrast, among the fish sensitized group, the raw extract demonstrated a higher correlation coefficient value (r_s = .2378, p = .4673) than the heated extract, but again this was not statistically significant (r_s = .0455, p = .8908).

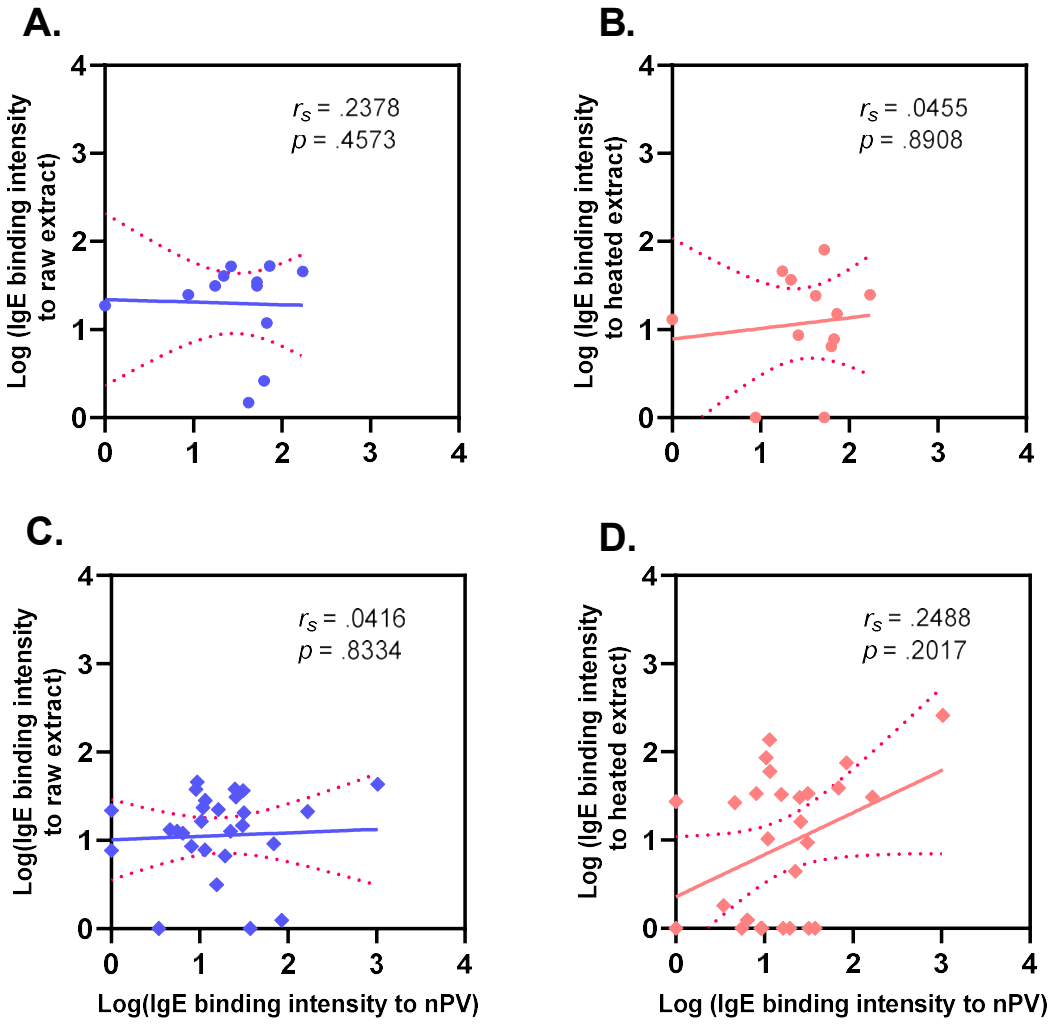

Figure 6.18 The correlation of participants' IgE reactivity to the **salmon extract** and the purified salmon **parvalbumin** (nPV) between two subgroups of participants. (A) the correlation between IgE response to the raw extract and the nPV among participants in the fish/seafood allergy group (n = 12); (B) the correlation between IgE response to the heated extract and the nPV among participants in the fish/seafood allergy group; (C) the correlation between IgE response to the raw extract and the nPV among participants in the shellfish allergy group (n = 28); (D) the correlation between IgE response to the heated extract and the nPV among participants in the shellfish allergy group. The Spearman correlation test was used to compute the correlation coefficient and p-value.

6.5 Discussion

Following our published protocol of the previous study on seafood allergy in Vietnam, the investigation of the clinical presentations and the allergen-specific IgE reactivity among adult participants with a history of fish and shellfish allergy in Australia was carried out. This is the first study to provide insights into the pathogenesis and the etiology of seafood allergy among Australian adults, and allows the assessment of putative cross-reactivity between fish, shellfish, as well as indoor allergens. Ingestion was the main pathway eliciting allergic disorders in this cohort. From the reported clinical presentations and symptom onset, it seems that most of the participants present with a type I food allergy. Allergy to shellfish is more frequent than to fish (15). In this study fish and shellfish allergic subjects demonstrated species-specific IgE reactivity, and the cross-reactivity to allergic components from exposure to indoor allergens was likely. Parvalbumin and tropomyosin are partly responsible for the cross-reactivity between fish and shellfish species as well as some non-food allergen sources. The contribution of other allergenic components from fish and shellfish to seafood sensitization was indicated.

Most of the study subjects (85.4%) presented with acute symptoms on the skin and around oral organs, included lip/tongue tingling, lip swelling, tight throat or itchy throat or mouth. 82.9% of individuals suffered an associated allergic disease apart from food allergy. This implies the closely causal relationship of allergic conditions and the hypersensitive prone in a certain subpopulation. For instance, food allergens were reported to trigger atopic dermatitis exacerbations during childhood (16). Individuals with childhood eczema were reported to be more likely to develop food allergy later in life (17). In this study, 80.0% of the participants developed their allergic conditions in adulthood, of which 68.7% of participants are shellfish allergic and the remaining 31.3% consist of participants with fish/seafood allergy. Some individuals indicated that they were able to tolerate seafood during their childhood and adolescence. Though seafood allergy is known as a chronic allergic disorder, it is unclear how these individuals lost their tolerance to allergenic seafood proteins when growing into adulthood (18).

About 38.5% of the healthy controls in this cohort demonstrated IgE reactivity to HDM, and more than half of the shellfish allergic subjects (56.8%) concurrently self-reported having dust mite allergy. HDM is one of the most important indoor allergen sources and mite sensitization is estimated to affect up to 1 - 2% of the world's population (19). However, interpopulation surveys of mite sensitization reported much higher rates. Sensitization to HDM among adults in Europe, from a survey in 15 developed countries, confirmed with SPT that sensitization to HDM was 21.7% (20), while the sensitization rate among Latino American women to *Dermatophagoides pteronyssinus* and *D. farinae* was 37% and 34%, respectively (21). In Australia, HDM sensitization frequency, confirmed by SPT, was reported to be as high as 62.5% to *D. pteronyssinus* and 54.2% to *D. farinae* (22). The close link between prolonged mite exposure and the development of allergic diseases such as allergic asthma or allergic rhinitis, were noted (23-25). Twenty-four allergen groups from HDM have previously been identified and characterized (19). Among those allergic components, mite tropomyosins are well known to correlate with the development of shellfish allergy among the sensitized population, due to the high amino acid sequence homology (82-100%) among invertebrate tropomyosins (26). Co-sensitization to tropomyosin from shellfish and HDM is frequently observed, especially among communities with high shellfish allergy in the tropics (12, 27). In the current cohort, more than half of the shellfish allergic participants (53.6%) reacted to HDM extract by grid-immunoblotting, and 63.4% of the same population were positive to the purified HDM tropomyosin (section 6.4.4). Thus, it is of importance to confirm the sensitization to tropomyosin Der p 10 in this cohort (e.g. by inhibition ELISA) to evaluate the likelihood of co-sensitization with seafood-derived tropomyosin, which may be clinicallly relevant.

Similarly, not all shellfish allergic individuals showed IgE reactivity to purified tropomyosin from HDM (66.7%) and cockroach (58.3%). Generally, this subpopulation demonstrated much higher sIgE levels to allergen components from HDM raw extract than to HDM tropomyosin ($p < .0001$), and to cockroach heated extract than to cockroach tropomyosin ($p < .0001$). Hence, tropomyosin might be one of the allergens responsible for the cross-reactivity between HDM/cockroach and shellfish sensitization, but there might be other, yet undefined, concomitant triggers.

When investigating IgE binding to allergenic proteins from vannamei prawn, it was discovered that HC had stronger sIgE reactivity and more positive responses (27/28 patients) than TM (Lit v 1), MLC (Lit v 3) and SCP (Lit v 4) ($p < .0001$). Tropomyosin is considered to be the most predominant allergen in crustacean and mollusk (28), and some studies suggest using tropomyosin as a biomarker for the *in vitro* diagnosis of prawn allergy (14, 29). However, the diagnostic value of tropomyosin to shellfish allergy seems to vary among studied populations. For example, in an investigation among 35 Brazilian prawn allergic patients, the authors demonstrated a correlation between prawn tropomyosin IgE measurements and challenge proven prawn allergy (30). Tropomyosin (rPen a 1) specific IgE measurement was also a good predictor of prawn allergy in Spain (14). Furthermore, co-sensitization to TM and SCP was effective in accurately predicting clinical relevance to prawn allergy, among prawn allergic patients from Spain, Brazil and the US (31). In contrast, studies among prawn allergic populations in Singapore, China and Japan, these correlations were not found (32-34). Clinical reactions to prawn allergy are known to be attributed to other allergen components in addition to tropomyosin, including HC, SCP, AK and Troponin C (35). The allergen HC was identified in the giant freshwater shrimp (*Macobrachium rosenbergii*) (28), vannamei prawn (*L. vannamei*) (36) and squid (*Todarodes pacificus)*(37). This allergen (MW: 75 kDa), which functions as an oxygen transporter in cells/tissues, was found to be associated with clinical cross-reactivity between crustaceans, mollusks and mites (38). In addition anaphylaxis due to the HC allergen in shrimp has been reported (39). Thus, HC might be an important allergen that needs more attention in the diagnosis of shellfish allergy in this population.

In the current study, nearly half of the participants with shellfish allergy tested negative for prawn sIgE by ImmunoCAP. The low specificity of the ImmunoCAP result might underly the diversity of prawn allergens among species. As per the manufacturer's declaration, the current ImmunoCAP prawn reagent contains a mixture of four prawn species (*P. borealis, P. monodon, M. barbata, M. joyneri*), but only the black tiger prawn (*P. monodon*) is consumed in the Asia Pacific region. Thus, this commercial test might not fully cover all putative allergens in this population.

Furthermore, from the participant interview results, it was noted that most of the subjects with a clinical history of reactions to seafood tended to avoid consuming

seafood since the very first episode (generally in their twenties). With the average age of the participants being 42.5±15.8 years, it could be estimated that the recruited participants may have been avoiding seafood for at least the last five years prior to the time they joined this study. In particular, four participants reported the last allergic episodes occurred more than 20 years ago. The allergen specific and total IgE levels could decrease with time. For instance, the total IgE and the levels of serum specific IgE to common aeroallergens (HDM, cat and grass) of 3, 206 European adults in a following-up allergy study indicated a decrease by 0.6% up to 7% as compared to the initial values recorded at the participants' younger age (40). Thus, this could be one reason for a low level of specific antibodies to the prawn in some of the subjects.

Among fish extracts, John dory, tipalia, and basa fish were the top three most IgE-reactive fish, whereas only a few participants had IgE against sea mullet, yellowfin tuna, cobia, and Indian mackerel. Generally, participants showed significantly less IgE binding to fish than to shellfish extracts ($p < .0001$). Also, the cohort with clinical history to fish demonstrated a higher IgE binding intensity to most of the analyzed fish extracts and purified fish tropomyosin (but not statistically significant), compared to the cohort without a fish allergic history. An allergen specific IgE level could reflect the exposure history of an individual to the allergen source. Theoretically, participants who never consume or are never exposed to the investigated allergens could lack pre-formed allergen-specific antibodies and lead to low IgE reactivity in serological tests. On the other hand, an elevated IgE level can be a sign of hypersensitivity as well as exposure to low levels of the allergen. In this investigation, two shellfish allergic participants showed a 1000-fold higher IgE level against fish extracts than the remaining group, although these participants reported no symptoms towards fish consumption. Specifically, subject #3 presented with lip or tongue tingling and tight throat during an allergic episode that occurred within 10 minutes after prawns/clams/calamari consumption, whereas subject #19 presented with hives, redness of the skin and body swelling in the first 30 minutes. Both subjects demonstrated high sIgE levels of 35.2 kU_A/L and 5.21 kU_A/L to prawn ImmunoCAP testing, respectively. Participant #19 also demonstrated high sIgE levels to salmon (f41) with 7.8 kU_A/L, and cod (f3) with 11.6 kU_A/L. Although no fish ImmunoCAP testing was performed on subject #3, it is highly likely that this participant could demonstrate high IgE to salmon and cod as well. Noticeably, these two individuals reported the comorbidity of asthma, allergic rhinitis

and dust mite allergy, thus they may fall in the subgroup of individuals who present with a high antibody reactivity but do not experience clinical symptoms. On the other hand, these cases might indicate the hypothesis of poor correlation of IgE levels to certain types of clinical food allergy.

However, IgE measurement has been used widely in the laboratory and clinic setting to predict food allergy status (41, 42). According to the current literature, an allergen-specific IgE level of greater than 0.35 kU_A/L is considered as a positive test result for that allergen (43). Through the analysis of many retrospective studies, Sampson (44) established new diagnostic values for six common foods: egg (6 kU_A/L), milk (32 kU_A/L), peanut (15 kU_A/L), soybean (65 kU_A/L), wheat (100 kU_A/L) and fish (20 kU_A/L), using the 95% predictive decision points, and accurately predicted food allergy status among 100 children and adolescents. Allergen-specific IgE measurement is thought to be a promising allergy diagnostic test, which could reduce unnecessary oral food challenges. However, to ensure the practical application of the allergen-specific antibody quantification, it is essential to have a good test/reagent that can cover all putative allergens in a community. Furthermore, relying on the test outcomes from solely one or two implicated species from a patient's report might not provide enough evidence to confirm the allergic status, and thus misdiagnosis is likely. In the current study, the author was unable to perform oral food challenges to confirm the evidence of shellfish and fish sensitization with clinical relevance. Thus, it cannot be ruled out that some participants are sensitized without having true clinical reactions. This has to be considered as a limitation of this study and a direction for future investigations of seafood allergy in Australia.

It is also important to note that the established IgE threshold values to food allergens by Sampson (39) varies with patient's age, gender and race (45, 46). IgE measurements might contain technical errors and bias coming from the selection of commercial systems to perform the assay (47). In addition an individual's serum may contain certain confounders, such as allergen specific IgG subclasses or bioactive components that could limit or inhibit the binding capacity of serum IgE antibodies to allergen components in the extracts (48). Furthermore, the analyzed extracts consist of heterogeneous allergen components which have been demonstrated to be sub-optimal in many commercial preparations. For example, measurements of parvalbumin

concentrations among 22 different fish indicated a stark difference of this allergen in different fish species (31). To overcome the current drawbacks from the natural allergen sources (extracts), the implementation of recombinant allergen molecules for *in vitro* allergy diagnosis was introduced (49). It is also possible that the knowledge gained from molecular allergen characterization may help to improve the allergy diagnosis and thus, will be followed-up in **Chapter 7** of this thesis.

In the current study, up to an 8000-fold difference in allergen-specific IgE reactivity was recorded among allergic participants and up to a 700-fold difference in an individual's IgE reactivity to the investigated seafood panel. This variation in IgE binding was even more significant when compared to the healthy control group. Utilizing the grid-immunoblotting design all investigated allergens and subjects were performed in a consistent system (the assay performed in a membrane at the same time with a consistency of primary and secondary antibodies used). The resulting signals were corrected using relevant blanks (extract and serum). Our findings would seem to reflect the variation and diversity of participants' IgE reactivity towards investigated extracts, rather than technical artefacts.

In summary, this study is the first comprehensive seafood allergy investigation among the adult population in Australia. Within a short period of time, we recruited a substantial number of participants with a history of seafood allergy in North Queensland. This would seem to imply that seafood allergy is common among Australian adults, especially in the tropical regions of the country. Participants demonstrated diverse patterns of IgE reactivity to crustacean and mollusk allergens. Black tiger prawn and clam were the most IgE reactive, whereas vannamei prawn and squid seemed to be less allergenic. A large proportion of shellfish allergic subjects and healthy controls appeared to be sensitized to HDM, suggesting the dominance of mite exposure in allergic individuals in this cohort. Besides tropomyosin, other allergens may play a role in provoking allergic disorders, for example hemocyanin. Participants with a shellfish allergic condition might also be sensitized to fish allergens and vice versa. This study is the first step towards enhancing the diagnosis and management of seafood allergy in the adult population in Australia.

6.6 References

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6.7 Chapter 6 summary

- Clinical history and blood samples of 40 seafood allergic patients and 28 healthy controls were collected to enable the investigation of seafood allergy in North Queensland, Australia

- Allergen-specific IgE reactivity to a comprehensive panel of most frequently consumed crustacean, mollusk and fish species were performed and analyzed.

- Allergen-specific IgE reactivity of participants to major allergens tropomyosin and parvalbumin was performed and analyzed.

- The binding of IgE to different allergens from vannamei prawn was studied, revealing the possible contribution of hemocyanin as a trigger for allergic reactions among seafood allergic subjects in Australian adults.

CHAPTER 7 IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF PUTATIVE CRUSTACEAN ALLERGENS

Manuscript in Preparation:

Thu T.K. Le, Sandip D. Kamath, Roni Nugraha, Elecia Johnson, Khanh V. Bui, Yen, T.H. Phan, Aya C. Taki, Hieu C. Chu and Andreas L. Lopata. A comparative investigation on crustacean allergen profiles among two shellfish allergic subpopulations in the Asia Pacific. Clinical and Translational Allergy.

7.1 Introduction

From the previous chapters (**Chapter 5** and **Chapter 6**), participants from Vietnam and Australia demonstrated considerable IgE reactivity to the heated protein extracts from crustacean, mollusk, and fish. Purified allergens, including natural tropomyosin (nTM), natural parvalbumin (nPV), recombinant Sarcoplasmic calcium-binding protein (rSCP), recombinant myosin light chain (rMLC) and recombinant hemocyanin (rHC), from 13 seafood species were employed to determine their IgE reactivity. However, it is known that many additional seafood allergens are not yet identified and characterized. Furthermore, seafood species from different geographic regions might contain different allergen profiles due to the variation in feeding, environmental temperature, and biological conditions (1).

In food allergy management, a comprehensive understanding of the putative food allergens and their allergenicity is crucial for disease diagnosis and management. Current diagnostic tests, including *in vivo* tests such as skin tests and *in vitro* tests such as IgE measurement, are directed from patient reports on allergy-eliciting foods and the current literature of the putative allergenic components in food products. However, environmental exposures and diets vary significantly across studied populations, meaning that the patterns of implicated food allergens is likely to differ greatly as well (2). Current approaches in food allergen investigation focus on identifying putative food components and confirming their allergic potency to a minimum of five allergic patients (3). This approach is useful for general food allergen surveillance and developing preventive management in food production and food labeling (4), but it might complicate food allergy diagnostics and lead to unnecessary over-care regarding food allergen avoidance among sensitized subjects.

Thus, this chapter seeks to identify the allergenic components in raw and heated crustacean that could trigger allergic reactions among seafood allergic participants in Vietnam and Australia using immunoblotting techniques and mass spectrometry. This study aims to determine the allergen recognition profile among participants from Vietnam and Australia and identify potential new seafood allergens. It is expected that findings from this study, along with the outcomes from the previous investigations on

IgE reactivity will provide objective evidence of putative allergens that dominate the seafood allergy among sensitized people in Vietnam and Australia.

7.2 Aims

- to identify the putative allergens from raw and heated crustacean extracts that demonstrate IgE recognition
- to molecularly characterize the suspected IgE binding proteins by mass spectrometry
- to compare the putative allergen profiles between shellfish allergic participants from Vietnam and Australia

7.3 Materials and methods

7.3.1 Seafood protein extraction

Three crustaceans were analyzed in this investigation including black tiger prawn (*P. monodon*), vannamei prawn (*L. vannamei*), and blue swimmer crab (*P. pelagicus).* The raw and heated shellfish protein extracts were prepared as described in section 5.3.5 of Chapter 5.

7.3.2 Patient sera

Participants with a history of seafood allergy, recruited from studies in Chapters 5 and 6, were investigated. The details of the participants are summarized in Table D5.2 and Table D6.1 (Appendix D).

7.3.3 Seafood protein profiling by SDS-PAGE

The protein profiles of crustacean extracts were examined by performing SDS-PAGE as described in Section 5.3.8 of Chapter 5. The Dual Double-Wide Mini-Vertical Electrophoresis system (CBS Scientific, California, USA) was used to separate the shellfish proteins. One hundred µg of protein extract was heated to 95^{o}C in a 5x sampling buffer containing Dithiothreitol (DTT) and 1xPBS (making a final volume of 150 µL sample) for 5 min. The sample was then loaded evenly across the well (12% acrylamide resolving gel, 1 mm thick). A volume of 2.5 µL Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA) was used as the protein marker. The proteins were separated at 100V for 20 min, then 220V until the dye front reached the bottom line of the cassette.

7.3.4 Immunoblotting analysis

The separated proteins were transferred onto the nitrocellulose membrane (Bio-Rad, USA) using the Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, USA) at 16V for 30

min and blocked with 1xCasein in PBS-T for 1h. The membrane was assembled in a surf blot apparatus (Idea Scientific, MN, USA) creating a total of 33 slots for the serum incubation. Patient serum was diluted 1:15 in 0.2x Casein in PBS-T loaded onto the membrane via the surf-blot channels, and incubated overnight at -4°C with gentle rocking. The next day, the sera were washed off and the membrane was washed three times with PBS-T to remove the unbound components from the sera. Patient IgE reactivity was detected by the Santa Cruz mouse anti-human IgE antibody (dilution 1:1000 in 0.2x Casein in PBS-T, incubation for 30 min at room temperature). For the detection of the mouse antibodies, 1:10,000 diluted goat anti-mouse IgG antibody (Dylight™ 800, Thermo, IL, USA) in 0.5x Casein in PBS-T was used. The binding was visualized using the Odyssey CLx Imager. Data were imported into the Image Studio™ software (version 5.2, Li-cor, NE, USA) to analyze the binding intensities. Two positive and three negative controls were used to compare the IgE binding reactivity. Details of the control group were described in Table D6.3 and D.6.4, Appendix D.

7.3.5 Protein digestion for mass spectrometry

To prepare the samples for mass spectrometric analysis, the raw and heated protein extracts were separated by SDS-PAGE. Five µg of the extracts (two replicates of each extract) were loaded onto a 12% polyacrylamide gel and proteins separated at 100 V for 20 min and 220 V until the dye front reached the bottom line of the cassette. The investigated protein bands were labeled and cut into pieces. In-gel tryptic digestion was conducted following the protocol of Jia et al. (5) with minor modification. The gel pieces were treated independently. First, the gel pieces were processed in destaining buffer (200 mM NH_4HCO_3, pH8, 50% acetonitrile) twice and dried in a SpeedVac on low heat. The gel pieces were resuspended in the reduction buffer (20 mM dithiothreitol (DTT), 25 mM NH_4HCO_3 for 1 h at 65°C. Next, the DTT was removed and gel pieces were alkylated with 50 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3 in darkness for 40 min at 37°C. The gel pieces were washed twice with the wash buffer (25 mM NH_4HCO_3) for 15 min at 37°C prior to completely drying in a SpeedVac. For the tryptic digest, the dried gel pieces were first rehydrated in the digest buffer (40 mM NH_4HCO_3, 10% acetonitrile) containing 20 µg/ml trypsin (Sigma-Aldrich, MO, USA) for 1 h at room temperature. An additional 20 µl of enzyme mix was added to

the sample and incubated overnight at 37 °C. The next day, the supernatants were removed and placed into new tubes. The remaining digested proteins in the gel pieces were acidified using 0.1% formic acid for 45 min at 37 °C three times. The original supernatant and extracts were combined and concentrated in a SpeedVac. The tryptic peptides were resuspended in 50 µl of 0.1% formic acid before sending out for mass spectrometry analysis at the Bio21 facility, Melbourne, Australia.

7.3.6 Mass spectrometry analysis

For the analysis of the digested samples, a Thermo LTQ Orbitrap Elite mass spectrometer coupled to an Ultimate 3000 RSLC nano-HPLC (Dionex Ultimate 3000) was used (the Bio21 Institute, Melbourne, Australia). The nanoLC system was equipped with an Acclaim Pepmap nano-trap column (Dionex-C18, 100 Å, 75 µm x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dionex-C18, 100 Å, 75 µm x 50 cm). Samples were injected onto the nano-trap column before the enrichment column was switched in-line with the analytical column. The LTQ Orbitrap Elite spectrometer was operated in the data-dependent mode with nanoESI spray voltage of 1.8 kV, a capillary temperature of 250°C and S-lens RF value of 55%. All spectra were acquired in positive mode with full scan MS spectra from m/z 300-1650 in the FT mode at 240,000 resolution. Automated gain control was set to a target value of $1.0^{-6,}$ and a lock mass of 445.120025 was used. The top 20 most intense peaks were subjected to rapid collision, induced dissociation (rCID) with a normalized collision energy of 30 and activation q of 0.25. A dynamic exclusion of 30 seconds was applied for repeated precursors.

7.3.7 Protein identification

The identification of peptides from acquired MS/MS spectra was performed using Mascot v2.5 (www.matrixscience.com) against the NCBI protein database for all shellfish species (Swissprot, 548873 sequences; 195617897 residues, as of April 2019). Search parameters include precursor mass tolerance of 200 ppm, fragment mass tolerance of 0.6 Da (CID). Carbamidomethyl (C) was set as a fixed modification; oxidation (M) and deamidated (NQ) were set as variable modifications. The cleavage enzyme was trypsin, and a maximum of 3 missed cleavages was accepted for protein

matching. A set of criteria was used to determine the most likely matches following the guidelines from Mascot and the protocol established by Koeberl (6). The exclusion criteria for each protein match were 1) identified protein has the Mascot score below 80 (for a confidence level of greater than 95%, P = 1/ (200*548873), S = -10LogP = 80); 2) likely contaminated components such as trypsin, human's keratin or bacterial-origin peptides; 3) identified protein with limited information about its origin and/or function; 4) identified protein contains only one peptide. If there are more than one species having matching peptides, further Mascot parameters were used to identify the best match such as emPAI value and expectation value.

7.3.8 Data analysis

For the analysis of immunoblotting for IgE-protein reactivity, the fluorescence signal of each band was digitized using an analog to digital converter that converts the analog signal to a digital scale expressed by an arbitrary fluorescence unit. The final intensity values were subtracted for the local background and exported as comma-limited text files into Microsoft Excel files. The imported data was analyzed using the SPSS Statistics version 25.0 for Windows (IBM Corp., Armonk, N.Y., USA) to plot the distribution of IgE reactivity bands by molecular weight and binding intensity for each extract.

To identify putative allergens by mass spectrometry, coomassie stained gels and immunoblots, containing the same extracts and run under identical conditions, were compared and bands from the protein gel that corresponded to the protein-of-interest in the immunoblot were cut out and the identity determined by tryptic digest and massspectrometry. A z-test was used to compare the difference in the IgE reactivity between two cohorts with an alpha level of 5%.

7.4 Results

7.4.1 Protein profiles of raw and heated extracts

The protein profiles of raw and heated shellfish extracts are presented in Figure D7.3 (Appendix D). In general, raw protein extracts contained heterogeneous proteins with a molecular weight range of 10 to 150 kDa. Most proteins in the heated extracts were within the molecular weight range of 10-20 kDa and 37-50 kDa.

7.4.2 IgE reactivity to raw and heated extracts

The IgE reactivity against crustacean proteins in the raw and heated extracts, among the Vietnamese and Australian cohorts, is presented in Figure 7.1, Figure 7.2 and Figure 7.3, utilizing BTP, vannamei and blue swimmer crab extracts, respectively. Overall, participants showed more IgE binding, and at higher intensities, to the raw extracts than the heated extracts. Participants from the Australian cohort displayed greater IgE reactivity than those from Vietnam. Vannamei prawn (raw extract) seemed to be the most reactive allergen source.

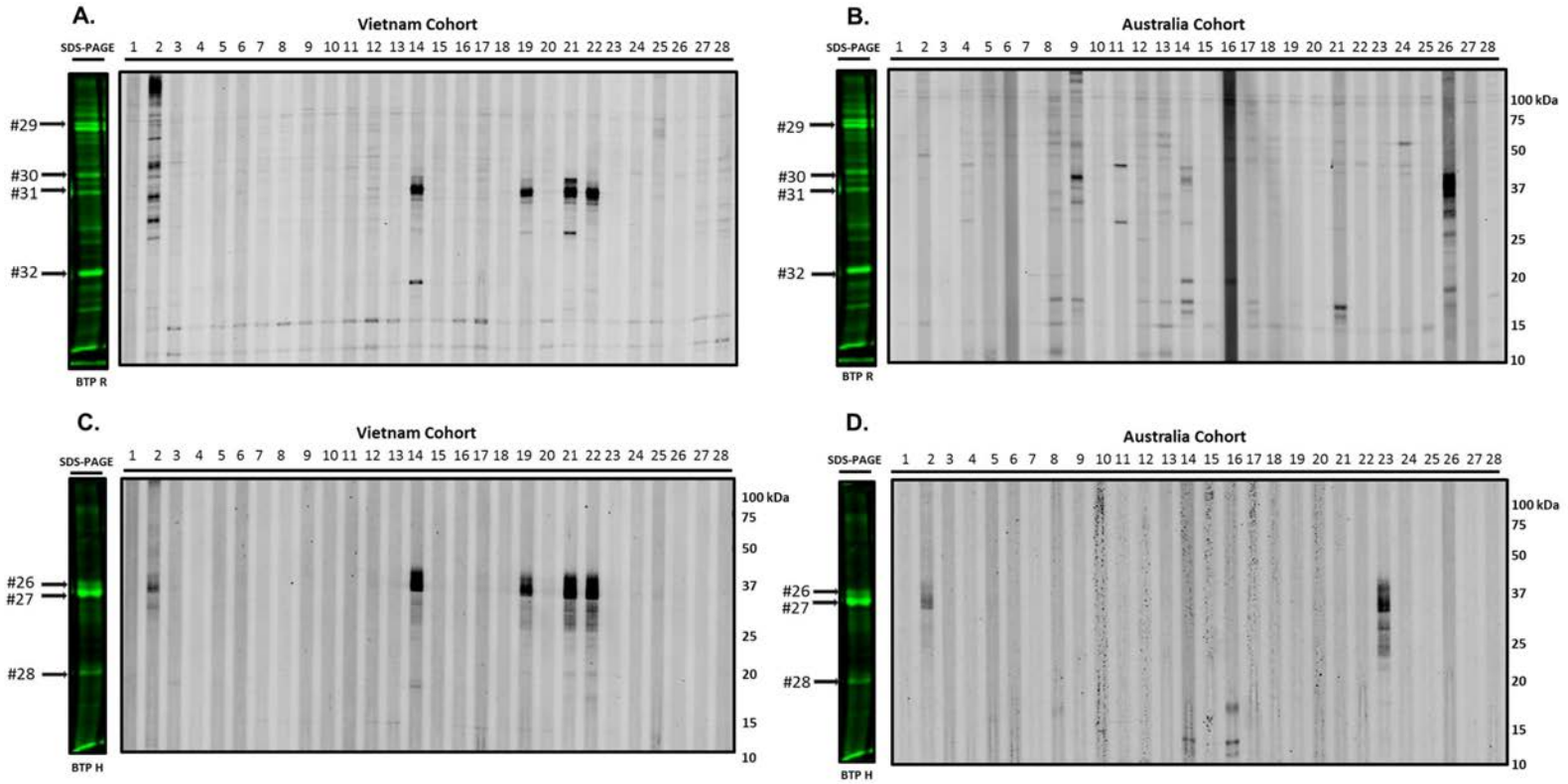

Figure 7.1 IgE reactivity to **black tiger prawn extracts** by immunoblotting.

Antibody reactivity against raw prawn extract among participants from the Vietnamese cohort (A) and the Australian cohort (B). Antibody reactivity against heated prawn extract among participants from the Vietnamese cohort (C) and the Australian cohort (D). The arrow and ID number (#) on the left-hand side of the gel indicate the protein bands that where excised for mass spectrometry. Sera from the participants were labeled from 1 to 28.

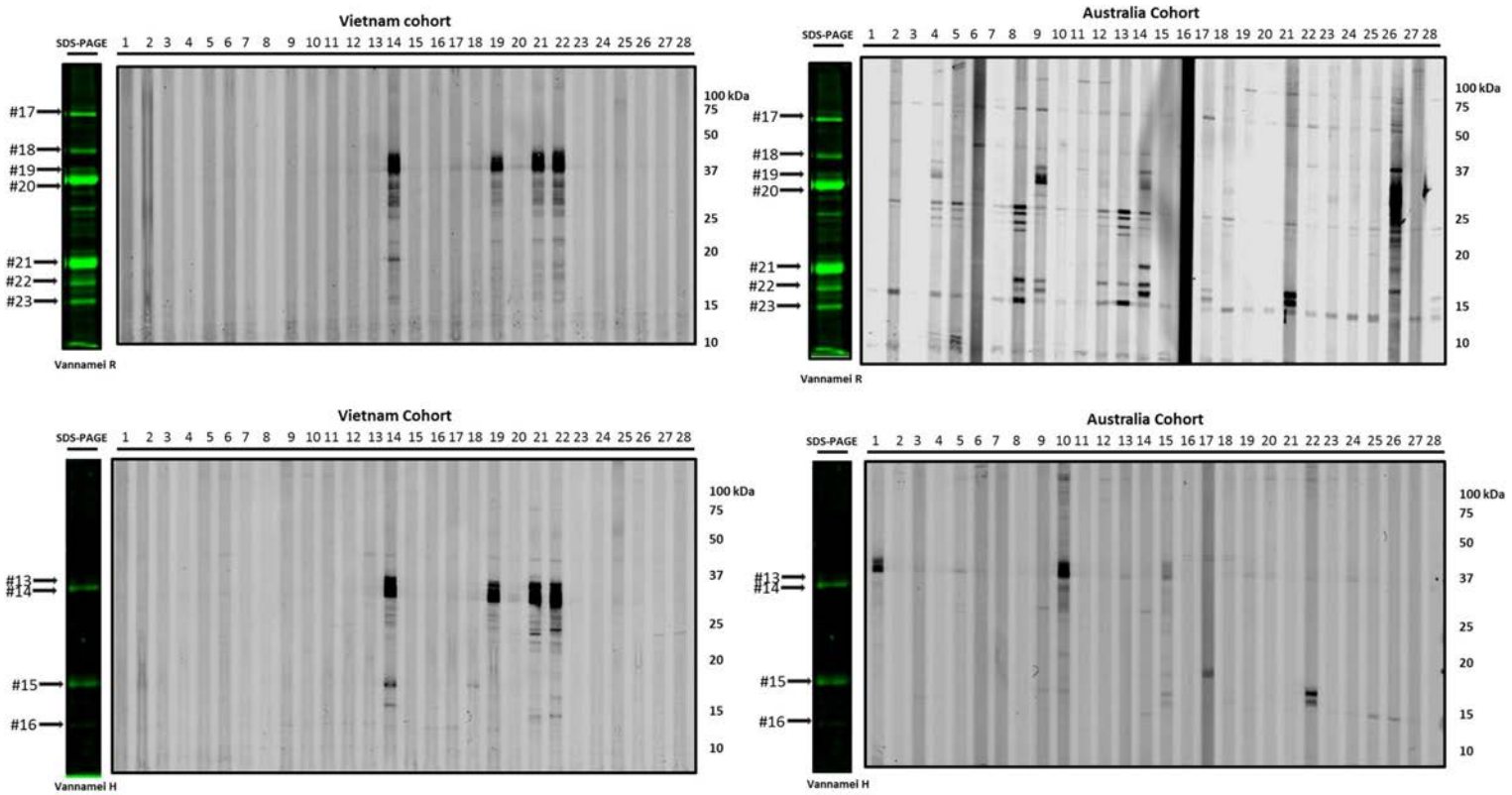

Figure 7.2 IgE reactivity to **vannamei prawn extracts** by immunoblotting.

Antibody reactivity against raw prawn extract among participants from the Vietnamese cohort (A) and the Australian cohort (B). Antibody reactivity against heated prawn extract among participants from the Vietnamese cohort (C) and the Australian cohort (D). The arrow and ID number on the left-hand side of the gel indicate the protein band that was cut for mass spectrometry. Sera from the participants were labeled from 1 to 28.

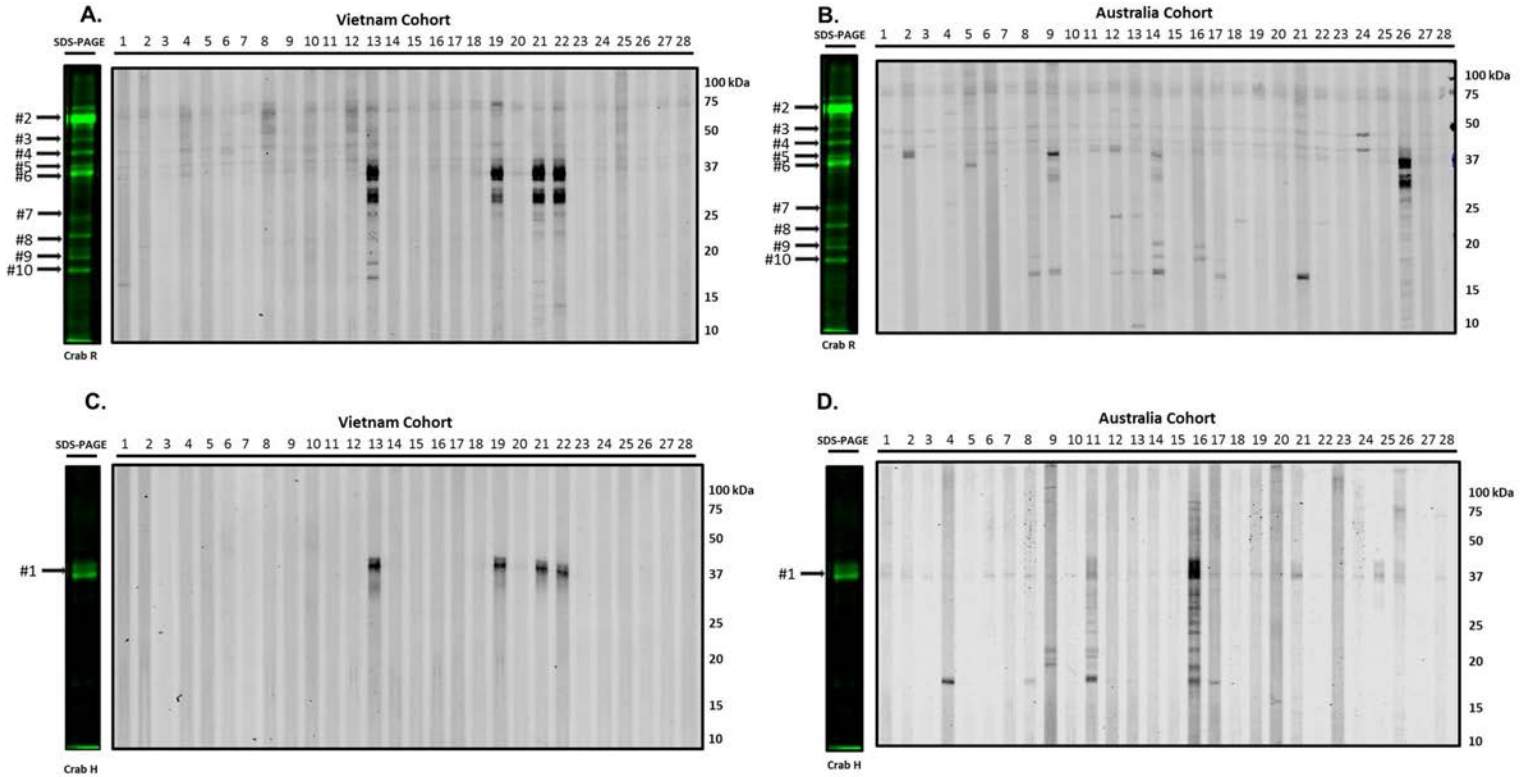

Figure 7.3 IgE reactivity to **blue swimmer crab** extracts by immunoblotting.

Antibody reactivity against raw prawn extract among participants from the Vietnamese cohort (A) and the Australian cohort (B). Antibody reactivity against heated prawn extract among participants from the Vietnamese cohort (C) and the Australian cohort (D). The arrow and ID number on the left-hand side of the gel indicate the protein band that was cut for mass spectrometry. Sera from the participants were labeled from 1 to 28.

7.4.3 Protein identification by mass spectrometry

A total of 32 protein bands were excised and analyzed by mass spectrometry (Figure 7.1, Figure 7.2 and Figure 7.3). The peptide matching outcomes are presented in Table 7.1, Table 7.2 and Table 7.3. All the investigated proteins demonstrated a confident match with proteins from the database. Overall, ten different proteins were identified, which are all known seafood allergens. TM and SCP were the most predominant IgE binding allergens in all crustacean extracts.

Table 7.1 List of identified proteins from **black tiger prawn** extract from matched known protein database (www.uniprot.org).

Band ID	Extract type	Experimental MW (kDa)	Protein identity	Sequence coverage (%)	Accession number (GenBank)	Mass	Score	emPAI	Calculated pI	Known allergens
26	Heated	35.5	**Tropomyosin**	82	A1KYZ2	32,830	1152	22.97	4.72	Pen m 1
27	Heated	34.5	**Tropomyosin**	87	A1KYZ2	32,830	1311	41.7	4.72	Pen m 1
28	Heated	18.2	**Tropomyosin**	36	P86704	32,734	119	0.79	4.7	Pan b 1
			Tropomyosin	44	A1KYZ2	32,830	119	0.96	4.72	Pen m 1
			SCP	51	P02636	22,251	84	0.76	4.63	-
29	Raw	78.1	**Myosin heavy chain**	2	P24733	223,824	222	0.06	5.6	-
			Arginine kinase	40	C7E3T4	40,400	188	0.73	6.05	Pen m 2
			Arginine kinase	30	P51545	40,250	122	0.48	6.36	-
			Enolase	14	P56252	47,525	84	0.14	5.85	-
			Arginine kinase	26	Q9NH48	40,656	80	0.26	6.34	-
30	Raw	41.2	**Enolase**	30	P56252	47,525	251	1.74	5.85	-
			Arginine kinase	55	C7E3T4	40,400	207	1.57	6.05	Pen m 2

			Actin	41	P86700	20,904	120	0.82	5.03	alpha-actin
			Actin	29	P18600	42,158	115	0.57	5.3	-
31	Raw	34.9	**Tropomyosin**	64	A1KYZ2	32,830	522	3.24	4.72	Pen m 1
			Tropomyosin	56	O61379	31,720	505	3.03	4.64	Pan s l
			Arginine kinase	53	C7E3T4	40,400	200	0.88	6.05	Pen m 2
			Enolase	21	P56252	47,525	167	0.31	5.85	-
32	Raw	18.5	**SCP**	70	P02636	22,251	423	8.49	4.63	-
			SCP	70	P02635	22,239	230	4.41	4.52	-
			SCP	50	P05946	21,783	186	1.36	4.61	Pon l 4
			Arginine kinase	55	C7E3T4	40,400	182	0.73	6.05	Pen m 2

Note: '-' indicates not determined. SCP: Sarcoplasmic calcium-binding protein

Table 7.2 List of the identified proteins from **vannamei prawn** extract from matched known protein database (www.uniprot.org).

Band ID	Extract type	Experimental MW (kDa)	Protein identity	Protein sequence coverage (%)	Accession number (GenBank)	Mass	Score	emPAI	Calculated pI	Known allergen
13	Heated	37.4	**Tropomyosin**	62	A1KYZ2	32,830	481	2.17	4.72	Pen m 1
14	Heated	36.2	**Tropomyosin**	73	A1KYZ2	32,830	1136	16.96	4.72	Pen m 1
15	Heated	18.7	**SCP**	58	P02636	22,251	166	1.33	4.63	-
			Myosin light chain	100	P86703	1,870	68	9.28	4.32	-
16	Heated	15.4	**Hemocyanin C chain**	2	P80096	75,997	48	0.09	5.37	-
17	Raw	70.7	**Hemocyanin C chain**	2	P80096	75,997	74	0.09	5.37	-
			Tropomyosin	41	A1KYZ2	32,830	115	0.33	4.72	Pen m 1
18	Raw	46.2	**Tropomyosin**	35	A1KYZ2	32,830	97	0.21	4.72	Pen m 1
			Actin	22	P83751	42,068	90	0.16	5.3	-
19	Raw	38.4	**Tropomyosin**	60	A1KYZ2	32,830	243	1.38	4.72	-
			Arginine kinase	50	C7E3T4	40,400	175	1.03	6.05	Pen m 2
20	Raw	34.2	**Tropomyosin**	87	A1KYZ2	32,830	2335	110.82	4.72	Pen m 1
21	Raw	18.5	**SCP**	68	P02636	22,251	454	9.92	4.63	-
22	Raw	16.9	**SCP**	46	P02636	22,251	143	1.02	4.63	-
23	Raw	15.3	**SCP**	22	P02636	22,251	89	0.32	4.63	-

Note: '-' indicates not determined. SCP: Sarcoplasmic calcium-binding protein.

Table 7.3 List of the identified proteins from blue swimmer crab extract from matched known protein database (www.uniprot.org).

Band ID	Extract type	Experimental MW (kDa)	Protein identity	Protein sequence coverage (%)	Accession number (GenBank)	Mass	Score	emPAI	Calculated pI	Known allergen
1	Heated	38.8	**Tropomyosin**	67	Q9N2R3	30,417	577	5.47	4.76	Cha f 1
2	Raw	70.7	**Hemocyanin subunit 2**	12	P84293	75,102	113	0.24	5.4	-
3	Raw	56	**Pyruvate kinase**	8	O62619	57,917	101	0.18	7.13	-
			Glucose-6-phosphate isomerase	6	P52029	62,585	101	0.11	6.63	-
4	Raw	48.6	**Enolase**	30	P56252	47,525	329	1.09	5.85	-
5	Raw	42	**Enolase**	25	P56252	47,525	312	0.71	5.85	-
			Actin	47	P86700	20,904	87	0.16	5.03	alpha-actin
6	Raw	39.1	**Enolase**	26	P56252	47,525	168	0.4	5.85	-
			Tropomyosin	28	O44119	32,887	80	0.21	4.74	Hom a 1
7	Raw	27.5	**Arginine kinase**	64	Q9NH49	40,632	718	4.17	6.19	-
8	Raw	24	**Triosephosphate isomerase A**	20	Q1MTI4	27,179	179	0.78	4.9	-
			Arginine kinase	49	Q9NH49	40,632	135	0.87	6.19	-
9	Raw	19.6	**Arginine kinase**	50	Q9NH49	40,632	93	0.6	6.19	-
			SCP	42	P05946	21,783	291	1.73	4.61	Pon l 4

10	Raw	18.2	**SCP**	49	P05946	21,783	287	2.63	4.61	Pon I 4
			Arginine kinase	62	Q9NH49	40,632	183	0.6	6.19	-

Note: '-' indicates not determined. SCP: Sarcoplasmic calcium-binding protein

7.4.4 Comparison of the identified allergens among two participant groups

The identified allergens were used to match the participants' IgE reactivity from the immunoblotting results. The distribution of allergen patterns among two populations is summarized in Table 7.4.

Table 7.4 The distribution of identified allergens between the two cohorts.

Species	Identified protein	IgE reactivity to identified proteins from the raw extract			IgE reactivity to analyzed proteins from the heated extract		
		% Vietnam cohort (n = 28)	% Australia cohort (n = 28)	Difference, p	% Vietnam cohort (n = 28)	% Australia cohort (n = 28)	Difference, p
Vannamei	Tropomyosin	14.3	46.4	**.0096**	21.4	35.7	.241
	SCP	10.7	82.1	**< .0001**	-	-	-
	Hemocyanin	-	-	**-**	10.7	25.0	.1666
	Myosin light chain	-	-	**-**	21.4	10.7	.2797
	Hemocyanin C chain	14.3	89.3	**< .0001**	14.3	14.3	1
Crab	Tropomyosin	28.6	14.3	.1967	57.1	14.3	**.0009**
	SCP	32.1	14.3	.1168	-	-	-
	Enolase	17.2	25.0	.4809	-	-	-
	Arginine kinase	7.1	14.3	.3918	-	-	-
	Hemocyanin	17.9	35.7	.1348	-	-	-
	Pyruvate kinase	10.7	10.7	1	-	-	-
	Triosephosphate isomer	14.3	14.3	1	-	-	-
BTP	Myosin heavy chain	78.6	96.4	**.0453**	-	-	-
	Tropomyosin	28.6	17.9	.3467	17.9	7.1	.2296
	SCP	21.4	17.9	.7389	-	-	-
	Arginine kinase	39.3	28.6	.4014	-	-	-

Note: '-' indicates no IgE reactivity recorded. BTP: black tiger prawn.

The z-test was used to compare the difference in IgE reactivity between two cohorts (alpha level = 5%).

7.5 Discussion

The implicated proteins that demonstrated IgE reactivity against black tiger prawn, vannamei prawn, and blue swimmer crab, raw and heated extracts, among shellfish allergic participants from two populations, were identified by mass spectrometry. Overall, all the allergens identified are previously known allergens reported in crustacean and mollusk and were identified with high certainty. Identified allergens were proteins with the molecular weight ranging from 17-75 kDa. Raw extracts present much greater allergen diversity as compared to the heated extracts. Shellfish allergic participants from Australia showed significantly more IgE reactivity to SCP, HC-C chain and myosin heavy chain (MHC) in the raw extracts as compared to individuals from Vietnam. However, a similar phenomenon was not seen in the heated extracts. TM was the major trigger for the IgE reactivity found in the heated extracts in the two cohorts.

The identification of TM as the most abundant allergen in crustacean raw and heated extracts in this investigation is in line with the current literature (7) and the findings in Chapter 5 and Chapter 6 of this thesis. Identified TM proteins in this study displayed a molecular weight of about 34.2-39.1 kDa. Only one protein band was visibile from vannamei with a molecular weight of 46.2 kDa. This protein indicated a good match with the TM from black tiger prawn and was included in the analysis. Though TM is known as a heat-stable protein (8), the IgE reactivity to TM decreased in the heated extracts. This scenario is seen with other heat-stable proteins in this investigation including SCP and enolase.

HC is an important protein participating in the respiratory function of crustacean and mollusk (9). HC has been demonstrated to be an allergen in the giant freshwater shrimp (*M. rosenbergii*) (7), vannamei prawn (*L. vannamei*) (10) and squid (*T. pacificus)*(11). In this study, HC was identified in vannamei and crab extracts. More shellfish allergic participants from Australia showed IgE reactivity to HC than those from Vietnam ($p < .0001$). HC sensitization was recently reported among adult shellfish allergic patients in Australia (12). The contribution of HC as an allergen was investigated in Chapter 5 and Chapter 6 where the recombinant HC seemed to be

more reactive than the natural TM from the same species. As discussed in the previous chapter, HC remains an important seafood allergen that requires further investigation.

SCP was the second dominant allergen found in prawn and crab extracts in this study. However, the two populations demonstrated different IgE recognition patterns to this allergen from the raw and heated extracts, and also between species. Australian shellfish allergic participants were more reactivity to SCP from vannamei prawn than those of crab and BTP. The IgE reactivity against SCP from BTP was confirmed at a frequency of 10% in a study among 21 Australian shellfish allergic adults (12). Interestingly, Vietnamese participants only responded to SCP in the raw extracts. It is unknown whether this variation comes from the difference of SCP concentration among the preparations or the difference in the specific IgE binding regions.

Other proteins identified among raw extracts were AK, enolase, pyruvate kinase and triosephosphate isomerase A. Several participants showed IgE reactivity, but no significant difference was seen among the two populations.

In summary 32 protein bands that demonstrated IgE reactivity against participants' sera were excised and identified using mass spectrometry in this study. Ten allergens were identified with high certainty. TM was the major allergen identified in the raw and heated crustacean extracts. In addition, SCP and HC were abundant and important allergens among shellfish allergic participants from Australia.

7.6 References

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7.7 Chapter 7 summary

- Ten allergens including tropomyosin (TM), sarcoplamic calcium-binding protein (SCP), hemocyanin (HC), hemocyanin C chain, myosin light chain (MLC), myosin heavy chain (MHC), enolase, arginine kinase (AK), pyruvate kinase and triosephosphate isomerase from raw and heated crustacean extracts were identified with high certainty.

- TM was the most abundant protein in the raw crustacean extracts. This identified protein has a molecular weight of about 34.2-39.1 kDa and IgE binding decreased in the heated extracts.

- Australian shellfish allergic participants demonstrated a statistically different IgE reactivity pattern against HC, SCP and MHC compared to those from Vietnam. No difference was seen in the IgE reactivity of the two cohorts to other identified allergens.

CHAPTER 8 GENERAL DISCUSSION AND FUTURE DIRECTIONS

8.1 General discussion

Food allergy is thought to dominate the Western society as a consequence of industrialized lifestyles (1). However, recent population-based reports on food allergy from the developing world revealed a paradoxical fact (**Chapter 1**). The wave of food allergy epidemics seems to be real and reaching Asia, Africa, and South America (2, 3). The highest food allergy prevalence in Asia was found to be 7.71% for shellfish among Taiwanese children (4-18 years) (4). The highest preavalence of fish allergy was reported at 2.29% among adolescents in the Philippines (5). The prevalence of challenge-proven food allergy in infants 0-24 months of age, raised more than double (from 3.5% to 7.7%) after a decade in Chonqing, China (6). Thus, the World Allergy Organization is calling for more national-scale food allergy investigations to evaluate the real status of food allergy worldwide (7).

Vietnam is one of the countries in Asia that currently lacks population-based data on food allergy. The first experimental chapters of this thesis (**Chapter 2** and **Chapter 3**) was to evaluate the frequency of food allergy cases among Vietnamese children and adults, and to identify any offending food allergen patterns. From a paper-based survey conducted in five regions across Vietnam, the author recruited 17, 659 respondents (response rate of 69.9%) and revealed a doctor-diagnosed food allergy rate of 6.7% and 4.6% among children and adults, respectively (8, 9). Crustacean, mollusk, and fish were reported as the leading triggers for allergic reactions in both children and adults, while the involvement of plant-derived allergen sources like peanut, tree nuts, soy, and wheat were less significant. Allergy to beef was reported among the Asian community for the first time, and the frequency in children and adults varies by geographic locations (urban *vs.* rural). In terms of contributing factors, food allergy was suggested to run in the family. Vietnamese children with a current atopic condition such as eczema, rhinitis or asthma exhibited a high rate of food allergy. Overall, the rural populations demonstrated significantly less rates of food allergy than their urban counterparts.

Following-up the population-based survey, a similar food allergy survey was performed and distributed via the internet. The web-based survey on food allergy in Vietnamese adults provided comparable data to the conventional paper-based survey regarding major study implications; including the prevalence rate of food allergy, the food allergen reactivity patterns, as well as contributing factors. The advantages and pitfalls of the paper-based and web-based surveys are discussed in detail in **Chapter 4**. The author suggests the application of the web-based surveys as a low-cost, rapid, labor-efficient and convenient platform for future epidemiological studies in Vietnam and elsewhere.

Current food allergy diagnostics suggest oral food challenge as the gold standard for confirming a clinical food allergy. The observational studies using surrogate measurements such as self-administrated questionnaires and *in vitro* measurement of IgE, has limitations, with a risk that they may overestimate the prevalence of true food allergy. In the context of Vietnam, due to the lack of medical facilities and trained staff, confirming clinical food allergy by oral food challenges in a large-scale population study is not feasible. Furthermore, in certain rural areas of Vietnam, questionnaire surveys might be a completely new practice. In this study, the survey questionnaire was designed as a short, general health-check questionnaire about food consumption and related health problems, to gain a satisfying response rate and reduce the participation bias. The EAACI guidelines on food allergy definitions were applied to identify the target subpopulations and generate the prevalence data. Furthermore, the survey was conducted across five different regions of Vietnam, representing up to half of the Vietnamese population and culture. Thus, the data from this study is reliable and is likely to reflect the current food allergy status in Vietnam.

From the population-based survey, seafood was reported as the leading allergy-triggering food and this finding was in line with previous reports on food allergy in the Asian community. To understand more about this food allergy type, further investigations on the clinical presentations and *in vitro* IgE reactivity against seafood allergens were conducted. From October 2018 to April 2019, a total of 39 participants were recruited at Bach Mai Hospital, the

biggest hospital in Vietnam. Of which, 34 participants have a clinical history to seafood and 2/34 subjects were child participants. Prawns and crabs were implicated as the most common allergy-inducing foods. Most of participants presented an acute onset with an average of 4 to 5 concurrent symptoms per episode. Nineteen participants had SPT to shellfish, fish and other aeroallergen sources and 34 individuals were invited to donate blood for the *in vitro* analysis of their immunological profile (**Chapter 5**). Five healthy controls were recruited and donated their blood for the study.

A similar study on seafood allergy was also conducted in North Queensland, Australia (**Chapter 6**). Participants were recruited from two intakes in November 2017 and May 2018. Subjects with a history of seafood consumption/exposure were invited to an interview with the clinician, followed by a blood donation. A total of 69 participants were recruited including 28 individuals with a shellfish allergy, eight subjects with mixed seafood allergy and four participants with fish allergy only. Twenty-eight healthy controls were recruited andtheir blood was collected. Overall, similar to the Vietnamese cohort, participants in Australia presented acute episodes of the allergic symptoms with the dominance of skin related symptoms and oral allergy syndrome. Prawns and crabs were the major implicated food items. The Australian cohort reported more anaphylactic events, where 14.5% of participants were taken to the emergency room. Regarding food allergy management, 15.7% of Australian participants currently carry an EpiPen® while none of the Vietnamese participants reported having an emergency life-saving kit.

All serum samples collected from the participants in Vietnam and Australia were used to analyze the IgE reactivity against heated seafood extracts and seafood allergen components. The seafood protein extracts selected for the investigation included commonly consumed seafood species from Vietnam and Australia. Certain common seafood species such as black tiger prawn (*Penaeus monodon)*, vannamei prawn (*Litopenaeus vannamei)*, blue swimmer crab (*Portunus pelagicus)*, basa fish (*Pangasius hypophthalmus)*, tilapia (*Oreochromis sp)*, barramundi (*Lates calcarifer)*, and Atlantic salmon (*Salmon*

salar) were used to compare the IgE reactivity between the two subpopulations. So far, this is the **first seafood allergy investigation** ever conducted in Vietnam and the **first comparative study** on seafood allergy across two countries in the Asia Pacific.

Generally, shellfish allergic participants from two cohorts demonstrated a similar IgE binding pattern to the investigated crustacean and mollusk species. Back tiger prawn was more IgE reactive than mud crab, blue swimmer crab and vannamei prawn. In the mollusk group, clam was the most IgE reactive species, whereas squid and Pacific oyster seemed to be less allergenic. IgE reactivity against black tiger prawn was significantly different from vannamei prawn among two cohorts ($p < .0001$). More Vietnamese participants (61.8%) reacted positively to the prawn tropomyosin than the Australian counterparts (39.0%) ($p < .0001$). Besides tropomyosin, the heat-sensitive allergen hemocyanin from vannamei prawn was indicated as a highly reactive component among the two cohorts. In addition, Vietnamese participants were more sensitive to prawn myosin light chain than the Australian subjects ($p < .0001$). Regarding indoor allergens, all participants demonstrated higher IgE reactivity against HDM extract compared to cockroach extract ($p < .0001$).

The *in vitro* IgE reactivity of the participants was analyzed against 13 different fish species that are commonly consumed in the Asia Pacific. Overall, the Vietnamese cohort elicited higher IgE binding against proteins in heated fish extracts than the Australian group, but this was not statistically significant. The comparison of IgE reactivity against different fish parvalbumins was conducted using sera from the fish allergic subjects, and the group with shellfish allergy with no clinical history to fish. Again, participants with a clinical history to fish presented higher IgE binding intensities, but this was not statistically significant. Noticeably, there were two cases where that the participants demonstrated a significantly high IgE reactivity to fish heated extract and one of them showed high IgE levels to salmon and cod by ImmunoCAP, but no clinical history to fish was reported. It is not confirmed whether these cases implicate the poor correlation of IgE levels to clinical fish allergy or are merely examples of a hyper

IgE reactivity condition in a certain sensitized subpopulation; this needs further investigation.

In this thesis, the SPT were performed in 19/34 (55.9%) participants in Vietnam and ImmunoCAP testing to prawn, salmon, and cod were performed among participants (all shellfish allergic participants were confirmed with prawn ImmunoCAP) in Australia. The outcomes from the above diagnostic tests, in general, did not show a good correlation with the participants' clinical history, nor the IgE reactivity generated from this study. The variability of allergen concentration and allergen components among different SPT reagents for fish allergy diagnostics was addressed previously (10). It is important to have a follow-up study among these cohorts to identify the real cause for the poor correlation and to improve the current diagnostics of seafood allergy.

Australia currently has the highest challenge-proven pediatric food allergy rate in the world (11), however, this study on food allergy among Australian adults is limited. Peanut, cow's milk, and egg are the most common food allergens among Australian infants and children (12) but it is not known whether the adult population in Australia suffers from the same food allergy pattern. The only available food allergy data among the Australian adult population was reported in 2002 from 1,141 adults aged 20-45. This study reported a rate of shellfish allergy, confirmed by SPT, of 0.53%, proceeded by peanut allergy (0.63%)(13). Another household survey in 2009 by Allen et al. (14) revealed shellfish (5.9%) among the top three common food allergens after cow's milk (8.3%) and peanut (6.9%). From these above reports, it seems that seafood allergy might be common among Australian adults. Therefore, it is essential to collect population-based data on seafood allergy in the adult population in order to complete the picture of food allergy prevalence in this country. The presented study reported numerous participants around Queensland with seafood allergy symptoms, Furthermore, this abnormal health condition among adults seems to not receive adequate attention as is displayed towards children. Within Queensland, food allergy diagnosis clinics are based in and around Brisbane, the state capital city. Most of the participants recruited in this study have been suffering from food allergy for a long time, without having an appropriate

diagnosis and/or intervention. This is partly due to the unavailability of allergy specialists in close proximity. The lack of medical services for food allergy, and the food allergy under-diagnosis issues addressed in this study will raise awareness in the food allergy management community and appropriate interventions are planned for the future.

In Vietnam, similar to many other developing countries, allergic diseases may be highly prevalent, but the study of food allergy and appropriate management systems have not received much attention from the general public; possibly due to other prevailing health burdens (i.e. infectious diseases). With an estimated 6 million people in Vietnam (data generated from the presented population-based survey) currently suffering from food allergies, it is crucial to call for an appropriate intervention at the national scale. The outcomes of this study were published in open-access journals to be able to disseminate the findings from this thesis to the advocates in Vietnam, so that appropriate interventions can be implemented for the improved quality of life for people with food allergy in this country.

In the last chapter of this thesis (**Chapter 7**), the confirmation of IgE reactivity to crustacean allergens was performed by immunoblotting and mass spectrometry. The aim of this chapter was to subjectively identify crustacean allergens (in particular, water-soluble proteins) that demonstrate IgE reactivity among recruited shellfish allergic participants. The author reported the predominance of IgE binding to tropomyosin in raw and heated crustacean extracts. Sarcoplasmic calcium-binding protein and hemocyanin are the two identified allergens that demonstrated more IgE reactivity in Australian participants than in the Vietnamese subjects. This study confirmed our understanding of the existing seafood allergens among sensitized populations in two investigated countries. Further investigation needs to be performed to transfer the findings into current diagnostic practice.

8.2 Future directions

From the investigations of food allergy and seafood allergy in Vietnam and Australia presented in this thesis, and taking into consideration the limitations of the studies that are discussed in each chapter, I would like to suggest several directions for future investigations as below:

a) Expansion of the food allergy survey to the remaining populations of Vietnam, for instance, the populations in Northern Vietnam, the populations in the mountainous regions and the minor ethnic communities. It is estimated that the variation in the climate conditions and dietary practices of these populations might impact the food allergy rates.

b) Following-up on investigations on other food allergies in Vietnam. For example, investigation of the etiology and pathogenesis of red meat allergy in Vietnam. Especially, investigating the association between the incidence of tick bites and red meat allergy among populations from rural areas. Further investigations on food allergy prevalence in children of other age groups (i.e. infants, school-age children, adolescents). This study will provide a whole picture of food allergy prevalence in Vietnam and advance the understanding of food allergy variation over a life course.

c) About four million people are working in the seafood industry in Vietnam. This population includes more than two million personnel working directly in the seafood processing plants across the country. As discussed in the first chapter, prolonged exposure to aerosolized seafood allergen particles is a trigger for the development of adult-onset seafood allergy. It is crucial to have a comprehensive study on occupational seafood allergy in Vietnam to address the impact of working conditions on the general health of the workers, and to apply appropriate interventions to improve tfood allergy management in general, as well as the and welfare of the seafood workers.

d) So far, there is limited information on food allergy available to the general population in Vietnam, especially information provided in the Vietnamese

language. With an estimation of about six million Vietnamese people suffering from food allergy, it is essential to have a food allergy management program in place in Vietnam. Suggestions for future initiatives include: developing a food allergy action plan and food allergen factsheets for the affected groups, compiling a localized food allergen handbook that can be accessible to the public (especially food service providers and food processing manufacturers), and establishing national guidelines for allergen labelling on food products.

e) Further investigation into the molecular characterization of other seafood allergens such as mollusk allergens, to profile the allergen pattern from these species in the two populations. Furthermore, it is important to have a study based on the heat-sensitive allergens present in the raw crustacean and mollusk extracts. These molecular analyses are the first step towards developing a better diagnostic tool for seafood allergy.

f) From the patient collection, it would be ideal to perform oral food challenges, to confirm clinical seafood allergy among the participants. Then, from the outcomes of the confirmed test, the researcher would be able to review all the diagnostic test data including the clinical symptoms, IgE levels to putative allergens, SPT results to define implicated diagnostic value, such as establishing the 95% positive predictive value, the negative predictive value, defining the specificity and sensitivity as well as to develop the predictive likelihood model, to support the diagnosis of seafood allergy.

g) For the management of seafood allergy in Australia, there is a need for a population-based survey on food allergy in the adult population to estimate the likely impacts of food allergy and lay the groundwork for a better food allergy management system in Australia. There is also a need for more allergy clinics in the remote areas of the country.

h) This survey reported that many participants with seafood allergy also react to allergen components from HDM and cockroach. However, it is not known which allergen components contribute to the cross-reactivity and to what extent. It is important to have further investigations among these populations to estimate the contribution of indoor allergens to seafood allergy incidence; thus, more appropriate interventions could be implemented. Some suggestions include performing immunoblotting against allergen components from HDM, conducting inhibition ELISA's to analyze the allergen components that contribute to the cross-reactivity between seafood and indoor allergens.

In conclusion, the research activities in this thesis provides valuable insight into the current status of food allergy in Vietnam, and the pathogenesis of seafood allergy among Vietnamese and Australian patients. Findings from this study contribute to the development of better therapeutics and effective management of seafood allergy in these countries as, well as in the Asia Pacific.

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APPENDICES

9.1 Appendix A – Ethics approvals

9.1.1 Appendix A1: Ethics Approval No H6437 for the population-based survey of food allergy in Vietnam.

This administrative form has been removed

9.1.2 Appendix A2: Ethics Approval No H7233 for the seafood allergy study in Vietnam.

This administrative form has been removed

9.1.3 Appendix A3: Ethics Approval No H6829 for the seafood allergy study in North Queensland, Australia.

This administrative form has been removed

9.2 Appendix B - Survey Questionnaire

9.2.1 Appendix B1. Questionnaire for the population-based survey of food allergy in Vietnamese children

I. General information of the child:

Gender: ☐ Male ☐ Female

Age:.....

Living location: .....

II. Questions:

1. Has your child ever had any symptoms as below when consuming foods (*tick which apply*)

☐ Hives (reddish, swollen, itchy areas on the skin)

☐ Eczema (a persistent dry, itchy rash)

☐ Redness of the skin or around the eyes

☐ Itchy mouth or ear canal

☐ Nausea or vomiting

☐ Diarrhea

☐ Stomach pain

☐ Nasal congestion or a runny nose

☐ Sneezing

☐ Slight, dry cough

☐ Odd taste in mouth

☐ Obstructive swelling of the lips, tongue, and/or throat

☐ Shortness of breath or wheezing

☐ Trouble swallowing

☐ Drop in blood pressure

☐ Loss of consciousness

☐ Chest pain

☐ A weak pulse

☐ No symptoms as above

2. Do any symptoms as above repeat when the child eat a specific food?

☐ Yes ☐ No

3. According to your observations, the cause of any allergy-like symptoms manifests, as listed in question 1, after eating which food group below?

☐ Crustacean (shrimp, crab, ……)

☐ Fish (Please specify: ……..……)

☐ Molluscs (squid, octopus, clam, snails……)

☐ Egg

☐ Wheat, wheat-based products

☐ Peanut

☐ Soy bean

☐ Tree nut: cashew, walnut, almond

☐ Milk and dairy products

☐ Beef meat

☐ Other food commodities (Please specify: ……)

☐ No allergy to any foods

4. Do you think that your child have suffered **food allergy**?

☐ Yes → please go to question 5

☐ No → please go to question 7

5. Has your child ever visited specialized doctor for food allergy?

☐ Yes ☐ No

6. Has your child been diagnosed to have **food allergy**?

☐ Yes ☐ No

7. Are you or is there any other member in your family have **other types of allergy** (pollen allergy, antibiotics allergy…)?

☐ Yes ☐ No

8. Are you or is there any other member in your family have **food allergy**?

☐ Yes ☐ No

9. If yes, please specify the food that you or other members in your family are allergic to:

……

……

……

10. If you suspect that your child has been suffering any of the above symptoms of food allergies, do you wish to allow your child to follow up the second phase of the project to investigate the food allergy causative factors?

☐ Yes ☐ No

9.2.2 Appendix B2. Questionnaire for the population-based survey of food allergy in Vietnamese adults (both paper-based survey and web-based survey)

I. General information of respondent:

Gender: ☐ Male ☐ Female

Age:.....

Living location: .....

II. Questions:

1. Have you ever had any symptoms as below when consuming foods (*tick which apply*)

☐ Hives (reddish, swollen, itchy areas on the skin)

☐ Eczema (a persistent dry, itchy rash)

☐ Redness of the skin or around the eyes

☐ Itchy mouth or ear canal

☐ Nausea or vomiting

☐ Diarrhea

☐ Stomach pain

☐ Nasal congestion or a runny nose

☐ Sneezing

☐ Slight, dry cough

☐ Odd taste in mouth

☐ Obstructive swelling of the lips, tongue, and/or throat

☐ Shortness of breath or wheezing

☐ Trouble swallowing

☐ Drop in blood pressure

☐ Loss of consciousness

☐ Chest pain

☐ A weak pulse

☐ No symptoms as above

2. Do any symptoms as above repeat when you eat a specific food?

☐ Yes ☐ No

3. According to your observations, the cause of any allergy-like symptoms manifests, as listed in question 1, after eating which food group below?

☐ Crustacean (shrimp, crab, ……)

☐ Fish (Please specify: ……)

☐ Molluscs (squid, octopus, clam, snail……)

☐ Egg

☐ Wheat, wheat-based products

☐ Peanut

☐ Soy bean

☐ Tree nut: cashew, walnut, almond

☐ Milk and dairy products

☐ Beef meat

☐ Other food commodities (Please specify:……)

☐ No allergy to any foods

4. Do you think that you have suffered **food allergy**?

☐ Yes → please go to question 5

☐ No → please go to question 7

5. Have you ever visited specialized doctor for food allergy?

☐ Yes ☐ No

6. Have you been diagnosed to have **food allergy**?

☐ Yes ☐ No

7. Are you or is there any other member in your family have **other types of allergy** (pollen allergy, antibiotics allergy…)?

☐ Yes ☐ No

8. Are you or is there any other member in your family have **food allergy**?

☐ Yes ☐ No

9. If yes, please specify the food that you or other members in your family are allergic to:

……

……

……

10. If you have been suffering any of the above symptoms of food allergies, do you wish to follow up the second phase of the project to investigate the food allergy causative factors?

☐ Yes ☐ No

9.2.3 Appendix B3. Screening questionnaire to recruit participants for the seafood allergy study

Patient ID: _ _ _ _

QUESTIONNAIRE FOR SEAFOOD ALLERGY STUDY

I. General information:

Name:.....

Date of birth:.....

Occupation:.....

Sex: ☐Male ☐Female

II. Clinical information:

1. Please specify the types of seafood which have previously caused the adverse reactions when you consumed.
   Please list the name of fish and/or tick appropriate boxes for the other types of seafood:

Fish	Crustacean	Mollusc	Other seafood
•	☐Shrimp	☐Squid	•
	☐Crab	☐Octopus	
•	☐Lobster	☐Scallops	•
		☐Clams	
•	•	☐Oysters	
		☐Snails	
•	•	•	
•		•	

2. Which symptoms have you previously had in relation to a seafood ingestion?

☐Nausea	☐Hives	☐Wheezing	☐Swelling of lips or face
☐Vomiting	☐Eczema	☐Congested or running nose	☐Lips or tongue tingling
☐Diarrhea	☐Itching	☐Coughing	☐Shock
☐Abdominal pain	☐Redness of skin	☐Chest pain	☐Faint or dizzy
☐Itchy throat or mouth			☐Drop in blood pressure

☐Other:
.....
.....

3. How long did it take for the allergic reaction to occur?

☐ In less than 10 minutes
☐ In 10 minutes to 30 minutes
☐ In 30 minutes to 1 hour
☐ In 1 to 2 hours
☐ In 2 hours to 12 hours
☐ After more than 12 hours
☐ Don't know/ Don't remember

4. Have you ever been diagnosed of seafood allergy?

☐Yes
☐No

5. How were you diagnosed with allergies before?

	☐Skin test	**☐Blood test**
Result:	☐Yes	☐Yes
	☐No	☐No

If possible, please specify:
.....
.....
.....

6. When was the first time you ate seafood?

☐ < 1 year old

☐ 1 – 5 years old

☐ 6 – 10 years old

☐ >10 years old

☐ Don’t know/ Don’t remember

☐ Never eaten

7. When was the first time you recognized the allergic reaction due to having seafood?

☐ < 1 year old

☐ 1 – 5 years old

☐ 6 – 10 years old

☐ >10 years old

☐ Don’t know/ Don’t remember

☐ Never eaten

☐ At the FIRST time eating that seafood

8. What did you do in the most SEVERE episode?

☐ Go to hospital

☐ Use antihistamines

☐ Go to pharmacy to buy drug

☐ Take no action

☐ Epinephrine/ Adrenaline

Others, please specify:

.....
.....
.....

9. Are you now able to eat seafood without any reactions?

☐ Still have reactions

☐ Eat now with no reaction

☐ Haven't eaten again

☐ Only react sometimes

☐ Don't know

10. Do you have any other medical problems at the moment?

☐Yes

☐No

Please specify:
.....
.....
.....

III. Family History

1. Do other people in your family have any of the following conditions?

☐ Seafood allergy

- ☐ Food allergy
- ☐ Hay Fever
- ☐ Eczema
- ☐ Drug allergy
- ☐ Asthma

2. Are there any other medical problems in your family?

- ☐ Heart disease
- ☐ Skin problems
- ☐ Lung problems
- ☐ Stomach problems
- ☐ Immune diseases

IV. Environmental history:

1. Are you living in?

☐City ☐Suburb ☐Rural

2. Do you have any pets?

☐Yes

☐No

3. If yes, what kind of pets do you have?
   Please specify:
   .....
   .....

Name of the interview/clinician:

Date of interview:

9.2.4 Appendix B4. Screening questionnaire for the blood collection of the seafood allergy study in Vietnam

Donor ID:_ _ _ _ _

BLOOD DONOR SCREENING QUESTIONNAIRE

This is your medical history form, to be completed prior to donating blood. All information provided will be kept confidential. This information will be used for the evaluation of your health and readiness to collect your blood sample. Please take your time and complete it carefully and thoroughly. Your answers will help us to decide whether you are suitable as a healthy donor for this study.

If you have questions or concerns, please feel free to ask.

FOR PARTICIPANT TO BE COMPLETED

I. General information:

Date of birth: .....

Sex: ☐Male ☐Female

II. Clinical information:

1. Are you feeling well today?

   ☐Yes ☐ No

2. Do you have any current medications?

   ☐Yes ☐No

   If yes, please specify: .....

3. Have you ever suffered from seafood allergy?

   ☐Yes ☐No

4. Have you ever suffered from any other food allergies except seafood allergy?

   ☐Yes ☐No

5. Have you had any adverse clinical symptoms due to seafood consumption?

   ☐Yes ☐ No

   If yes, please specify: .....

6. Have you suffered from any allergic diseases (i.e. asthma, atopic dermatitis...)

☐Yes ☐No

If yes, please specify: .....

FOR OFFICE USE ONLY

Collection blood sample: ☐Yes ☐No

Name of the clinician/ nurse: .....

Date: .....

9.2.5 Appendix B5. Interview questionnaire for the seafood allergy study in Vietnam

9.2.6 Appendix B6. Screening questionnaire to recruit participants for the seafood allergy study in Australia

Participant ID:

QUESTIONNAIRE FOR SEAFOOD ALLERGY STUDY

I. Participant Information:			
First name:	Last name:	Gender: ○ Male ○ Female	Date of birth:
Ethnicity:	○ Australia	○ Other, please specify:	

II. Clinical Information:

1. Please specify the types of seafood causing the adverse reactions when consumed. Please list the name of the seafood and/or tick appropriate boxes where apply

FISH	**CRUSTACEAN**	**MOLLUSC**	**OTHER SEAFOOD**
☐Atlantic Salmon	☐Prawns	☐Squid	

☐Barramundi	☐Crabs	☐Cuttlefish	
☐Mackerels	☐Lobster	☐Calamari	
☐Whitings	☐Bugs	☐Octopus	
☐Tuna	☐Freshwater Crayfish	☐Scallops	
☐Dories		☐Clams	
☐Mullet		☐Oysters	
☐King fish		☐Mussels	
☐Breams		☐Abalones	
☐Billfish		☐Sea snails	
☐Freshwater Fish			

2. Which symptoms have you previously had in relation to a seafood ingestion?

☐Nausea/vomiting	☐Nausea/vomiting	☐Nausea/vomiting	☐Nausea/vomiting
☐Diarrhea	☐Diarrhea	☐Diarrhea	☐Diarrhea
☐Abdominal pain	☐Abdominal pain	☐Abdominal pain	☐Abdominal pain
☐Itchy throat or mouth	☐Itchy throat or mouth	☐Itchy throat or mouth	☐Itchy throat or mouth
☐Hives/urticaria	☐Hives/urticaria	☐Hives/urticaria	☐Hives/urticaria
☐ Flare of eczema	☐ Flare of eczema	☐ Flare of eczema	☐ Flare of eczema
☐Itching	☐Itching	☐Itching	☐Itching
☐Redness of the skin	☐Redness of the skin	☐Redness of the skin	☐Redness of the skin
☐Congested or running nose	☐Congested or running nose	☐Congested or running nose	☐Congested or running nose
☐Wheezing	☐Wheezing	☐Wheezing	☐Wheezing
☐Coughing	☐Coughing	☐Coughing	☐Coughing
☐Lips or tongue tingling	☐Lips or tongue tingling	☐Lips or tongue tingling	☐Lips or tongue tingling

☐Swelling of lips or face	☐Swelling of lips or face	☐Swelling of lips or face	☐Swelling of lips or face
☐Swelling elsewhere	☐Swelling elsewhere	☐Swelling elsewhere	☐Swelling elsewhere
☐Tight throat	☐ Tight throat	☐ Tight throat	☐ Tight throat
☐Tight chest/ chest pain	☐ Tight chest/ chest pain	☐ Tight chest/ chest pain	☐ Tight chest/ chest pain
☐Shock	☐Shock	☐Shock	☐Shock
☐Faint or dizzy	☐Faint or dizzy	☐Faint or dizzy	☐Faint or dizzy
☐Drop in blood pressure	☐Drop in blood pressure	☐Drop in blood pressure	☐Drop in blood pressure
☐Cough or tightness of throat on inhalation of cooking fumes from seafood	☐Cough or tightness of throat on inhalation of cooking fumes from seafood	☐Cough or tightness of throat on inhalation of cooking fumes from seafood	☐Cough or tightness of throat on inhalation of cooking fumes from seafood
☐Other symptoms from inhalation of cooking fumes from seafood. Please specify:	☐Other symptoms from inhalation of cooking fumes from seafood. Please specify:	☐Other symptoms from inhalation of cooking fumes from seafood. Please specify:	☐Other symptoms from inhalation of cooking fumes from seafood. Please specify:

☐Other symptoms:	☐Other symptoms:	☐Other symptoms:	☐Other symptoms:
3. How long did it take for the allergic reaction to occur?			
○ Less than 10 minutes	○ Less than 10 minutes	○ Less than 10 minutes	○ Less than 10 minutes
○ 10 minutes to 30 minutes	○ 10 minutes to 30 minutes	○ 10 minutes to 30 minutes	○ 10 minutes to 30 minutes
○ 30 minutes to 1 hour	○ 30 minutes to 1 hour	○ 30 minutes to 1 hour	○ 30 minutes to 1 hour
○ 1 to 2 hours	○ 1 to 2 hours	○ 1 to 2 hours	○ 1 to 2 hours
○ 2 hours to 12 hours	○ 2 hours to 12 hours	○ 2 hours to 12 hours	○ 2 hours to 12 hours

○ More than 12	○ More than 12	○ More than 12	○ More than 12
○ Don't know/ Don't remember	○ Don't know/ Don't remember	○ Don't know/ Don't remember	○ Don't know/ Don't remember
4. When was the first time you ate seafood that you can recall or that you have been told about?			
○ < 1 year old	○ < 1 year old	○ < 1 year old	○ < 1 year old
○ 1-5 years old	○ 1-5 years old	○ 1-5 years old	○ 1-5 years old
○ 6-10 years old	○ 6-10 years old	○ 6-10 years old	○ 6-10 years old
○ >10 years old	○ >10 years old	○ >10 years old	○ >10 years old
○ Don't know/ Don't remember	○ Don't know/ Don't remember	○ Don't know/ Don't remember	○ Don't know/ Don't remember
○ Never eaten	○ Never eaten	○ Never eaten	○ Never eaten
5. When was the first time you recognised having an allergic reaction due to eating the seafood?			
○ <1 year old	○ <1 year old	○ <1 year old	○ <1 year old

○ 1-5 years old	○ 1-5 years old	○ 1-5 years old	○ 1-5 years old
○ 6-10 years old	○ 6-10 years old	○ 6-10 years old	○ 6-10 years old
○ >10 years old	○ >10 years old	○ >10 years old	○ >10 years old
○ Don't know/ Don't remember	○ Don't know/ Don't remember	○ Don't know/ Don't remember	○ Don't know/ Don't remember
○ Never eaten	○ Never eaten	○ Never eaten	○ Never eaten
○ The first time eating that	○ The first time eating that	○ The first time eating that	○ The first time eating that
6. What did you do in your most SEVERE allergic episode to seafood?			
○ Go to hospital	○ Go to hospital	○ Go to hospital	○ Go to hospital
○ Use antihistamines	○ Use antihistamines	○ Use antihistamines	○ Use antihistamines
○ Go to pharmacy for	○ Go to pharmacy for	○ Go to pharmacy for	○ Go to pharmacy for
○ Take no action	○ Take no action	○ Take no action	○ Take no action

○ Epinephrine/Adrenaline	○ Epinephrine/Adrenaline	○ Epinephrine/Adrenaline	○ Epinephrine/Adrenaline
Others, please specify:			
7. Have you ever been diagnosed with seafood allergy by a doctor?		○ Yes	○ No
8. How were you diagnosed with these allergies? (tick all that apply)			
Results:	☐Skin Test	☐Blood Test	☐Food Challenge Test (in hospital or in doctors rooms)
	○ Yes	○ Yes	○ Yes
	○ No	○ No	○ No
If possible, please specify:			
9. Do you have other known allergies?			

☐Asthma	○ Yes	○ No
☐Allergic rhinitis (hay fever)	○ Yes	○ No
☐EoE (Eosinophilic oesophagitis)	○ Yes	○ No
☐Allergy to dust mite	○ Yes	○ No
☐Other food allergy *If yes, please specify*:	○ Yes	○ No
10. Do you have any other medical problems at the moment?	○ Yes	○ No
If yes, please specify:		
11. Are you able to eat tinned fish products such as tinned tuna or tinned salmon?		

○ Yes		
○ No		
○ Don't know		
12. Are you now able to eat the seafood that caused you the allergic reaction without any reactions?		
○ Still have adverse reactions		
○ Eat now with no reaction		
○ Haven’t eaten again		
○ Only react sometimes		
○ Don't know		
III. Family history and environmental information		
1. Do food allergies or allergic diseases (eczema, hayfever and asthma) run in your immediate family?	○ Yes	○ No

If yes, please specify:			
2. Are you living in?	○ Inner city	○ Suburb	○ Rural
3. Do any of these apply to you?		○ Smoking	○ Second-hand smoking
4. Do you have any pets?		○ Yes	○ No
If yes, what kind of pets do you have? Please specify: -----End of the questionnaire-----			

Name of the interview/clinician:

Date of interview:

9.3 Appendix C -Buffer and Solutions

Preparation of 2L worth of 1x PBS (Phosphate buffered saline)

Compound	Weight
Sodium Chloride (NaCl)	16g
Potassium Chloride (KCl)	0.4g
Sodium Phosphate dibasic (Na_2HPO_4)	2.88g
Potassium dihydrogen phosphate (KH_2PO_4)	0.48g

pH adjusted to pH7.2 using 5M NaCl and filtered through 0.2µm membrane.

PBS-T wash buffer

1xPBS with 0.05% Tween-20

SDS-PAGE Reagents

Solution B

Tris-HCl (2 M, pH 8.8)..... 75 ml

10% SDS in Milli-Q H_2O ..... 4 ml

Milli-Q H_2O ..... 21 ml

Solution C

Tris-HCl (1 M, pH 6.8) ..... 50 ml

10% SDS in Milli-Q H_2O ..... 4 ml

Milli-Q H_2O ..... 46 ml

5 x Protein sample loading dye

Tris-HCl (1 M, pH 6.8) ..... 0.6 ml

50% Glycerol 5ml

10% SDS ..... 2 ml

Dithiothreitol (1 M) ..... 1 ml

1% Bromophenol blue ..... 1 ml

Milli-Q H_2O ..... up to 10 ml

12% SDS-PAGE gel recipe

Resolving gel

40% Acrylamide ..... 6 ml

Solution B ..... 5 ml

Milli-Q H_2O ..... 8.9 ml

10% Ammonium persulphate ..... 100 µl

TEMED ..... 10 µl

Stacking gel

40% 29:1 Acrylamide ..... 0.93 ml

Solution C ..... 2.5 ml

Milli-Q H_2O ..... 6.5 ml

10% Ammonium persulphate ..... 100 µl

TEMED ..... 10 µl

1 x Gel Electrophoresis running buffer

Tris ---------- 3 g/l

Glycine ---------- 14.4 g/l

SDS ---------- 1 g/l

Milli-Q H_2O ---------- up to 1 l

SDS-PAGE gel destaining solution

Methanol (AR grade) ..... 500 ml

Glacial acetic acid ..... 100 ml

Milli-Q H_2O ..... 400 ml

Immunoblotting Buffers

Transfer buffer

Tris ..... 1.164 g

Glycine ..... 0.58 g

10% SDS ..... 750 µl

Methanol ..... 40 ml

Milli-Q H_2O ..... up to 200 ml

Protein Purification Buffers

Phosphate buffer

Na_2HPO_4 ..... 1.44 g

KH_2PO_4 ..... 0.24 g

NaCl ..... 17.5 g

KCl ..... 0.2 g

Milli-Q H_2O ..... 1 l

Mix to dissolve and adjust pH to 7.4.

Stock solution

Imidazole ..... 0.068 g

Phosphate buffer ..... 50 ml

Equilibration buffer

Stock solution ..... 1 ml

Milli-Q H_2O ..... up to 50 ml

Wash buffer

Stock solution ..... 5 ml

Milli-Q H_2O ..... up to 50 ml

Elution buffer

Stock solution ..... 30 ml

Milli-Q H_2O ..... up to 50 ml

9.4 Appendix D – Supplementary Tables and Figures

9.4.1 Table D3.1 Reported clinical adverse reactions caused by food consumption in adults (*n*=6,563) in descending order of prevalence.

Symptom	Response	
	n	%
Diarrhoea	**4,153**	**16.7**
Nausea or vomiting	**3,047**	**12.2**
Stomach pain	**2,650**	**10.6**
Hives	2,317	9.3
Sneezing	1,954	7.8
Odd taste in mouth	1,795	7.2
Nasal congestion or a running nose	1,708	6.9
Slight, dry cough	1,655	6.6
Trouble swallowing	1,299	5.2
Itchy mouth or ear canal	1,056	4.2
Chest pain	831	3.3
Shortness of breath or wheezing	600	2.4
Drop in blood pressure	426	1.7
Eczema	402	1.6
Redness of the skin or around the eyes	349	1.4
Swelling of the lips. Tongue and/or throat	307	1.2
Weak pulse	224	0.9
Loss of consciousness	142	0.6
Total	**24,915**	**100.0**

9.4.2 Table D3.2 Causative food groups evoking adverse reactions in this survey reported from 6,563 affected participants in descending order of prevalence.

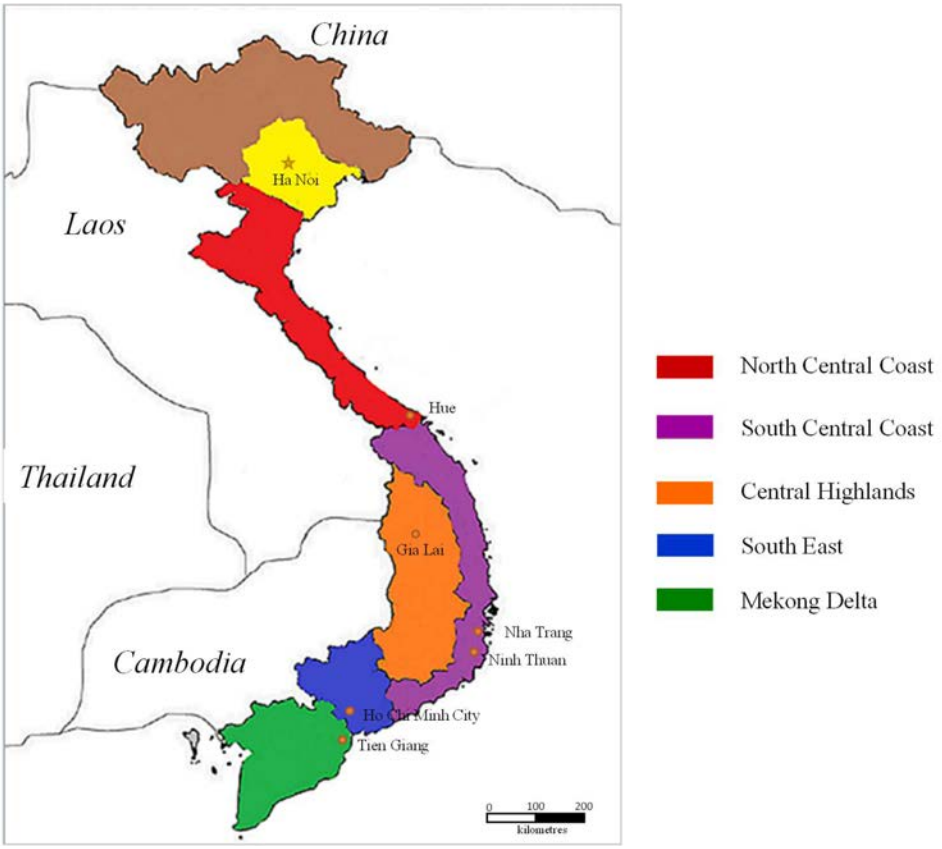
Food group	n	% among food groups	% among participants
Crustacean	**1,835**	**28.0**	**24.9**
Fish	**995**	**15.2**	**13.5**
Molluscs	**994**	**15.1**	**13.5**
Other foods	750	11.4	10.2
Milk	701	10.7	9.5
Beef	504	7.7	6.8
Wheat	372	5.7	5.0
Peanut	371	5.7	5.0
Tree nut	337	5.1	4.6
Egg	279	4.3	3.8
Soy	241	3.7	3.3
Total	**7,379**	**100.0**	**100.0**

9.4.3 Table D3.3 The number of participants visited health service by clinical symptoms and the percentage of participants seek medical advice by clinical symptoms (in descending order) among participants reported clinical symptoms caused by food consumption (*n*=6,563).

Symptom	Number of participants seek medical advice (n)	Percentage of participants seek medical advice
Loss of consciousness	**51**	**94.4**
Redness of the skin or around eyes	**119**	**84.4**
Eczema	**130**	**75.1**
Weak pulse	71	73.2
Drop in blood pressure	116	64.8
Swelling of the lips, tongue and/or throat	87	63.5
Shortness of breath or wheezing	148	57.1
Hives	609	55.9
Chest pain	159	43.7
Itchy	203	39.3
Trouble swallowing	225	38.5
Stomach pain	435	38.1
Slight, dry cough	273	38.1
Nausea or vomiting	515	37.5
Nasal congestion or a runny nose	285	36.1
Sneezing	319	35.6
Odd taste in mouth	262	31.5
Diarrhoea	578	30.9

9.4.4 Table D3.4 Weighted data of FA survey in Vietnamese adults

	Adults			Adults (adjusted)		
	Male	**Female**	**Total**	**Male**	**Female**	**Total**
Number of cases (n)	2,955	6,084	9,039	4,519	4,520	9,039
Reported adverse reactions to food consumption	2,028	4,535	6,563	3,101	3,369	6,470
Clinical symptom repetition	901	2,248	3,149	1,378	1,670	3,048
Self-reported FA	291	838	1,629	656	891	1,547
Approaching medical services for FA diagnosis	147	339	617	286	319	605
Doctor-diagnosed FA	118	311	527	222	284	506



9.4.5 Figure D3.1 Map of study locations in inlands of Vietnam.

The survey was conducted at different regions from three participating universities: Gia Lai, Nha Trang, Ninh Thuan and Ho Chi Minh City and Mekong Delta. Number of participants from each region: North Central Coast (n=91), South Central Coast (n=3,753), Central Highlands (n=617), South East (n=4,249) and Mekong Delta (n=329). The figure was generated from Maphill (www.maphill.com/vietnam), modified and annotated with Microsoft® PowerPoint for Windows.

9.4.6 Table D5.1 List of allergens and protein extracts used in the study

No.	Class	Common name	Scientific name	nTM	rTM	rMLC	rSCP	rHC	nPV	Heated extract	Raw extract
1	Crustacean	Vannamei	*Litopeaneaus vannamei*	x		x	x	x		x	
2		Black tiger prawn	*Penaeus monodon*	x						x	
3		Blue swimmer crab	*Portunus pelagicus*	x						x	
4		Mud crab	*Scylla serrata*							x	
5		Soft shell crab	*Scylla serrata*							x	
6	Mollusk	Clam	*Meretrix lyrata*							x	
7		Oyster	*Crassostrea gigas sp.*	x						x	x
8		Scallop	*Chlamys nobilis*							x	
9		Squid	*Loligo formosa*	x						x	
10		Cuttlefish	*Sepia pharaonis*	x						x	
11		Octopus	*Octopus aegina*	x						x	
12		Abalone	*Haliotidae*	x						x	
13		Snail	*Cerithidea obtusa*							x	
14	Bony fish	Tilapia	*Oreochromis sp*		x					x	
15		Basa fish	*Pangasius hypophthalmus*						x	x	
16		Atlantic cod	*Gadus morhua*						x	x	
17		Barramundi	*Lates calcarifer*						x	x	
18		Yellowtail scad	*Atule mate*							x	

19		Goby fish	*Pseudapocryptes elongatus*							x	
20		Atlantic salmon	*Salmon salar*						x	x	
21		Yellowfin tuna	*Thunnus albacares*							x	
22		Indian Mackerel	*Rastrelliger kanagurta*							x	
23		Featherback fish	*Notopterus notopterus*							x	
24		Asian swamp eel	*Fluta albas*							x	
25		Round scad	*Decapterus punctatus*							x	
26		Walking catfish	*Clarias macrocephalus*							x	
27		Blue mackerel	*Scomber australasicus*							x	
28	**Other allergen sources**	Anisakis	*Anisakis simplex*		*x*						
29		Mealworm	*Tenebrio molitor*							x	x
30		Cockroach	*Blattella germanica*		x					x	x
31		HDM	*Dermatophagoides pteronyssinus*		x						x

'x': included in the study; nTM, in-house purified tropomyosin; rMLC, recombinant myosin light chain; rSCP, recombinant sasco plasmic calcium-binding proteins; rHC, recombinant hemocyanin; rTM, recombinant tropomyosin; nPV, in-house purified parvalbumin.

9.4.7 Table D5.2 Demographic data, clinical history of patients in the study

No.	Sex	Age (years)	Species implicated	History of clinical implications	Clinical reaction onset (hours)	Other allergic conditions	ImmunoCAP Prawn
1	M	24	Crab	I, W, SL, TT	0.5	AR	**1.44**
2	M	30	Prawn, crab	H, I, SL	0.5		**0.19**
3	M	37	Mollusk	H, I, SL, R	1		**0.1**
4	F	39	Prawn, clam	NV, H, I, W, LT, SL	0.25	AR	**4.66**
5	M	13	Prawn	H, I, R, C, SL	0.1	AR	**0.28**
6	M	32	Prawn	H, I, W	2		**1.74**
7	M	34	Prawn, squid	I, W, SL	0.5	AR	**32**
8	M	14	Prawn, crab	ITM, I, C	0.1	A, AR	**40.3**
9	F	56	Prawn, crab	I, SL	2		**0.87**
10	F	35	Crab	H, W, S	1	AR	**20.4**
11	M	8	Crab	C	0.5		**0.34**
12	M	25	Prawn, crab	NV, H, I, TCP	1	AR	**0.03**
13	F	17	Prawn, crab	NV, AP	1		**3.69**
14	M	24	Prawn, crab	H, I			**1.54**
15	M	29	Prawn, crab	NV, AP, H, I, R, C, SL, TCP, F			**38.4**
16	F	59	Prawn, crab	H, I, LT, SL, TCP	0.5	CE	**0.05**
17	F	24	Prawn	NV, AP, I	0.1		**16**

18	F	36	Prawn, crab	D, AP, ITM, H, I	0.5	AR	**0.2**
19	F	39	Mollusk	H, I	0.5	CE	**3.88**
20	M	29	Prawn, crab	NV, D, AP, H, I, LT, S, F, DBP	1		**0.22**
21	F	44	Prawn, crab	NV, D, AP, I, H, I, R, W, LT, SL, TT, F	0.1		**43.6**
22	F	22	Prawn, crab, outer skin of prawn, cricket	NV, ITM, H, I, W, LT, SL		AR	**3.7**
23	M	28	Fish, sea snail	NV, D, AP, ITM, H, I, W, SL, SE	0.5	AR, OFA	**2.16**
24	M	30	Outer skin of prawn, crab	AP, ITM, H, I, W, SL	1	A, AR, OFA	**2.49**
25	M	22	Prawn, crab	ITM, H, I, SL	0.5	CE	**13.1**
26	F	53	Prawn, crab	ITM, H, I, C, LT, SL, TCP	0.1	AR, AC, OFA	**0.19**
27	F	29	Prawn, cockroach, HDM	H, I	0.5		**0.12**
28	M	25	Prawn, crab	AP, I	0.5		**2.86**
29	F	39	Mollusk	H			**9.29**
30	M	46	Prawn	H, I, SL, DBP	4		**8.75**
31	F	29	Oyster, mollusk	H, I, C, LT, SL, TT, TCP		CE	**2.23**

32	F	14	Prawn outer skin, crab	H, I			**31.9**
33	F	28	Prawn				**3.92**
34	F	37	Prawn, crab, lobster	NV, D, AP, ITM, H, I, R, LT, SL		CE	**0.5**

F, Female; M, Male; NV, Nausea/vomiting; D, Diarrhea; AP, Abdominal pain; ITM, Itchy throat or mouth; H, Hives/urticaria; E, Flare of eczema; I, Itching; R, Redness of the skin; C, Congested or running nose; W, Wheezing; C, Coughing; LT, Lip or tongue tinging; SL, swelling of lips or face; SE, Swelling elsewhere; TT, tight throat; TCP, tight chest/chest pain; S, Shock; F, Faint/dizzy; DBP, a drop in blood pressure; A, Asthma; AR, Allergic rhinitis; AC, Allergic conjunctivitis; CE, childhood eczema; OFA, Other food allergies.

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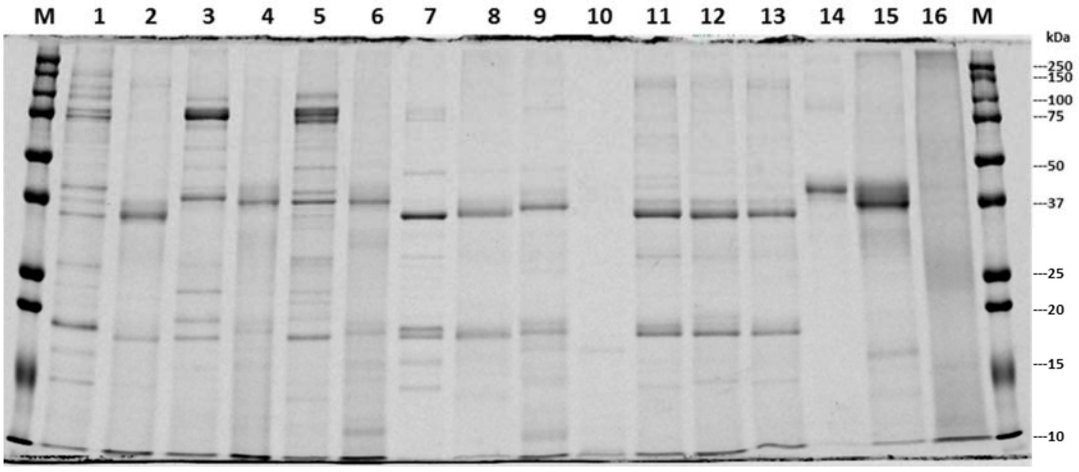
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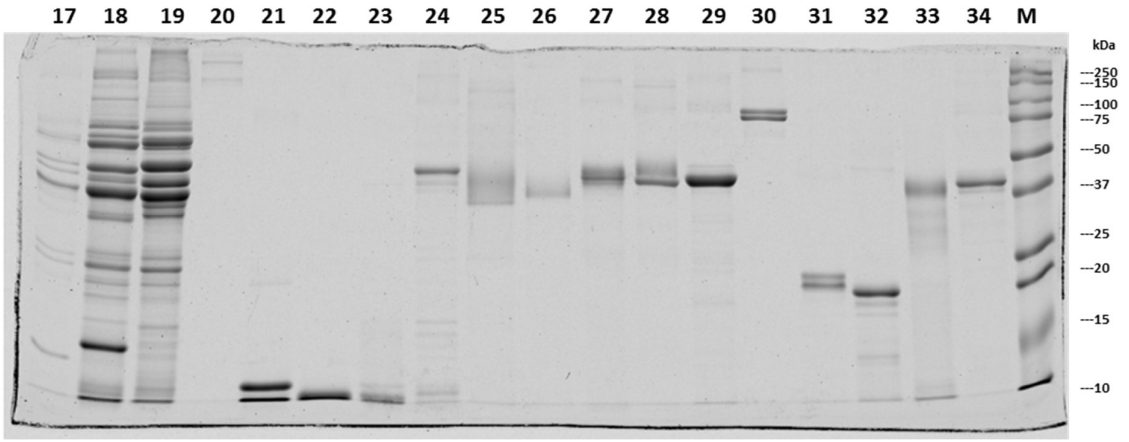
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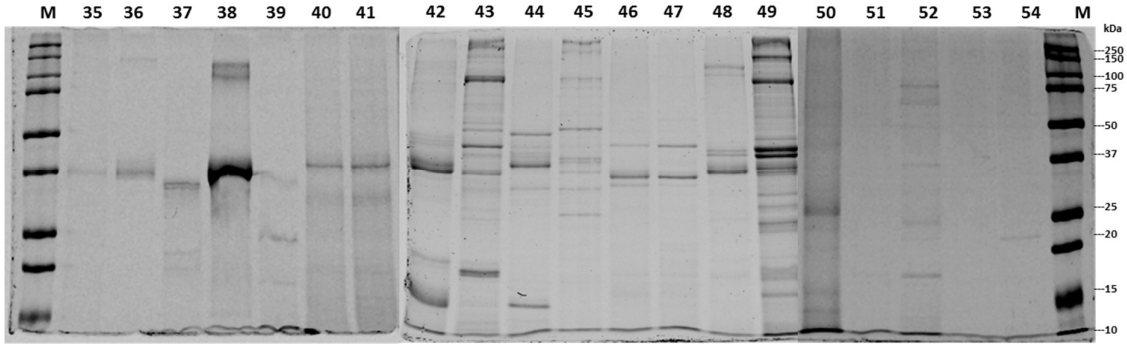
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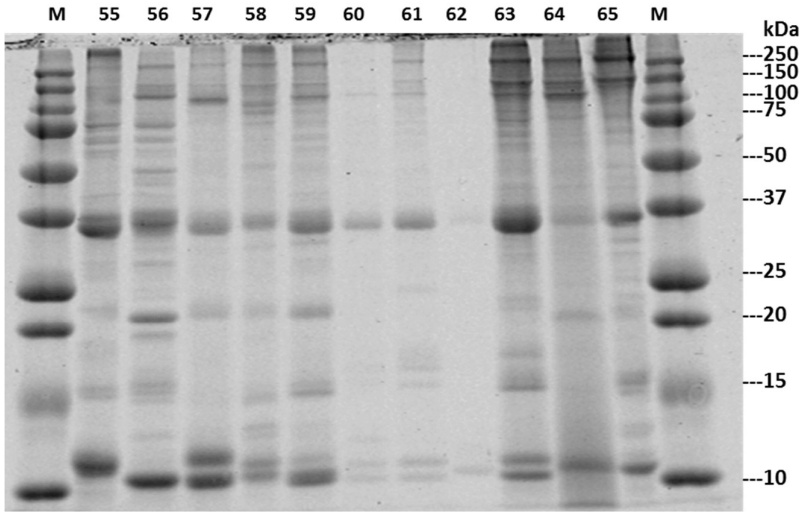
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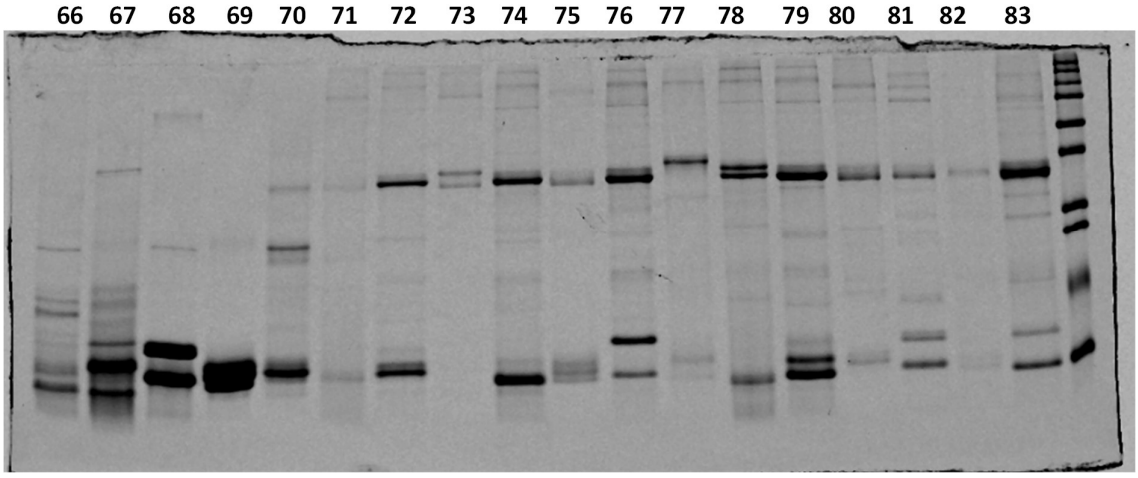
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1	Black tiger prawn R	21	nPV Barramundi	41	Softshell crab H	61	Snake-skin gourami
2	Black Tiger prawn H	22	Basa nPV	42	Scallop H	62	Walking catfish
3	Blue swimmer crab R	23	Salmon nPV	43	Scallop R	63	Asian swamp eel
4	Blue swimmer crab H	24	Tilapia nTM	44	Cuttlefish H	64	Tinfoil barb fish
5	Mud crab R	25	Abalone nTM	45	Cuttlfish R	65	Blue-barred parrot fish
6	Mud crab H	26	Oyster nTM	46	Clam H	66	nPV Yellowfin tuna
7	Vannamei R	27	HDM nTM	47	Clam R	67	nPV Atlantic salmon
8	Vannamei H	28	Cockroach rTM	48	Abalone H	68	nPV Barramundi
9	Yabby prawn H	29	Anisakis nTM	49	Abalone R	69	nPV Basa
10	Mealworm H	30	Vannamei rHC	50	HDM Dp raw	70	nPV Cod
11	Banana prawn H	31	Vannamei rSCP	51	Mealworm H	71	Indian Mackerel
12	Endeavour prawn H	32	Vannamei rMLC	52	Mealworm R	72	Yellowfin tuna
13	King prawn H	33	Squid nTM	53	Cockroach H	73	Sea mullet
14	Black tiger prawn nTM	34	Vannamei nTM	54	Cockroach R	74	Barramundi
15	Blue swimmer crab nTM	35	Oyster H	55	Featherback fish H	75	Snapper
16	HDM Dp H	36	Oyster R	56	Yellowtail scad	76	Salmon
17	Crocodile R	37	Octopus nTM	57	Climbing perch	77	Spanish mackerel
18	Beef R	38	Cuttlefish nTM	58	Red tilaipa	78	Coral trout
19	Chicken R	39	Snail H	59	Pointed tail goby	79	Cod
20	Fish collagen	40	Soft shell crab R	60	Sand goby	80	Basa
						81	nPV Barramundi
						82	nPV Salmon
						83	nPV Tuna

9.4.8 Figure D5.1 Protein profiles of the investigated seafood and allergens. H: heated extract, R: raw extract, nTM: natural tropomyosin, rTM: recombinant tropomyosin, nPV: natural parvalbumin, rHC: recombinant hemocyanin, rSCP: recombinant sasco-plasmic calcium-binding protein, rMLC: recombinant myosin light chain, HDM: house dust mite.

9.4.9 Table D6.1 Demographic data and clinical history of seafood allergic patients in this study

No.	ID	Sex	Age (years)	Ethnicity	Pathway	History of clinical reactions	Species implicated	Clinical reaction onset (hours)	Other allergic conditions
1	JCU106	F	51	Caucasian	Ingestion	NV, H, I, R, W, LT, SL	Prawns, crabs	0.5	A, AR, DM
2	JCU108	M	33	Asian	Ingestion	NV, F	Prawns	>12	
3	JCU139	M	37	Caucasian	Ingestion	LT, TT	Prawns, calamari, clams	0.2	A, AR, DM, OFA
4	JCU113	F	28	Asian	Ingestion	R, LT, SL	Prawns, crabs, mussels	0.2	AR, CE
5	JCU114	M	70	Caucasian	Ingestion	NV	Prawn, freshwater crayfish	1 - 2	A, HDM
6	JCU115	F	34	Caucasian	Ingestion	ITM, H, LT, SL, TT, TCP	Prawns, lobster, calamari	0.2	A, AR, OFA
7	JCU116	F	54	Caucasian	Ingestion	H, LT, SL, TT	Prawns, crabs, bugs	0.5	A, AR
8	JCU125	F	30	Asian	Ingestion	NV, AP, ITM, I, R, SL	Prawns	0.2	A
9	JCU132	M	30	Caucasian	Ingestion	NV, H, W, C	Prawns	0.5	AR, HDM
10	JCU138	M	62	Caucasian	Contact	R	Prawns	>12	A, AR, DM, OFA
11	JCU141	F	52	Caucasian	Ingestion	NV, D, AP	Prawns, crabs, lobsters	2 - 12	
12	JCU161	F	46	Caucasian	Ingestion	NV, F	Prawns	2 - 12	A, AR, OFA
13	JCU128	F	40	Caucasian	Ingestion	NV, ITM, LT, TCP	Prawns, calamari	0.5	
14	JCU160	F	20	Caucasian	Ingestion	I, R, SL	Prawns	>12	
15	JCU162	M	70	Caucasian	Contact	ITM, H, R, SL	Prawns, octopus	0.2	A, AR, OFA
16	JCU163	F	26	Caucasian	Ingestion	NV, ITM, I, TCP	Prawns, bugs, oyster	0.5	AR, DM, CE
17	JCU164	M	32	Asian	Ingestion	ITM, H, R, TCP	Prawns, crabs, lobster, bugs	0.5	AR, DM, OFA
18	JCU165	F	26	Asian	Ingestion	ITM, H, I, R, LT, SL, TT, TCP	Prawns, calamari	0.5	AR, DM, CE

19	JCU166	F	30	Caucasian	Ingestion	H, R, SE	Prawns	0.5	A, AR, DM, CE
20	JCU169	M	29	Latin American	Ingestion	ITM, H, I, R, LT	Prawns, crabs, lobster	0.2	A, AR, DM, CE, OFA
21	JCU171	F	43	Caucasian	Ingestion	NV, ITM, I, R, SL, TCP	Prawns, freshwater crayfish, scallop, oyster	0.5	AR, DM
22	JCU172	F	71	Caucasian	Ingestion	NV, D, AP, I, TCP	Prawns, lobster, oyster	1	AR
23	JCU175	F	61	Caucasian	Ingestion	NV, AP, ITM, H, W, C, LT, SL, TT, TCP	Prawns, bugs	0.5	AR, DM
24	JCU176	F	24	Asian	Ingestion	I, R	Prawns	0.5	AR, DM
25	JCU177	M	58	Caucasian	Ingestion	NV, ITM, I, SL, SE	Prawns	0.5	
26	JCU178	M	35	Caucasian	Ingestion	ITM, H, I, R, C, LT	Prawns	0.5	A, AR, CE
27	JCU179	M	28	Asian	Ingestion	ITM, H, I	Prawns, crabs, lobster, bugs	1	DM, CE
28	JCU180	M	26	Caucasian	Ingestion	ITM, C, LT	Prawns, crabs	0.5	
29	JCU148	F	17	Caucasian	Contact	I	Prawns, crabs, bugs, calamari, scallops	0.5	CE
30	JCU112	F	33	Caucasian	Ingestion	ITM, E, R, LT, SL, SE, TT	Prawns, calamari	0.2	AR, DM
31	JCU119	F	31	Caucasian	Ingestion	NV, ITM, I, SL	Prawns, crabs	0.2	A, AR, DM
32	JCU131	F	45	Caucasian	Contact	ITM, R, LT, SL	Prawns	0.2	AR, DM, CE
33	JCU168	F	49	Caucasian	Ingestion	NV, D, AP	Prawns	1	A, AR, DM
34	JCU174	F	29	Caucasian	Ingestion	NV, ITM, I, R	Prawns	0.5	AR, DM, OFA
35	JCU126	F	49	Caucasian	Ingestion	NV, D, AP, F	Prawns, squid, scallops	0.5	A, AR, HDM, CE, OFA
36	JCU144	F	50	Caucasian	Ingestion	ITM, H, W, C, LT, TT, TCP	Atlantic salmon, mackerel, whitings, tuna, prawns, crabs, lobsters,	0.2	A, HDM, OFA

							calamari, scallops, oysters, mussels		
37	JCU170	M	78	Caucasian	Ingestion	ITM, C, SL, TT	Barramundi, prawns	1	A
38	JCU133	F	66	Caucasian	Ingestion	ITM, H, I, R, LT, TT	Barramundi, mackerel, whitings	0.2	
39	JCU140	M	51	Caucasian	Ingestion	SL, SE	Atlantic salmon	0.2	
40	JCU147	M	43	Caucasian	Contact	R, SE, TT		>12	A
41	JCU173	F	57	Caucasian	Ingestion	NV, ITM, I, C, LT, SL, TT, TCP	Tuna, mullet, breams	0.5	CE

F, Female; M, Male; NV, Nausea/vomiting; D, Diarrhea; AP, Abdominal pain; ITM, Itchy throat or mouth; H, Hives/urticaria; E, Flare of eczema; I, Itching; R, Redness of the skin; C, Congested or running nose; W, Wheezing; C, Coughing; LT, Lip or tongue tinging; SL, swelling of lips or face; SE, Swelling elsewhere; TT, tight throat; TCP, tight chest/chest pain; S, Shock; F, Faint/dizzy; A, Asthma; AR, Allergic rhinitis; HDM, House dust mite allergy; CE, childhood eczema; OFA, Other food allergies.

9.4.10 Table D6.2 List of allergens and protein extracts used in the study

No.	Class	Common name	Scientific name	rTM	nTM	rMLC	rSCP	rHC	nPV	Heated extract	Raw extract
1	Crustacean	Vannamei prawn	*Litopeaneaus vannamei*		x	x	x	x		x	
2		Black tiger prawn	*Penaeus monodon*		x					x	
3		Blue swimmer crab	*Portunus pelagicus*		x					x	
4		Mud crab	*Scylla serrata*		x					x	
5		King prawn	*Melicertus latisulcatus*							x	
6		Banana prawn	*Fenneropenaeus merguiensis*							x	
7		Endeavour prawn	*Metapenaeus endeavour*							x	
8		Yabby	*Cherax destructor*							x	
9	Mollusk	Clam	*Meretrix lyrata*							x	
10		Oyster	*Crassostrea gigas sp.*		x					x	x
11		Scallop	*Chlamys nobilis*							x	
12		Squid	*Loligo formosa*		x					x	
13		Cuttlefish	*Sepia pharaonis*		x					x	
14		Octopus	*Octopus aegina*		x					x	
15		Abalone	*Haliotidae*		x					x	

16	Bony fish	Tilapia	*Oreochromis sp*	x						x	
17		Basa fish	*Pangasius hypophthalmus*						x	x	
18		Atlantic cod	*Gadus morhua*						x	x	
19		Barramundi	*Lates calcarifer*						x	x	
20		Atlantic salmon	*Salmon salar*						x	x	
21		Yellowfin tuna	*Thunnus albacares*						x	x	
22		Indian mackerel	*Rastrelliger kanagurta*							x	
23		Snapper	*Lutjanus campechanus*							x	
24		Sea mullet	*Mugil cephalus*							x	
25		Cobia	*Rachycentron canadum*							x	
26		Coral trout	*Plectropomus leopardus*							x	
27		John dory	*Zeus faber*							x	
28		Spanish mackerel	*Scomberomorini*							x	
29	Other allergen	Anisakis	*Anisakis siimplex*	x							
30		Cockroach	*Blattella germanica*	x						x	x
31		HDM	*Dermatophagoides pteronyssinus*	x							x

‘x’: included in the study; nTM, in-house purified tropomyosin; nPV, in-house purified parvalbumin; rMLC, recombinant myosin light chain; rSCP, recombinant sasco plasmic calcium-binding proteins; rHC, recombinant hemocyanin; nPV, in-house purified parvalbumin.

9.4.11 Table D6.3 Clinical data of the control group

9.4.12 Table D6.4 Clinical information of the positive controls

Patient ID	Implicated species	Other food allergy	Shrimp RAST (kUA/L)	Lobster RAST	Crab RAST	Oyster RAST	Mussel RAST	Squid RAST	Cod RAST	Tuna RAST	Tuna SPT	Salmon	SPT Salmon (mm)	HDM RAST
PC1	Flounder, prawn, crab	No	9.5	9.43	2.42	0.92	0.61	-	0.01	0.12	-	-	-	14.1
PC2	Crustaceans/molluscs	No	3.63	3.43	5.55	1.11	1.09	-	0.03	0.18	0.07	-	-	5.03
PC3	Barramundi	No	-	-	-	-	-	-	-	-	-	9.01	8	-
PC4	White fish	No	-	-	-	-	-	-	-	-	-	3.43	11	-
PC5	White fish	No	-	-	-	-	-	-	-	-	-	11.5	6	-

'-' indicates not determined.

9.5 Appendix E – Statistical analysis

9.5.1 Table E5.1 The Dunn's multiple comparison test of the IgE reactivity to mollusk proteins

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
Abalone vs. Snail	104	Yes	****	<0.0001	A-B
Abalone vs. Octopus	57	No	ns	0.1337	A-C
Abalone vs. Cuttlefish	52.5	No	ns	0.2617	A-D
Abalone vs. Squid	167	Yes	****	<0.0001	A-E
Abalone vs. Clam	-43.5	No	ns	0.8757	A-F
Abalone vs. Scallop	35.5	No	ns	>0.9999	A-G
Abalone vs. Oyster	167.5	Yes	****	<0.0001	A-H
Snail vs. Octopus	-47	No	ns	0.5593	B-C
Snail vs. Cuttlefish	-51.5	No	ns	0.3019	B-D
Snail vs. Squid	63	No	ns	0.0508	B-E
Snail vs. Clam	-147.5	Yes	****	<0.0001	B-F
Snail vs. Scallop	-68.5	Yes	*	0.0195	B-G
Snail vs. Oyster	63.5	Yes	*	0.0467	B-H
Octopus vs. Cuttlefish	-4.5	No	ns	>0.9999	C-D
Octopus vs. Squid	110	Yes	****	<0.0001	C-E
Octopus vs. Clam	-100.5	Yes	****	<0.0001	C-F
Octopus vs. Scallop	-21.5	No	ns	>0.9999	C-G
Octopus vs. Oyster	110.5	Yes	****	<0.0001	C-H
Cuttlefish vs. Squid	114.5	Yes	****	<0.0001	D-E
Cuttlefish vs. Clam	-96	Yes	****	<0.0001	D-F
Cuttlefish vs. Scallop	-17	No	ns	>0.9999	D-G
Cuttlefish vs. Oyster	115	Yes	****	<0.0001	D-H
Squid vs. Clam	-210.5	Yes	****	<0.0001	E-F
Squid vs. Scallop	-131.5	Yes	****	<0.0001	E-G
Squid vs. Oyster	0.5	No	ns	>0.9999	E-H
Clam vs. Scallop	79	Yes	**	0.0026	F-G
Clam vs. Oyster	211	Yes	****	<0.0001	F-H
Scallop vs. Oyster	132	Yes	****	<0.0001	G-H

9.5.2 Table E5.2 The Dunn's multiple comparison test result of the IgE reactivity to indoor allergens

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
HDM R vs. rTM HDM	126.5	Yes	****	<0.0001	A-B
HDM R vs. Cockroach R	165.5	Yes	****	<0.0001	A-C
HDM R vs. Cockroach H	72.5	Yes	***	0.001	A-D
HDM R vs. rTM Cockroach	146.5	Yes	****	<0.0001	A-E
HDM R vs. Mealworm R	128	Yes	****	<0.0001	A-F
HDM R vs. Mealworm H	26	No	ns	>0.9999	A-G
rTM HDM vs. Cockroach R	39	No	ns	0.6001	B-C
rTM HDM vs. Cockroach H	-54	No	ns	0.0511	B-D
rTM HDM vs. rTM Cockroach	20	No	ns	>0.9999	B-E
rTM HDM vs. Mealworm R	1.5	No	ns	>0.9999	B-F
rTM HDM vs. Mealworm H	-100.5	Yes	****	<0.0001	B-G
Cockroach R vs. Cockroach H	-93	Yes	****	<0.0001	C-D
Cockroach R vs. rTM Cockroach	-19	No	ns	>0.9999	C-E
Cockroach R vs. Mealworm R	-37.5	No	ns	0.7409	C-F
Cockroach R vs. Mealworm H	-139.5	Yes	****	<0.0001	C-G
Cockroach H vs. rTM Cockroach	74	Yes	***	0.0007	D-E
Cockroach H vs. Mealworm R	55.5	Yes	*	0.0386	D-F
Cockroach H vs. Mealworm H	-46.5	No	ns	0.19	D-G
rTM Cockroach vs. Mealworm R	-18.5	No	ns	>0.9999	E-F

rTM Cockroach vs. Mealworm H	-120.5	Yes	****	<0.0001	E-G
Mealworm R vs. Mealworm H	-102	Yes	****	<0.0001	F-G

9.5.3 Table E5.3 The Dunn's multiple comparison test result of the IgE reactivity to fish extracts

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
Basa fish vs. Barramundi	76.5	No	ns	>0.9999	A-B
Basa fish vs. Atlantic cod	41	No	ns	>0.9999	A-C
Basa fish vs. Tilapia	9.5	No	ns	>0.9999	A-D
Basa fish vs. Yellowfin tuna	76	No	ns	>0.9999	A-E
Basa fish vs. Indian mackerel	185	Yes	****	<0.0001	A-F
Basa fish vs. Asian swamp eel	137	Yes	**	0.0065	A-G
Basa fish vs. Blue mackerel	195.5	Yes	****	<0.0001	A-H
Basa fish vs. Featherback fish	169.5	Yes	****	<0.0001	A-I
Basa fish vs. Walking catfish	206.5	Yes	****	<0.0001	A-J
Basa fish vs. Round scad	143.5	Yes	**	0.0029	A-K
Basa fish vs. Goby fish	-42.5	No	ns	>0.9999	A-L
Basa fish vs. Yellowtail scad	43.5	No	ns	>0.9999	A-M
Basa fish vs. Atlantic salmon	-2	No	ns	>0.9999	A-N
Barramundi vs. Atlantic cod	-35.5	No	ns	>0.9999	B-C
Barramundi vs. Tilapia	-67	No	ns	>0.9999	B-D
Barramundi vs. Yellowfin tuna	-0.5	No	ns	>0.9999	B-E
Barramundi vs. Indian mackerel	108.5	No	ns	0.151	B-F
Barramundi vs. Asian swamp eel	60.5	No	ns	>0.9999	B-G
Barramundi vs. Blue mackerel	119	No	ns	0.0511	B-H

Barramundi vs. Featherback fish	93	No	ns	0.6387	B-I
Barramundi vs. Walking catfish	130	Yes	*	0.0149	B-J
Barramundi vs. Round scad	67	No	ns	>0.9999	B-K
Barramundi vs. Goby fish	-119	No	ns	0.0511	B-L
Barramundi vs. Yellowtail scad	-33	No	ns	>0.9999	B-M
Barramundi vs. Atlantic salmon	-78.5	No	ns	>0.9999	B-N
Atlantic cod vs. Tilapia	-31.5	No	ns	>0.9999	C-D
Atlantic cod vs. Yellowfin tuna	35	No	ns	>0.9999	C-E
Atlantic cod vs. Indian mackerel	144	Yes	**	0.0027	C-F
Atlantic cod vs. Asian swamp eel	96	No	ns	0.4903	C-G
Atlantic cod vs. Blue mackerel	154.5	Yes	***	0.0007	C-H
Atlantic cod vs. Featherback fish	128.5	Yes	*	0.0178	C-I
Atlantic cod vs. Walking catfish	165.5	Yes	***	0.0001	C-J
Atlantic cod vs. Round scad	102.5	No	ns	0.2698	C-K
Atlantic cod vs. Goby fish	-83.5	No	ns	>0.9999	C-L
Atlantic cod vs. Yellowtail scad	2.5	No	ns	>0.9999	C-M
Atlantic cod vs. Atlantic salmon	-43	No	ns	>0.9999	C-N
Tilapia vs. Yellowfin tuna	66.5	No	ns	>0.9999	D-E
Tilapia vs. Indian mackerel	175.5	Yes	****	<0.0001	D-F
Tilapia vs. Asian swamp eel	127.5	Yes	*	0.0199	D-G
Tilapia vs. Blue mackerel	186	Yes	****	<0.0001	D-H
Tilapia vs. Featherback fish	160	Yes	***	0.0003	D-I

Tilapia vs. Walking catfish	197	Yes	****	<0.0001	D-J
Tilapia vs. Round scad	134	Yes	**	0.0093	D-K
Tilapia vs. Goby fish	-52	No	ns	>0.9999	D-L
Tilapia vs. Yellowtail scad	34	No	ns	>0.9999	D-M
Tilapia vs. Atlantic salmon	-11.5	No	ns	>0.9999	D-N
Yellowfin tuna vs. Indian mackerel	109	No	ns	0.1437	E-F
Yellowfin tuna vs. Asian swamp eel	61	No	ns	>0.9999	E-G
Yellowfin tuna vs. Blue mackerel	119.5	Yes	*	0.0484	E-H
Yellowfin tuna vs. Featherback fish	93.5	No	ns	0.6115	E-I
Yellowfin tuna vs. Walking catfish	130.5	Yes	*	0.0141	E-J
Yellowfin tuna vs. Round scad	67.5	No	ns	>0.9999	E-K
Yellowfin tuna vs. Goby fish	-118.5	No	ns	0.0539	E-L
Yellowfin tuna vs. Yellowtail scad	-32.5	No	ns	>0.9999	E-M
Yellowfin tuna vs. Atlantic salmon	-78	No	ns	>0.9999	E-N
Indian mackerel vs. Asian swamp eel	-48	No	ns	>0.9999	F-G
Indian mackerel vs. Blue mackerel	10.5	No	ns	>0.9999	F-H
Indian mackerel vs. Featherback fish	-15.5	No	ns	>0.9999	F-I
Indian mackerel vs. Walking catfish	21.5	No	ns	>0.9999	F-J
Indian mackerel vs. Round scad	-41.5	No	ns	>0.9999	F-K
Indian mackerel vs. Goby fish	-227.5	Yes	****	<0.0001	F-L
Indian mackerel vs. Yellowtail scad	-141.5	Yes	**	0.0037	F-M
Indian mackerel vs. Atlantic salmon	-187	Yes	****	<0.0001	F-N

Asian swamp eel vs. Blue mackerel	58.5	No	ns	>0.9999	G-H
Asian swamp eel vs. Featherback fish	32.5	No	ns	>0.9999	G-I
Asian swamp eel vs. Walking catfish	69.5	No	ns	>0.9999	G-J
Asian swamp eel vs. Round scad	6.5	No	ns	>0.9999	G-K
Asian swamp eel vs. Goby fish	-179.5	Yes	****	<0.0001	G-L
Asian swamp eel vs. Yellowtail scad	-93.5	No	ns	0.6115	G-M
Asian swamp eel vs. Atlantic salmon	-139	Yes	**	0.0051	G-N
Blue mackerel vs. Featherback fish	-26	No	ns	>0.9999	H-I
Blue mackerel vs. Walking catfish	11	No	ns	>0.9999	H-J
Blue mackerel vs. Round scad	-52	No	ns	>0.9999	H-K
Blue mackerel vs. Goby fish	-238	Yes	****	<0.0001	H-L
Blue mackerel vs. Yellowtail scad	-152	Yes	***	0.001	H-M
Blue mackerel vs. Atlantic salmon	-197.5	Yes	****	<0.0001	H-N
Featherback fish vs. Walking catfish	37	No	ns	>0.9999	I-J
Featherback fish vs. Round scad	-26	No	ns	>0.9999	I-K
Featherback fish vs. Goby fish	-212	Yes	****	<0.0001	I-L
Featherback fish vs. Yellowtail scad	-126	Yes	*	0.0236	I-M
Featherback fish vs. Atlantic salmon	-171.5	Yes	****	<0.0001	I-N
Walking catfish vs. Round scad	-63	No	ns	>0.9999	J-K
Walking catfish vs. Goby fish	-249	Yes	****	<0.0001	J-L
Walking catfish vs. Yellowtail scad	-163	Yes	***	0.0002	J-M
Walking catfish vs. Atlantic salmon	-208.5	Yes	****	<0.0001	J-N

Round scad vs. Goby fish	-186	Yes	****	<0.0001	K-L
Round scad vs. Yellowtail scad	-100	No	ns	0.3408	K-M
Round scad vs. Atlantic salmon	-145.5	Yes	**	0.0022	K-N
Goby fish vs. Yellowtail scad	86	No	ns	>0.9999	L-M
Goby fish vs. Atlantic salmon	40.5	No	ns	>0.9999	L-N
Yellowtail scad vs. Atlantic salmon	-45.5	No	ns	>0.9999	M-N

9.5.4 Table E5.4 The Dunn's multiple comparison test result of the IgE reactivity to fish parvalbumin

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
Salmon nPV vs. Basa nPV	-16	No	ns	0.7971	A-B
Salmon nPV vs. Barramundi nPV	-29	Yes	*	0.0387	A-C
Salmon nPV vs. Atlantic cod nPV	-15	No	ns	0.953	A-D
Basa nPV vs. Barramundi nPV	-13	No	ns	>0.9999	B-C
Basa nPV vs. Atlantic cod nPV	1	No	ns	>0.9999	B-D
Barramundi nPV vs. Atlantic cod nPV	14	No	ns	>0.9999	C-D

9.5.5 Table E6.1 The Dunn's multiple comparison test results of the IgE reactivity to crustacean extract

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
Mud crab vs. Yabby	-51	No	ns	0.1511	A-B
Mud crab vs. Endeavour prawn	34	No	ns	>0.9999	A-C
Mud crab vs. Vannamei prawn	99	Yes	****	<0.0001	A-D
Mud crab vs. King prawn	58	Yes	*	0.0435	A-E
Mud crab vs. Banana prawn	-2	No	ns	>0.9999	A-F
Mud crab vs. Blue swimmer crab	74	Yes	**	0.0015	A-G
Mud crab vs. BTP	-44	No	ns	0.4586	A-H
Yabby vs. Endeavour prawn	85	Yes	****	<0.0001	B-C
Yabby vs. Vannamei prawn	150	Yes	****	<0.0001	B-D
Yabby vs. King prawn	109	Yes	****	<0.0001	B-E
Yabby vs. Banana prawn	49	No	ns	0.2104	B-F
Yabby vs. Blue swimmer crab	125	Yes	****	<0.0001	B-G
Yabby vs. BTP	7	No	ns	>0.9999	B-H
Endeavour prawn vs. Vannamei prawn	65	Yes	*	0.0109	C-D
Endeavour prawn vs. King prawn	24	No	ns	>0.9999	C-E
Endeavour prawn vs. Banana prawn	-36	No	ns	>0.9999	C-F
Endeavour prawn vs. Blue swimmer crab	40	No	ns	0.8147	C-G
Endeavour prawn vs. BTP	-78	Yes	***	0.0006	C-H
Vannamei prawn vs. King prawn	-41	No	ns	0.7085	D-E
Vannamei prawn vs. Banana prawn	-101	Yes	****	<0.0001	D-F
Vannamei prawn vs. Blue swimmer crab	-25	No	ns	>0.9999	D-G
Vannamei prawn vs. BTP	-143	Yes	****	<0.0001	D-H
King prawn vs. Banana prawn	-60	Yes	*	0.0298	E-F
King prawn vs. Blue swimmer crab	16	No	ns	>0.9999	E-G
King prawn vs. BTP	-102	Yes	****	<0.0001	E-H
Banana prawn vs. Blue swimmer crab	76	Yes	***	0.0009	F-G
Banana prawn vs. BTP	-42	No	ns	0.6145	F-H
Blue swimmer crab vs. BTP	-118	Yes	****	<0.0001	G-H

9.5.6 Table E6.2 The Dunn's multiple comparison test results of the IgE reactivity to mollusk extracts

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
Cuttlefish vs. Abalone	-18	No	ns	>0.9999	I-J
Cuttlefish vs. Squid	95	Yes	****	<0.0001	I-K
Cuttlefish vs. Octopus	30	No	ns	>0.9999	I-L
Cuttlefish vs. Clam	-35	No	ns	0.638	I-M
Cuttlefish vs. Pacific oyster	104	Yes	****	<0.0001	I-N
Cuttlefish vs. Scallop	34	No	ns	0.7444	I-O
Abalone vs. Squid	113	Yes	****	<0.0001	J-K
Abalone vs. Octopus	48	No	ns	0.0627	J-L
Abalone vs. Clam	-17	No	ns	>0.9999	J-M
Abalone vs. Pacific oyster	122	Yes	****	<0.0001	J-N
Abalone vs. Scallop	52	Yes	*	0.0272	J-O
Squid vs. Octopus	-65	Yes	**	0.0012	K-L
Squid vs. Clam	-130	Yes	****	<0.0001	K-M
Squid vs. Pacific oyster	9	No	ns	>0.9999	K-N
Squid vs. Scallop	-61	Yes	**	0.0034	K-O
Octopus vs. Clam	-65	Yes	**	0.0012	L-M
Octopus vs. Pacific oyster	74	Yes	****	<0.0001	L-N
Octopus vs. Scallop	4	No	ns	>0.9999	L-O
Clam vs. Pacific oyster	139	Yes	****	<0.0001	M-N
Clam vs. Scallop	69	Yes	***	0.0004	M-O
Pacific oyster vs. Scallop	-70	Yes	***	0.0003	N-O

9.5.7 Table E6.3 The Dunn's multiple comparison test results of the participants' IgE reactivity to the vannamei prawn allergen components

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
rMLC vs. rSCP	-29.5	No	ns	0.1266	A-B
rMLC vs. nTM	-35	Yes	*	0.031	A-C
rMLC vs. rHC	-70.5	Yes	****	<0.0001	A-D
rMLC vs. Heated extract	-80	Yes	****	<0.0001	A-E
rSCP vs. nTM	-5.5	No	ns	>0.9999	B-C
rSCP vs. rHC	-41	Yes	**	0.0053	B-D
rSCP vs. Heated extract	-50.5	Yes	***	0.0002	B-E
nTM vs. rHC	-35.5	Yes	*	0.027	C-D
nTM vs. Heated extract	-45	Yes	**	0.0014	C-E
rHC vs. Heated extract	-9.5	No	ns	>0.9999	D-E

9.5.8 Table E6.4 The Dunn's multiple comparison test results of the participants' IgE reactivity to indoor allergen components

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
HDM vs. HDM rTM	77.5	Yes	****	<0.0001	A-B
HDM vs. Cockroach R	91.5	Yes	****	<0.0001	A-C
HDM vs. Cockroach H	33.5	Yes	*	0.0464	A-D
HDM vs. Cockroach rTM	77.5	Yes	****	<0.0001	A-E
HDM rTM vs. Cockroach R	14	No	ns	>0.9999	B-C
HDM rTM vs. Cockroach H	-44	Yes	**	0.002	B-D
HDM rTM vs. Cockroach rTM	0	No	ns	>0.9999	B-E
Cockroach R vs. Cockroach H	-58	Yes	****	<0.0001	C-D
Cockroach R vs. Cockroach rTM	-14	No	ns	>0.9999	C-E
Cockroach H vs. Cockroach rTM	44	Yes	**	0.002	D-E

9.5.9 Table E6.5 The Dunn's multiple comparison test results of the participants' IgE reactivity to the heated fish extracts

Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	Adjusted P Value	
John dory vs. Coral trout	107.5	No	ns	0.1582	A-B
John dory vs. Basa fish	73	No	ns	>0.9999	A-C
John dory vs. Atlantic cod	57.5	No	ns	>0.9999	A-D
John dory vs. Cobia	239	Yes	****	<0.0001	A-E
John dory vs. Atlantic salmon	110.5	No	ns	0.118	A-F
John dory vs. Yellowfin tuna	182.5	Yes	****	<0.0001	A-G
John dory vs. Snapper	163	Yes	***	0.0002	A-H
John dory vs. Indian mackerel	269	Yes	****	<0.0001	A-I
John dory vs. Spanish mackerel	103.5	No	ns	0.2313	A-J
John dory vs. Sea mullet	196	Yes	****	<0.0001	A-K
John dory vs. Barramundi	198	Yes	****	<0.0001	A-L
John dory vs. Tilapia	10	No	ns	>0.9999	A-M
Coral trout vs. Basa fish	-34.5	No	ns	>0.9999	B-C
Coral trout vs. Atlantic cod	-50	No	ns	>0.9999	B-D
Coral trout vs. Cobia	131.5	Yes	*	0.0125	B-E
Coral trout vs. Atlantic salmon	3	No	ns	>0.9999	B-F
Coral trout vs. Yellowfin tuna	75	No	ns	>0.9999	B-G
Coral trout vs. Snapper	55.5	No	ns	>0.9999	B-H
Coral trout vs. Indian mackerel	161.5	Yes	***	0.0003	B-I
Coral trout vs. Spanish mackerel	-4	No	ns	>0.9999	B-J
Coral trout vs. Sea mullet	88.5	No	ns	0.8629	B-K
Coral trout vs. Barramundi	90.5	No	ns	0.7311	B-L
Coral trout vs. Tilapia	-97.5	No	ns	0.3997	B-M
Basa fish vs. Atlantic cod	-15.5	No	ns	>0.9999	C-D
Basa fish vs. Cobia	166	Yes	***	0.0001	C-E
Basa fish vs. Atlantic salmon	37.5	No	ns	>0.9999	C-F
Basa fish vs. Yellowfin tuna	109.5	No	ns	0.1302	C-G
Basa fish vs. Snapper	90	No	ns	0.7623	C-H
Basa fish vs. Indian mackerel	196	Yes	****	<0.0001	C-I
Basa fish vs. Spanish mackerel	30.5	No	ns	>0.9999	C-J

Basa fish vs. Sea mullet	123	Yes	*	0.0323	C-K
Basa fish vs. Barramundi	125	Yes	*	0.0259	C-L
Basa fish vs. Tilapia	-63	No	ns	>0.9999	C-M
Atlantic cod vs. Cobia	181.5	Yes	****	<0.0001	D-E
Atlantic cod vs. Atlantic salmon	53	No	ns	>0.9999	D-F
Atlantic cod vs. Yellowfin tuna	125	Yes	*	0.0259	D-G
Atlantic cod vs. Snapper	105.5	No	ns	0.1915	D-H
Atlantic cod vs. Indian mackerel	211.5	Yes	****	<0.0001	D-I
Atlantic cod vs. Spanish mackerel	46	No	ns	>0.9999	D-J
Atlantic cod vs. Sea mullet	138.5	Yes	**	0.0055	D-K
Atlantic cod vs. Barramundi	140.5	Yes	**	0.0043	D-L
Atlantic cod vs. Tilapia	-47.5	No	ns	>0.9999	D-M
Cobia vs. Atlantic salmon	-128.5	Yes	*	0.0176	E-F
Cobia vs. Yellowfin tuna	-56.5	No	ns	>0.9999	E-G
Cobia vs. Snapper	-76	No	ns	>0.9999	E-H
Cobia vs. Indian mackerel	30	No	ns	>0.9999	E-I
Cobia vs. Spanish mackerel	-135.5	Yes	**	0.0078	E-J
Cobia vs. Sea mullet	-43	No	ns	>0.9999	E-K
Cobia vs. Barramundi	-41	No	ns	>0.9999	E-L
Cobia vs. Tilapia	-229	Yes	****	<0.0001	E-M
Atlantic salmon vs. Yellowfin tuna	72	No	ns	>0.9999	F-G
Atlantic salmon vs. Snapper	52.5	No	ns	>0.9999	F-H
Atlantic salmon vs. Indian mackerel	158.5	Yes	***	0.0004	F-I
Atlantic salmon vs. Spanish mackerel	-7	No	ns	>0.9999	F-J
Atlantic salmon vs. Sea mullet	85.5	No	ns	>0.9999	F-K
Atlantic salmon vs. Barramundi	87.5	No	ns	0.9364	F-L
Atlantic salmon vs. Tilapia	-100.5	No	ns	0.3051	F-M
Yellowfin tuna vs. Snapper	-19.5	No	ns	>0.9999	G-H
Yellowfin tuna vs. Indian mackerel	86.5	No	ns	>0.9999	G-I
Yellowfin tuna vs. Spanish mackerel	-79	No	ns	>0.9999	G-J
Yellowfin tuna vs. Sea mullet	13.5	No	ns	>0.9999	G-K
Yellowfin tuna vs. Barramundi	15.5	No	ns	>0.9999	G-L

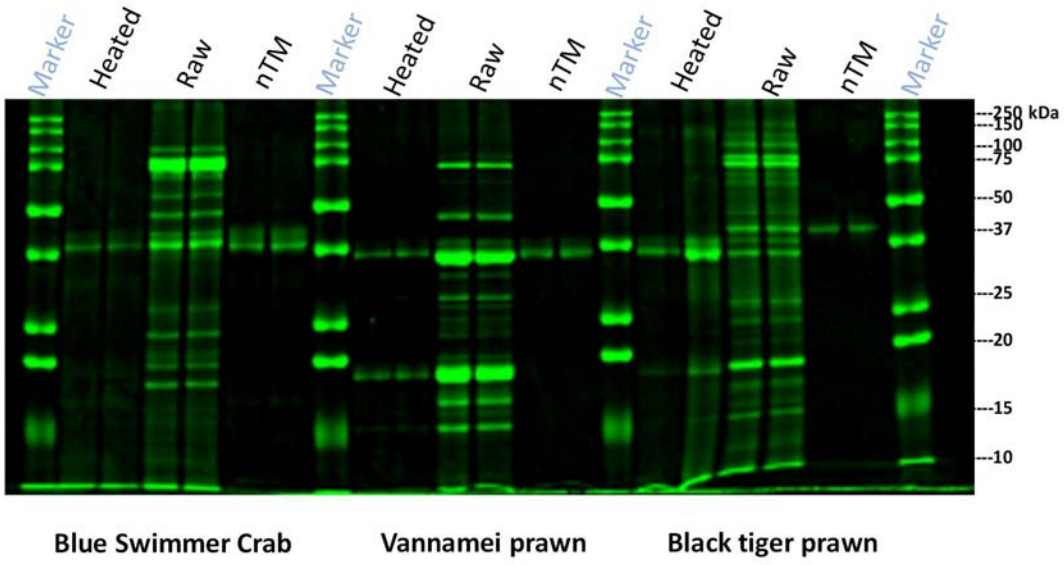
Yellowfin tuna vs. Tilapia	-172.5	Yes	****	<0.0001	G-M
Snapper vs. Indian mackerel	106	No	ns	0.1826	H-I
Snapper vs. Spanish mackerel	-59.5	No	ns	>0.9999	H-J
Snapper vs. Sea mullet	33	No	ns	>0.9999	H-K
Snapper vs. Barramundi	35	No	ns	>0.9999	H-L
Snapper vs. Tilapia	-153	Yes	***	0.0009	H-M
Indian mackerel vs. Spanish mackerel	-165.5	Yes	***	0.0002	I-J
Indian mackerel vs. Sea mullet	-73	No	ns	>0.9999	I-K
Indian mackerel vs. Barramundi	-71	No	ns	>0.9999	I-L
Indian mackerel vs. Tilapia	-259	Yes	****	<0.0001	I-M
Spanish mackerel vs. Sea mullet	92.5	No	ns	0.6176	J-K
Spanish mackerel vs. Barramundi	94.5	No	ns	0.5202	J-L
Spanish mackerel vs. Tilapia	-93.5	No	ns	0.567	J-M
Sea mullet vs. Barramundi	2	No	ns	>0.9999	K-L
Sea mullet vs. Tilapia	-186	Yes	****	<0.0001	K-M
Barramundi vs. Tilapia	-188	Yes	****	<0.0001	L-M

9.6 Appendix F – Molecular characterization of crustacean allergens

9.6.1 Table F7.1 All the proteins from two cohorts identified by mass spectrometry

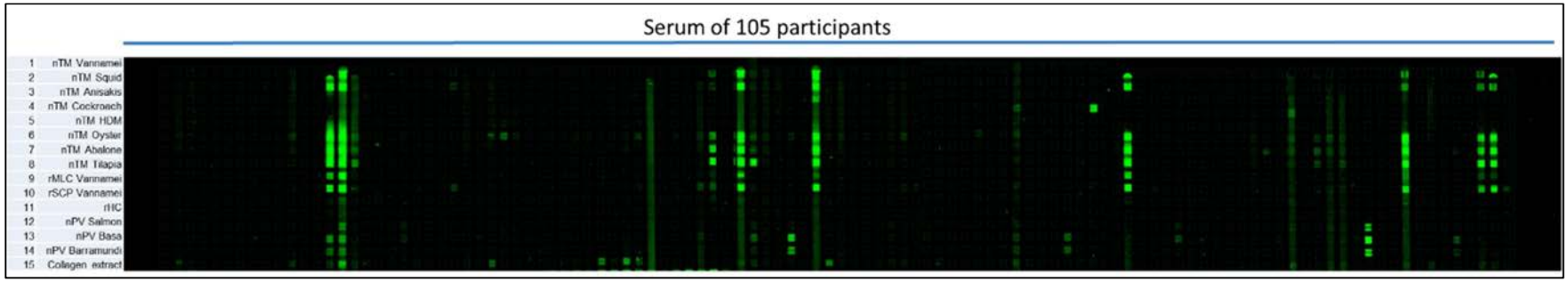
Seq	ID	Species	Treatment	Immunoblot MW (kDa)	Match protein	Entry ID	Mass	Mascot score	emPAI	Calculated pI	Organism	Scientific name	Protein sequence coverage (%)
1	42	Octopus	R	44.2	**Actin**	ACTC_BRABE	41,963	1080	6.75	5.3	Amphioxus	*Branchiostom*	60
2	43	Octopus	R	36.5	**Actin**	ACTM_APLCA	42,081	413	2.62	5.3	California sea hare	*Aplysia calif*	54
3	44	Octopus	R	33.8	**Actin**	ACTM_APLCA	42,081	311	2.35	5.3	California sea hare	*Aplysia calif*	48
4	45	Octopus	R	28.2	**Actin**	ACTM_APLCA	42,081	991	7.31	5.3	California sea hare	*Aplysia calif*	56
5	48	Oyster	R	35.8	**Actin**	ACTC_BRABE	41,963	214	0.98	5.3	Amphioxus	*Branchiostom*	42
6	49	Abalone	R	42.9	**Actin**	ACT_PLAMG	42,077	948	5.14	5.3	Sea scallop	*Placopecten*	57
7	56	Scallop	R	44.8	**Actin**	ACT_PLAMG	42,077	981	7.96	5.3	Sea scallop	*Placopecten*	65
8	60	Scallop	H	45.7	**Actin**	ACT_PLAMG	42,077	686	3.9	5.3	Sea scallop	*Placopecten*	63
9	71	Oyster	H	44	**Actin**	ACT_PLAMG	42,077	645	3.54	5.3	Sea scallop	*Placopecten*	62
10	7	Crab	R	27.5	**Arginine kinase**	KARG_CALSI	40,632	718	4.17	6.19	Blue crab	*Callinectes s*	64
11	28	BTP	H	18.2	**Arginine kinase**	KARG_PENMO	40,400	188	0.73	6.05	BTP	*Penaeus mon*	40
12	50	Abalone	R	39.9	**Arginine kinase**	KARG_HALMK	40,245	1517	6.19	5.73	Giant abalone	*Haliotis mad*	37
13	4	Crab	R	48.6	**Enolase**	ENO_HOMGA	47,525	329	1.09	5.85	European lobster	*Homarus vul*	30
14	5	Crab	R	42	**Enolase**	ENO_HOMGA	47,525	312	0.71	5.85	European lobster	*Homarus vul*	25
15	6	Crab	R	39.1	**Enolase**	ENO_HOMGA	47,525	168	0.4	5.85	European lobster	*Homarus vul*	26
16	30	BTP	R	41.2	**Enolase**	ENO_HOMGA	47,525	251	1.74	5.85	European lobster	*Homarus vul*	30
17	38	Cuttlefish	R	51.1	**Enolase**	ENO_DORPE	47,738	1128	5.95	5.78	Longfin inshore squid	*Loligo pealei*	48
18	39	Cuttlefish	R	37.5	**Enolase**	ENO_DORPE	47,738	556	1.55	5.78	Longfin inshore squid	*Loligo pealei*	36
19	16	Vannamei	H	15.4	**Hemocyanin C chain**	HCYC_PANIN	75,997	48	0.09	5.37	California spiny lobster	*Palinurus int*	2
20	2	Crab	R	70.7	**Hemocyanin subunit 2**	HCY2_CARAE	75,102	113	0.24	5.4	Green crab	*Carcinus me*	12
21	34	Squid	R	36.8	**Myosin heavy chain**	MYS_ARGIR	223,824	153	0.08	5.6	Bay scallop	*Aequipecten*	6
22	35	Squid	R	51	**Myosin heavy chain**	MYS_ARGIR	223,824	239	0.11	5.6	Bay scallop	*Aequipecten*	5
23	55	Scallop	R	98.9	**Myosin heavy chain**	MYS_ARGIR	223,824	221	16	5.6	Bay scallop	*Aequipecten*	12
24	59	Scallop	H	100	**Myosin heavy chain**	MYS_ARGIR	223,824	1370	1.75	5.6	Bay scallop	*Argopecten i*	27
25	3	Crab	R	56	**Pyruvate kinase**	KPYK_DROME	57,917	101	0.18	7.13	Fruit fly	*Drosophila m*	8
26	9	Crab	R	19.6	**Sarcoplasmic calcium-binding protein**	SCP1_ASTLP	21,783	291	1.73	4.61	Turkish narrow-clawed crayfish	*Pontastacus*	42
27	10	Crab	R	18.2	**Sarcoplasmic calcium-binding protein**	SCP1_ASTLP	21,783	287	2.63	4.61	Turkish narrow-clawed crayfish	*Pontastacus*	49
28	15	Vannamei	H	18.7	**Sarcoplasmic calcium-binding protein**	SCPA_PENSP	22,251	166	1.33	4.63	Penoeid shrimp	*Penaeus sp.*	58
29	21	Vannamei	R	18.5	**Sarcoplasmic calcium-binding protein**	SCPA_PENSP	22,251	454	9.92	4.63	Penoeid shrimp	*Penaeus sp.*	68
30	22	Vannamei	R	16.9	**Sarcoplasmic calcium-binding protein**	SCPA_PENSP	22,251	143	1.02	4.63	Penoeid shrimp	*Penaeus sp.*	46
31	23	Vannamei	R	15.3	**Sarcoplasmic calcium-binding protein**	SCPA_PENSP	22,251	89	0.32	4.63	Penoeid shrimp	*Penaeus sp.*	22
32	32	BTP	R	18.5	**Sarcoplasmic calcium-binding protein**	SCPA_PENSP	22,251	423	8.49	4.63	Penoeid shrimp	*Penaeus sp.*	70
33	58	Scallop	R	18.7	**Sarcoplasmic calcium-binding protein**	SCP_MIZYE	20,189	313	1.94	4.65	Japanese scallop	*Patinopecten*	48
34	63	Scallop	H	19.8	**Sarcoplasmic calcium-binding protein**	SCP_MIZYE	20,189	280	3	4.65	Japanese scallop	*Patinopecten*	56
35	8	Crab	R	24	**Triosephosphate isomerase A**	TPISA_DANRE	27,179	179	0.78	4.9	Zebrafish	*Brachydanio*	20
36	1	Crab	H	38.8	**Tropomyosin**	TPM_CHAFE	30,417	577	5.47	4.76	Crucifix crab	*Cancer feria*	67
37	13	Vannamei	H	37.4	**Tropomyosin**	TPM_PENMO	32,830	481	2.17	4.72	BTP	*Penaeus mon*	62
38	14	Vannamei	H	36.2	**Tropomyosin**	TPM_PENMO	32,830	1136	16.96	4.72	BTP	*Penaeus mon*	73
39	17	Vannamei	H	70.7	**Tropomyosin**	TPM_PENMO	32,830	115	0.33	4.72	BTP	*Penaeus mon*	41
40	18	Vannamei	R	46.2	**Tropomyosin**	TPM_PENMO	32,830	97	0.21	4.72	BTP	*Penaeus mon*	35
41	19	Vannamei	R	38.4	**Tropomyosin**	TPM_PENMO	32,830	243	1.38	4.72	BTP	*Penaeus mon*	60
42	20	Vannamei	R	34.2	**Tropomyosin**	TPM_PENMO	32,830	2335	110.82	4.72	BTP	*Penaeus mon*	87
43	26	BTP	H	35.5	**Tropomyosin**	TPM_PENMO	32,830	1152	22.97	4.72	BTP	*Penaeus mon*	82
44	27	BTP	H	34.5	**Tropomyosin**	TPM_PENMO	32,830	1311	41.7	4.72	BTP	*Penaeus mon*	87
45	31	BTP	R	34.9	**Tropomyosin**	TPM_PENMO	32,830	522	3.24	4.72	BTP	*Penaeus mon*	64
46	36	Squid	H	39.5	**Tropomyosin**	TPM2_BIOGL	32,663	195	1.9	4.58	Freshwater snail	*Biomphalaria*	26
47	40	Cuttlefish	H	47.4	**Tropomyosin**	TPM_HELAS	32,731	71	0.62	4.58	Brown garden snail	*Cornu aspers*	22
48	41	Cuttlefish	H	38	**Tropomyosin**	TPM_HELAS	32,731	702	1.63	4.58	Brown garden snail	*Cornu aspers*	22
49	46	Octopus	H	34.4	**Tropomyosin**	TPM_HELAS	32,731	687	3.26	4.58	Brown garden snail	*Cornu aspers*	25
50	51	Abalone	R	36.4	**Tropomyosin**	TPM_HALRU	32,811	274	2.17	4.6	California red abalone	*Haliotis rufe*	36
51	52	Abalone	H	41.5	**Tropomyosin**	TPM_HALDV	32,860	1096	25.39	4.55	Abalone	*Haliotis diver*	58
52	53	Abalone	H	39.7	**Tropomyosin**	TPM_HALDV	32,860	1048	18.77	4.55	Abalone	*Haliotis diver*	55
53	54	Abalone	H	34.5	**Tropomyosin**	TPM_HALDV	32,860	1291	18.77	4.55	Abalone	*Haliotis diver*	57
54	57	Scallop	R	36.2	**Tropomyosin**	TPM_CHLNI	32,522	990	9.31	4.56	Akazara scallop - Japanese scall	*Chlamys nipp*	50
55	61	Scallop	H	37	**Tropomyosin**	TPM_CHLNI	32,522	1869	64.4	4.56	Akazara scallop - Japanese scall	*Chlamys nipp*	60
56	69	Squid	R	39.1	**Tropomyosin-2**	TPM2_BIOGL	32,663	355	1.17	4.58	Freshwater snail	*Biomphalaria*	28
57	70	Squid	R	36.1	**Tropomyosin**	TPM_HELAS	32,731	132	1.17	4.58	Brown garden snail	*Cornu aspers*	19
58	72	Oyster	H	40.1	**Tropomyosin**	TPM_CHLNI	32,522	199	1.18	4.56	Akazara scallop - Japanese scall	*Chlamys nipp*	19
59	62	Scallop	H	22.2	**Troponin C**	TNNC_MIZYE	17,628	469	7.18	4.49	Japanese scallop	*Patinopecten*	48

9.6.2 Figure F7.2 Protein profile of crustacean extracts performed by SDS-PAGE

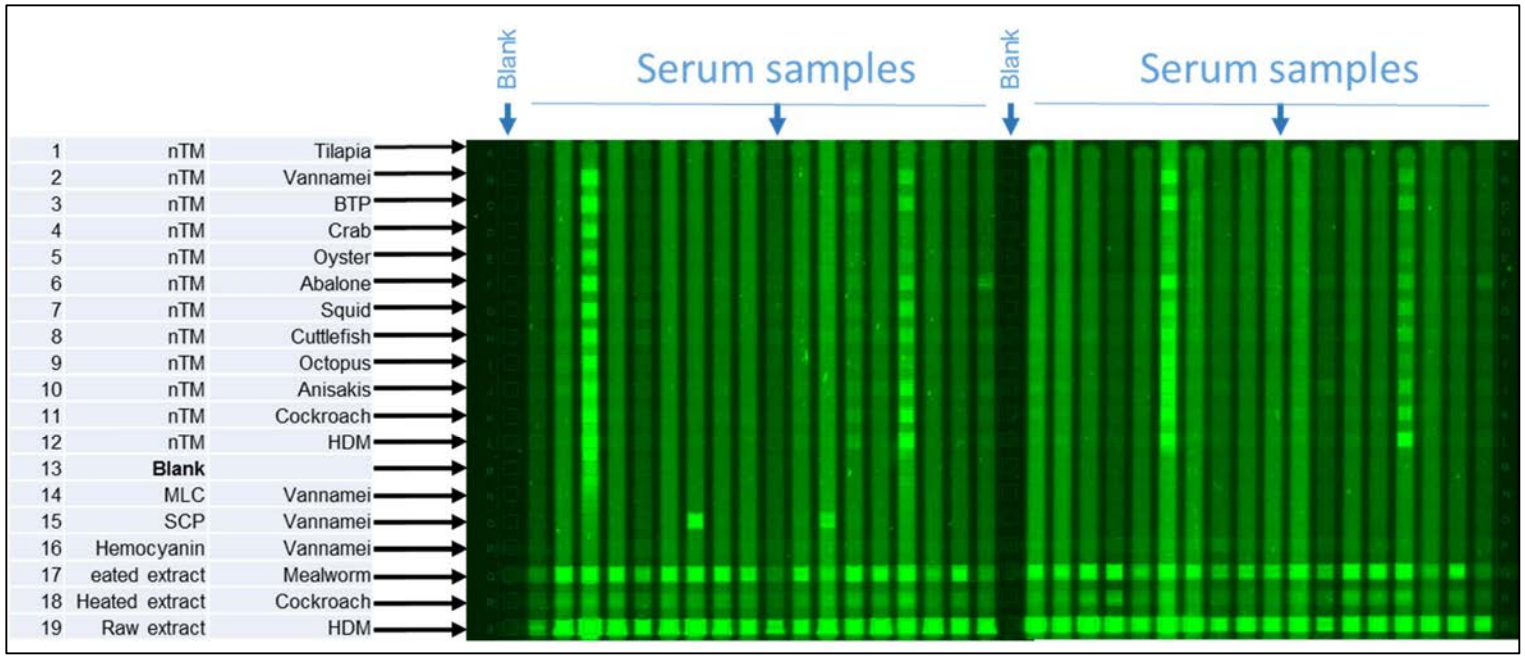


9.7 Appendix G – Grid-Immunoblotting outcomes

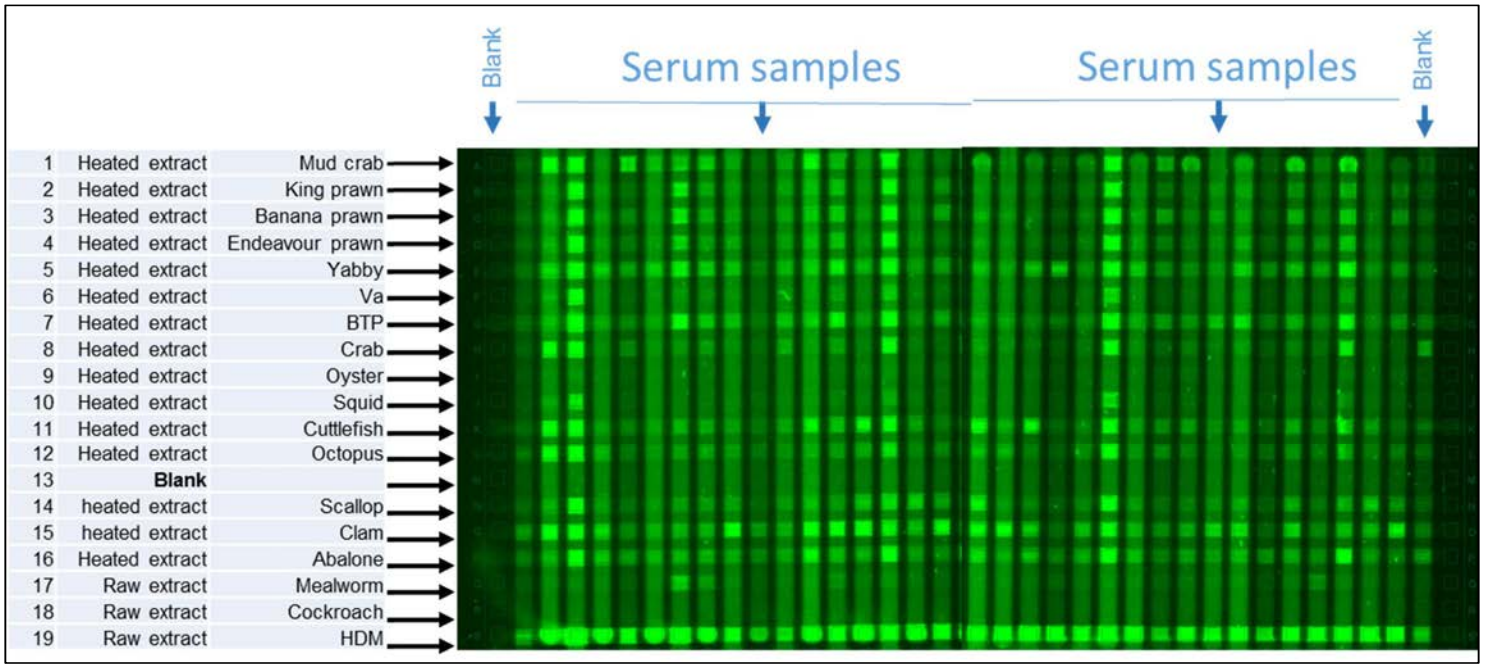
Figure G1: IgE reactivity of all participants against investigated allergens/protein extracts (*n* = 104).



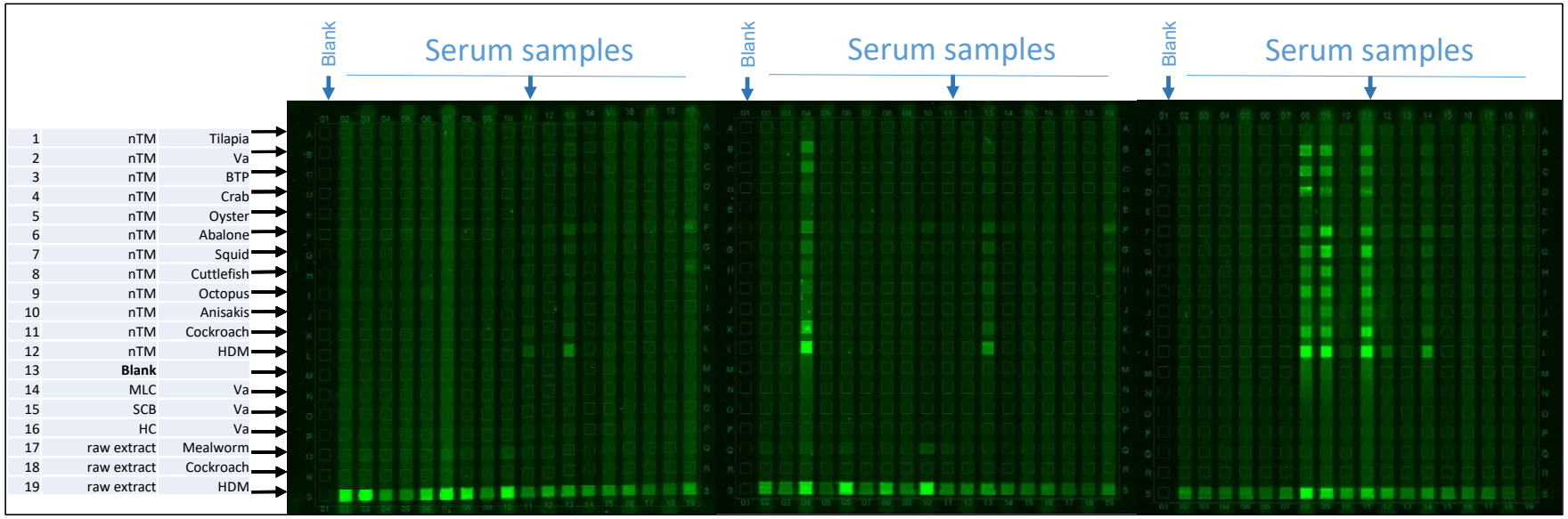
9.7.1 Figure G2: IgE reactivity of the Australian participants against tropomyosins (*n = 36)*.



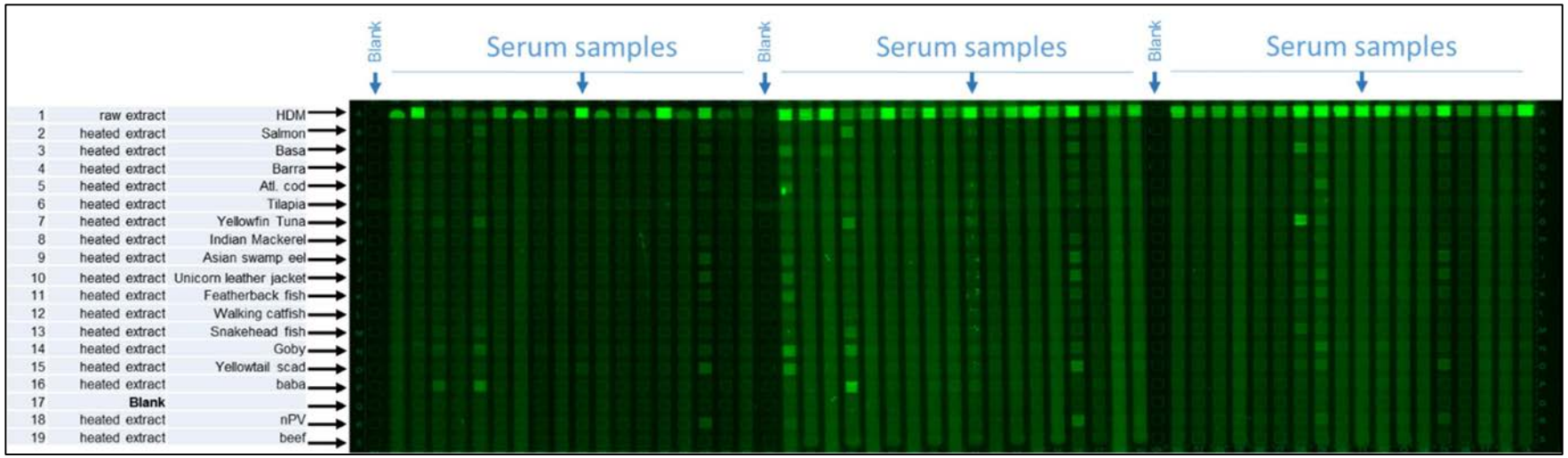
9.7.2 Figure G3: IgE reactivity of the Australian participants against investigated allergens/protein extracts (*n* = 36).



9.7.3 Figure G4: IgE reactivity of the Vietnamese participants against investigated allergens/protein extracts (*n* = 50).



9.7.4 Figure G5: IgE reactivity of participants in Vietnam against investigated fish allergens/protein extracts (*n* = 50).



9.7.5 Figure G6: IgE reactivity of the Australian participants against investigated fish allergens/protein extracts (*n* = 50).