UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Clinical and Experimental Sciences

Mast cell mediators: their subcellular localization, bacterial influence on their release and expression and their potential value as markers for predicting the severity of allergic reactions

by

Rana Salah Abadalkareem

Thesis for the degree of PhD

September 2018

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

Infection, inflammation and immunity

Thesis for the degree of Doctor of Philosophy

MAST CELL MEDIATORS: THEIR SUBCELLULAR LOCALIZATION, BACTERIAL INFLUENCE ON THEIR RELEASE AND EXPRESSION AND THEIR POTENTIAL VALUE AS MARKERS FOR PREDICTING THE SEVERITY OF ALLERGIC REACTIONS

Rana Salah Abadalkareem

Allergic reactions involve the explosive release of inflammatory mediators from mast cells including the proteases tryptase, carboxypeptidase A3 (CPA3) and chymase; and there have been suggestions that dipeptidyl peptidase I (DPPI) could be secreted as well. Our aim has been to investigate the levels of mast cell proteases and a panel of pro-inflammatory cytokines in serum during allergic reactions provoked by drugs or food to identify and replicate distinct clinico-immunological endotypes of allergic reactions using topological data analysis. In addition, we have investigated factors that can modulate the release and expression of mast cell mediators and their subcellular localization.

Patients who had suffered drug- or food-induced allergic reactions were recruited from two separate centers (Southampton, UK and Doha, Qatar). Healthy individuals who had no history of allergic diseases were recruited to serve as controls. Detailed clinical assessment and measurements of mast cell proteases and pro-inflammatory cytokines were performed. The clinical and biochemical parameters were analyzed using topological data analysis. In addition, cells of the LAD2 mast cell line were employed to investigate the effect of bacterial infection on the release and expression of mast cell mediators. This cell line was also employed to investigate the subcellular localization of mast cell proteases through the application of immunocytochemical analysis.

There were higher levels of CPA3 in baseline serum samples from patients with more severe historical allergic reactions than in those from healthy individuals ($p< 0.0001$). CPA3 levels of ≥ 6.5 ng/ml were associated with severe allergic reactions in patients with drug allergies (AUC: 0.61, 95% CI: 0.51-0.71, $p= 0.048$), and levels of ≥ 3 ng/ml in those with food allergies (AUC: 0.74, 95% CI: 0.62-0.86, $p= 0.002$). Higher serum levels of CPA3 were seen also in patients with one or more concomitant atopic conditions (including atopic dermatitis, hay fever and asthma) compared to those in healthy subjects ($p< 0.0001$). A strong association was found between the severity of allergic reactions and concomitant atopic illnesses ($p< 0.0001$).

Topological data analysis allowed identification of four novel clinico-immunological clusters those with: (I) high CPA3 and IL-13 levels, females, drug reactions, more severe historical reactions; (II) high CPA3, low IL-13, males, food reactions, more severe historical reactions; (III) low CPA3 and IL-13 levels, young, females, food reactions, mild historical reactions; and (IV) low CPA3, high IL-13, females, drug reactions, mild historical reactions. These clusters were replicated in both geographical cohorts.

In separate studies with cultured LAD2 cells, it was found that *S. aureus* infection could inhibit IgE- and non-IgE-dependent mast cell degranulation and down-regulate gene expression for major cytokines involved in anti-bacterial defense mechanisms (including that for TNFα, IL-8, and IL-1β). Bacterial exposure also altered the phosphorylation of protein kinases downstream of FcεRI engagement. At the subcellular level, DPPI was observed inside the granules of mast cells and colocalized with tryptase, CPA3 and chymase.

Our finding that DPPI may be present in association with other mast cell proteases within the granules suggests that DPPI is secreted upon mast cell stimulation and may have extracellular roles in immune modulation. DPPI could thus represent a novel marker for mast cell activation that can enhance the diagnosis of allergic reactions when measured in combination with tryptase and CPA3. The potential for bacterial infection to interfere with mast cell responses could reduce the susceptibility to allergic reactions and as the mechanisms involved deserve consideration as a novel therapeutic approach to prevent development of severe reactions. Serum levels of CPA3 have the potential to predict the severity of allergic reactions to drugs or food. Identification of four multidimensional endotypes underlines the connection between CPA3 and IL-13 levels and their association with clinical features of patients who have drug or food allergies. Their use as biomarkers can help to identify those at particular risk of allergic reactions and allow optimal interventions to be undertaken.

Table of Contents

List of Tables

List of Figures

DECLARATION OF AUTHORSHIP

I, Rana Salah Abadalkareem declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

Mast cell proteases: Markers for confirming a diagnosis of allergic drug reaction and for predicting susceptibility.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed: .....

Date: .....

Acknowledgement

Firstly, I would like to express my sincere gratitude to my supervisor Dr. Andrew Walls for the continuous support during the last four years, for his patience, motivation, and immense knowledge. His guidance helped me through all the time of research and writing of this thesis. I would like also to thank Dr. Efrem Eren and Dr. Ahmed Abdelmotelb for their insightful comments and encouragement.

My sincere thanks also go to Dr. Laurie Lau for teaching me the principles and techniques involved in ELISA and the antibody production. Without his precious support, it would have been very difficult to conduct this research.

I thank Dr. Tak Chin and staff in the Asthma and Allergy Clinic for their help with patient recruitment and in sample collection. I am greatly appreciative to David Johnston for his assistance in the confocal imaging.

I would like to acknowledge the time and effort dedicated by Dr. Joost Brandsma to help me in the topological data analysis. I was able to cross the hurdle of data analysis with Dr. Brandsma's kind help.

I must not forget to thank my colleagues in the immunopharmacology group for their supportive comments especially during hard times of this PhD journey. Their sense of humor had frequently eased the stress of working late in the laboratory.

I would like to thank my husband for his love and encouragement throughout my study. I am very proud of my beloved daughter for being so patient and understanding. Most importantly, I would like to thank my father and mother for their continuous support and encouragement through my entire life. This accomplishment would not have been possible without them.

Definitions and Abbreviations

ACE	Angiotensin converting enzyme
BAT	The basophil activation test
BCA	bicinchoninic acid
BSA	Bovine serum albumin
CPA3	Carboxypeptidase A3
diH_2O	Deionized water
DMEM	Dulbecco's modified eagles medium
DPPI	Dipeptidyl peptidase I
ELISA	Enzyme-linked immunosorbent assay
EGTA	ethylene glycol tetra acetic acid
FAAN	Food Allergy and Anaphylaxis Network
FBS	Foetal bovine serum
FcεRI	Fc-epsilon receptor I
GA	General anaesthetics
HRP	Horseradish peroxidase
IDT	Intradermal testing
IgE	Immunoglobulin E
IgG	Immunoglobulin G
LA	Local anaesthetics

MC_{TC}	Mast cell tryptase-positive chymase-positive
MC_T	Mast cell tryptase-positive
MRI	Magnetic resonance imaging
NIAID	National Institute of Allergy and Infectious Diseases
NICE	National Institute for Health and Care Excellence
NMBA	Neuromuscular blocking agents
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.05% Tween-20
PCA	Principle component analysis
PLS	Papillon-Lafevre syndrome
RAST	Radioallergosorbent test
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPT	Skin prick testing
TDA	Topological data analysis
TMB	Tetramethylbenzidine

Chapter 1: Introduction

1.1 Allergic reactions

Allergic reactions represent a condition of increased reactivity to a certain stimulus that can be drugs, food, insect bite, or even environmental triggers including pollens, animal dander and dust mite. The prevalence of allergic reactions has increased multi-fold in recent decades and many efforts have been directed to define the underlying mechanisms and predisposing factors that increase the susceptibility to allergic reactions. Demographic associations have been described, but the underlying molecular and cellular processes are less well understood. For example, children are more likely to develop allergic reactions to food, whereas adults and older age group tend to have drug-induced allergic reactions. The diagnosis of allergic reactions can be challenging because they are often of rapid onset and unexpected. In addition, they can present with a wide range of symptoms and signs, which can be localized to skin or progress to a life-threatening respiratory and cardiovascular involvement. Furthermore, there are major limitations to the laboratory tests that can be employed in a routine clinical practice to confirm the allergic nature of a reaction. Moreover, there is an unmet medical need for a laboratory test that can predict susceptibility to severe allergic reactions and can help to decrease the morbidity and mortality associated with them.

Allergic reactions occur following primary sensitization, which is associated with the generation of specific IgE antibodies that become attached to high affinity IgE receptors (FcεRI) on mast cells and basophils (1). When the allergen is re-introduced, it can interact and stimulate these receptor- IgE molecules on sensitized mast cells and basophils. The cross-linking of receptors leads to the release of various inflammatory mediators. These mediators contribute to the development of a diverse range of symptoms manifested in allergic reactions.

The contribution of mast cells to health and disease has been an area of active research since their discovery. They are multifunctional cells that historically have been associated with allergic diseases, though roles in defense mechanisms and inflammatory responses have come to be increasingly appreciated (2-4). Mast cell stimulation results in degranulation and release of many mediators but those with particular potential as markers of allergic reactions are the proteases: tryptase, chymase, carboxypeptidase A3 (CPA3), and dipeptidyl peptidase I (DPPI). Being unique to mast cells, determination of serum levels of these mediators in patients with allergic reactions would confirm an immunological process. Tryptase measurement is well-established as a confirmatory test when severe form of allergic reaction is suspected (5), yet frequently increased

levels cannot be detected. Baseline levels of tryptase have been reported as a predictive test for severity of allergic reactions (6, 7), but with low sensitivity. Therefore, there is a need to develop new tests that can be used either separately or in combination with those for tryptase to allow not only more effective diagnosis but also prediction of severity of allergic reactions. There is also a need to combine the clinical and immunological features to define subcategories (endotypes) of allergic reactions.

1.2 Epidemiology of allergic reactions

The true incidence of allergic reactions including anaphylaxis is difficult to know and worldwide epidemiological data are lacking a consistent approach. It has been reported in a systematic review that included 5,843 European anaphylaxis studies that the incidence rates for all-cause anaphylaxis ranged from 1.5 to 7.9 per 100,000 person/year, with an estimate that 0.3% (95% CI 0.1-0.5) of the population could experience anaphylaxis during their lives (8). In the United States, a study on the prevalence estimates of anaphylaxis caused by drugs, food, latex or insect stings has reported that 1.24% to 16.8% of the total population might suffer from anaphylaxis and 0.002% could suffer a fatal reaction (9). The trend for hospital admissions and fatalities of anaphylaxis in England and Wales has been investigated over a 21-year period (1992-2012). The study has reported an increase in the hospital admissions from 1.0 to 7.0 per 100,000 person/year (10). A parallel United States-based study in which emergency department visits for anaphylaxis from 2005 through 2014 were investigated, has shown an increase in the incidence rate from 14.2 to 28.6 per 100,000 population per year, with the highest increase among children aged five to 17 years during the ten-year time period (11).

Drugs, food, and insect stings are the most common triggers for allergic reactions (8, 9). A cohort study of 382 patients for whom a diagnosis of anaphylaxis was confirmed from 1996 to 2005, has shown that 27% of anaphylaxis cases were caused by drug allergy and 24% of them triggered by food, whereas 12% were associated with insect bites. Antibiotics have been identified as the predominant drugs causing anaphylaxis followed by nonsteroidal anti-inflammatory drugs (NSAIDs) and anti-hypertensive medications (12). Looking at hospital discharges for cases of anaphylaxis in England from 1991 to 1995, it has been reported that the most common causes of anaphylaxis were therapeutic drugs (62%), food (15%) and insect venom (11%) (13). Among 112 anaphylaxis fatalities in Australia between 1997 and 2005, it has been reported that drugs were the culprit trigger for 58% of fatalities followed by insect stings (18%) and food (6%). In the study, it has been shown that antibiotics were the drugs implicated in 24% of the fatalities followed by

anaesthetics (5%) and NSAIDs (3%) (14). Antibiotics have been identified as the culprit drugs for fatal drug-induced anaphylaxis in 41% of 368 fatalities recorded in the United States from 1999 to 2010, followed by radiocontrast agents (30%), antineoplastic drugs (12%), opiates (3%), antihypertensive agents (4%), NSAIDs (3%) and anaesthetics (3%) (15).

Peanut has been reported to be the offending allergen in 60% of fatalities from food-induced anaphylaxis followed by fish (20%) (14). In England and Wales, it has been reported that allergy to peanut or tree nuts was the cause of death in 73% of cases assessed between 1992 and 2012 (10). Cases of anaphylaxis who attended the emergency department in the United States have been reported to be caused mainly by food (27.1%), whereas only 12% and 4% were medication- and insect venom-related, respectively (11). However, the study did not include details about the types of medications or food causing the allergic reactions. It is noteworthy that the differences seen in the reported rates of drug-, food- or insect bite-associated allergic reactions could be related to ecological factors, dietary factors, or methodological differences in data retrieval.

The risk of medication- or insect bite-related anaphylaxis has been reported to be significantly higher in subjects older than 60 (10, 11, 14), in contrast to the rate of food-induced anaphylaxis which has been seen predominantly in younger age group (5-17 years; (11)) with the peak incidence of fatalities seen in the second and third decades (10, 14). The widespread use of medications for the treatment of chronic diseases or repeated infections in those older than 60 might explain the increased incidence of anaphylaxis among this age group. On other hand, it has been reported that food allergy is most prevalent among preschool children (1, 16), therefore food-induced allergic reactions are more common among children than drug-related allergic reactions. In addition, the increased risk of death from food-induced anaphylaxis in teens and young adults has been reported to be related to risk-taking behaviour. In a total of 174 subjects aged 13-21 years, it has been reported that 54% purposefully ingested unsafe food and 42% were willing to eat food that may contain the allergen (17). The high risk of insect bite-induced anaphylaxis among those older than 60 could be attributed to their engagement in outdoor activities. However, it is important to emphasize that there is a risk of drug allergy in early years of life. It has been reported that the use of antibiotics during infancy can affect the immune system and lead to the development of atopic diseases by the age of 6 years (18).

It has been suggested that females were more likely to have allergic reactions than males in adults (10, 11, 13, 19, 20), whereas males were at a higher risk of anaphylaxis than females among children (10, 19, 21). Adult females have been reported to outnumber males in hospital admissions for anaphylaxis with females accounting for 65% (10, 13, 19). In addition, females have been found to have a higher incidence of self-reported allergic reactions than males (10.2% vs.

5.3%) (22). On the other hand, boys less than 15 years have been reported to suffer from anaphylaxis at a ratio of 1.7:1 compared to females of same age group (10, 19). However, no significant differences have been reported in the severity of clinical manifestations and mortality between genders (10).

There are few studies that have considered cutaneous as well as systemic manifestations to determine the incidence of allergic reactions. Analysis of the symptom profile of anaphylaxis in 2012 paediatric and adult patients from Germany, Austria and Switzerland has shown that the most common manifestations were cutaneous (84%) followed by cardiovascular (72%) and respiratory (68%) symptoms (23). Worm *et al* have also reported that children were less likely to have circulatory symptoms and that the risk of development of hypotension or cardiovascular shock increased with age. In a retrospective study of case records from 1149 emergency department visits of systemic hypersensitivity reactions, it was reported that there was a predominance of skin involvement compared to cardiovascular or respiratory symptoms (24). It also identified age as a risk factor for severe allergic reactions, which included cardiovascular and respiratory symptoms. Consistent with this, in a study of 64 patients under the age of 18 years with history of anaphylaxis, it was found that the most common symptoms experienced were mucocutaneous (94%) and respiratory symptoms (84%), followed by gastrointestinal (42%) and cardiovascular (25%) involvement (21). The high rates for skin manifestations could be attributed to the ease of identification in cases of anaphylaxis compared to the other symptoms which are more liable to be misinterpreted.

Concomitant atopic illnesses have been reported to increase the risk of anaphylaxis. In a case-control study of patients with coexisting asthma aged from 10 to 79 years, it has been reported that the risk of anaphylaxis was two-fold greater in non-severe asthmatics (relative risk of 2, 95% CI= 1.65-2.6) and three-fold greater in the severe asthmatic patients (relative risk of 3.3, 95% CI= 2.47-3.47) compared to non-asthmatic subjects (12). The study has also reported a significant increase in the risk of anaphylaxis in association with atopic dermatitis among the asthma-free participants (odd ratio = 2.83, 95% CI= 1.5-5.29). Within the asthma cohort, the risk of anaphylaxis has been reported to increase significantly in patients who had concomitant allergic rhinitis (odd ratio= 1.76, 95% CI= 1.35-2.3) or atopic dermatitis (odd ratio= 1.69, 95% CI= 1.13-2.51). A population study on the characteristics of food allergy and anaphylaxis included 5149 participants who were less than 18 years has suggested that subjects with asthma were more likely to have severe reactions compared to non-asthmatics (33% vs 21%, p <0.0001). Furthermore, it has been reported that that 75% of fatalities caused by food-triggered anaphylaxis had asthma (10).

There have been suggestions that ethnicity may influence the susceptibility to allergic reactions (15, 25, 26). In a retrospective observational study of emergency department attendances at three hospitals in Birmingham, it was reported that the incidence of anaphylaxis in the South Asian population was nearly double that in White population (25). In an epidemiologic study in the Unites States from 1999 to 2010, it has been reported that fatalities from food-related anaphylaxis were strongly associated with the belonging to the African American race (15). An Australian study on the risk of anaphylaxis and allergic diseases among 57,000 children has reported that children with Asian-born mothers had higher prevalence of food allergy and eczema than those with non-Asian mothers. In addition, it has been shown that Australian-born Asian children were at greater risk of food- and non-food-induced anaphylaxis than Australian-born non-Asian children (26). These studies give an indication of probable genetic and environmental factors that influence the risk for allergic reactions. In addition, cultural differences might affect the degree of exposure for the allergens. In agreement, the ISAAC studies have reported a world-wide variation in the prevalence of allergic diseases including asthma, allergic rhinitis and atopic dermatitis (27-29). However, it is of note that measurements of any biomarkers have not been included in the aforementioned studies. Therefore, careful assessment of the clinical as well as immunological characteristics is mandatory to investigate the effect of ethnicity on the risk of anaphylaxis

From the above, it can be seen that allergic reactions are not uncommon on a worldwide scale. The most common triggers are drugs, food and insect bites. Old age and female gender are risk factors for drug- or insect bite- induced allergic reactions. Allergic reactions to food, on the other hand, are frequently encountered among boys younger than 18 years. Cutaneous manifestations are commonly associated with allergic reactions, though systemic symptoms should not be missed and careful clinical assessment is critical to achieve proper management. The presence of concomitant atopic conditions and ethnicity may influence the susceptibility for severe allergic reactions. However, further research is required to investigate the molecular and cellular mechanisms underlying the differences described.

1.3 Symptoms of allergic reactions

Clinical features of allergic reactions vary considerably, ranging from cutaneous and/or mucocutaneous to respiratory, gastrointestinal and/or cardiovascular symptoms. Anaphylaxis has been defined as a life-threatening generalized allergic reaction that can potentially lead to death after contact with the allergen (30, 31). A retrospective analysis of the presenting features of

allergic reactions has been used to construct a grading system for anaphylaxis ((24); Table 1.1). Five hundred forty-five cases with skin features including urticaria, erythema, periorbital oedema and angioedema has been classified as mild reactions. Respiratory, cardiovascular or gastrointestinal features found in 441 cases has been defined as moderate reactions. The strong association of hypotension or hypoxia with signs of neurologic compromise including incontinence, collapse and confusion presented in 139 cases has been defined as severe allergic reactions.

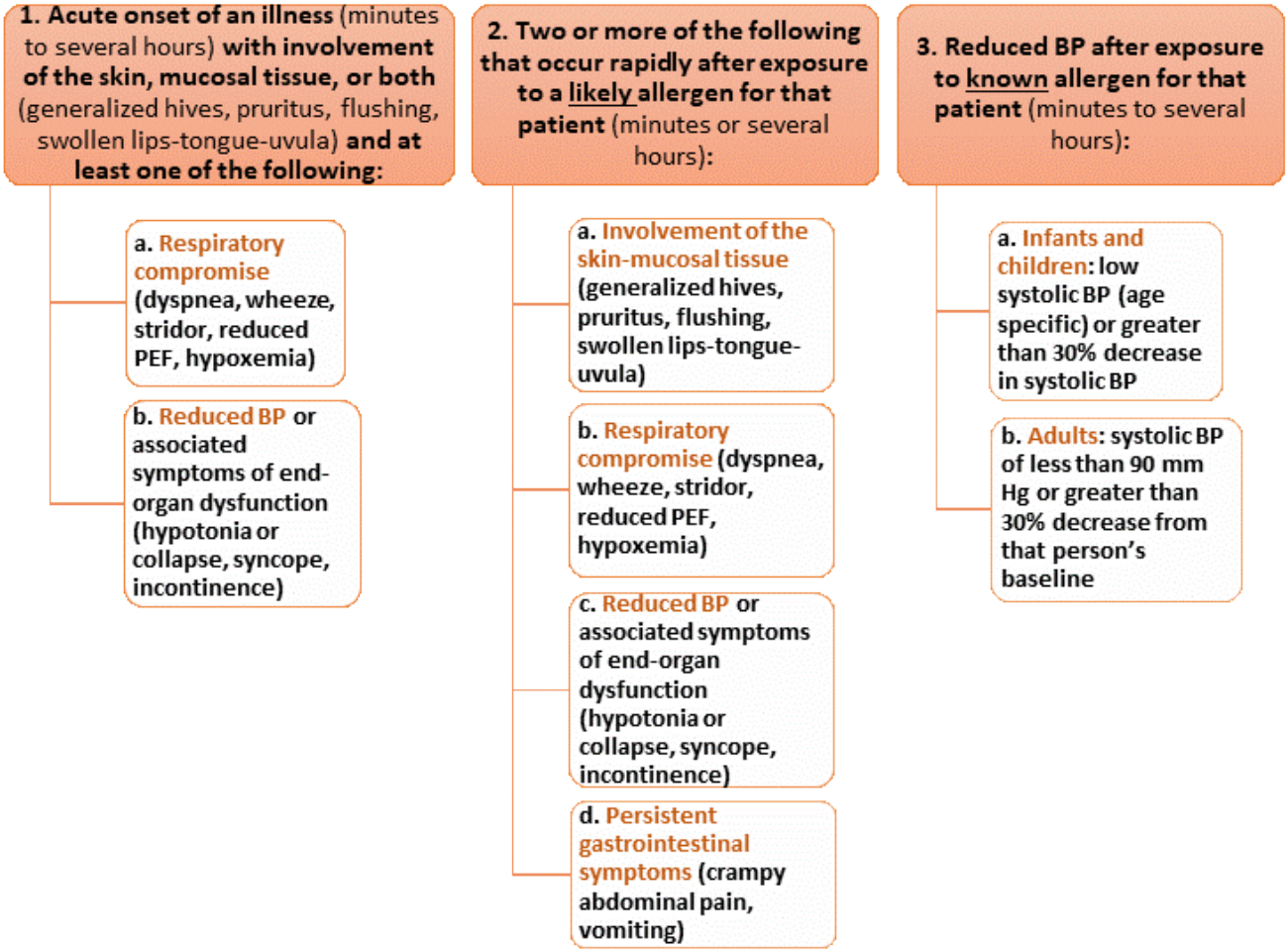
A symposium of the National Institute of Allergy and Infectious Diseases (NIAID) and Food Allergy and Anaphylaxis Network (FAAN) held in 2005 has updated the criteria for clinical diagnosis of anaphylaxis ((30); Figure 1.1). The first feature is an acute onset of skin and/or mucosal involvement with respiratory compromise or reduced blood pressure. The second criterion is the rapid onset of two or more symptoms occurring following the exposure to a potential allergen for that patient. The symptoms include skin and/or mucosal involvement, respiratory compromise, reduced blood pressure or persistent gastrointestinal symptoms. The third criterion is reduced blood pressure following exposure to a known allergen for that patient. When these criteria were applied in a retrospective cohort study of patients who presented to the emergency department with allergic reactions or anaphylaxis, it was found that anaphylaxis was identified with a sensitivity of 96.7 % (95% CI= 88.8%-99.1%) and a specificity of 82.4% (95% CI= 75.5%-87.6%; (32)).

Symptoms and signs of allergic reactions usually develop within minutes after exposure to the offending allergen, however reactions may occasionally take up to one-hour post exposure to develop (33). In a retrospective study of 123 cases of anaphylaxis over a period of 5 years, it was reported that the median time from exposure to development of symptoms and signs of an allergic reaction for all identifiable agents was 10 minutes (34). In a large case series of fatal anaphylaxis during 1992 through 1998, it has been reported the median time to respiratory or cardiac arrest was 5, 15 and 30 minutes for parenteral medications, insect venom and food, respectively (35).

Clinical manifestations of allergic reactions usually resolve after discontinuation of drug or food. In more serious conditions where prompt treatment is required, symptoms resolve within hours. However, 15-20% of the reactions has been reported to follow a biphasic course characterised by remission of symptoms for one to eight hours followed by recurrence of laryngeal oedema or hypotension (36, 37). Therefore, patients should be kept under careful observation following the incidence of severe allergic reactions.

Table 1.1 Grading system for anaphylaxis (adapted from Brown *et al* 2006 (24))

Grade	Defining symptoms and signs
Mild (Cutaneous and subcutaneous features)	Generalised erythema, urticaria, periorbital oedema or angioedema
Moderate (Cardiovascular, respiratory or gastrointestinal involvement)	Dyspnoea, stridor, wheeze, nausea, vomiting, dizziness, diaphoresis, chest or throat tightness, or abdominal pain
Severe (Hypoxia, hypotension or neurologic compromise)	Cyanosis, hypotension, confusion, collapse, loss of consciousness, or incontinence



PEF= Peak expiratory flow
BP= Blood pressure

Figure 1.1 Clinical criteria for diagnosing anaphylaxis (Adapted from Sampson *et al* 2006 (30))

1.4 Pathophysiology of allergic reactions

Allergic reactions could be precipitated by immunological and non-immunological mechanisms. The immunological reactions can be further classified into IgE-dependent and IgE-independent reactions (38). IgE-mediated reactions occur when an individual, who is sensitized, is exposed to the offending allergen. The allergen binds to IgE antibody, which cross-links with the high affinity IgE receptor (FcεRI) on mast cells and basophils. From this point, a series of events orchestrated by mast cells will occur resulting in the symptoms of allergy. Stimulation of mast cells triggers the release of various inflammatory mediators such as histamine, proteases, leukotrienes, prostaglandins, and cytokines. The major effects of these compounds include vasodilation, increased vascular permeability, bronchospasm, hypersecretion of mucous and eosinophil recruitment (39). IgE-independent mechanism involves the activation of mast cell through IgG, complement components, Toll-like receptors ligands, neuropeptides, cytokines, and chemokines (40). Non-immunological reactions occur when some agents trigger the release of mediators from mast cells directly. Examples include physical factors (heat or cold), opioids, NSAIDs and vancomycin (38).

In the present study the focus is on immediate IgE-mediated allergic reactions. Mast cells are considered unique cells having a central role in allergic reactions through the release of a plethora of compounds. The mechanism of IgE-mediated reactions and the biology of mast cells and their mediators must now be considered in order to provide a better understanding of allergic reactions.

1.4.1. Mechanism of IgE-mediated allergic reactions

Initial exposure to the allergen is important for development of subsequent allergic reactions. The allergens are antigenic molecules that are recognised as foreign by the immune system in atopic individuals (41). The most common allergens are drugs, food, insect venom, pollens, and dust mite excretion. Sensitization may occur when an individual is exposed to the allergen via skin contact, inhalation, or ingestion. The allergen is taken up by the antigen presenting cells (APCs) and transported to the adjacent lymphoid tissue, where processed and presented on major histocompatibility complex (MHC) class II to naïve T cells (42). The antigen-MHC class II complex induces the proliferation and maturation of naïve T cells to T helper (T_H) cells, which can

differentiate into T_H1, T_H2, or T_H17 cells. During allergic sensitization, T_H are differentiated to T_H2 (43). It has been suggested that activated T_H secret IL-4, which binds its T cell receptor and mediate the differentiation down T_H2 pathway in an autocrine manner (44).

Allergic sensitization also activates naïve B cells to differentiate and proliferate into plasma cells. The allergen binds to B cell receptors leading to upregulation of MHC class II, which stimulate T_H2 cells. The latter secrete IL-4 and IL-13 leading to antibody isotype switching to IgE production. Specific IgE antibodies migrate from the lymph nodes through the circulation and tissues and bind to FcεRI on mast cells (45).

Following sensitization, allergic response occurs when the allergen is re-introduced to that individual. The allergen cross-links with IgE- FcεRI complexes on mast cells leading to a sequence of downstream events including an increase in intracellular calcium and subsequently to degranulation (46). Mast cells release preformed as well as newly synthesized mediators causing the symptoms of an allergic reaction (45).

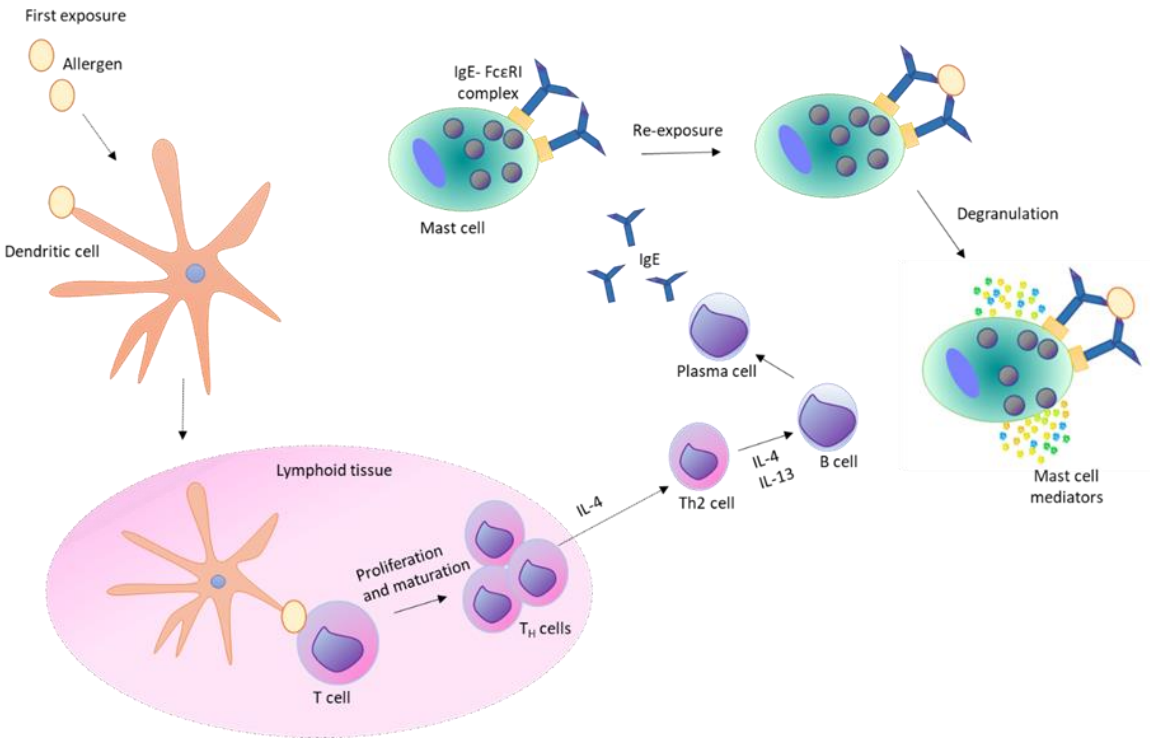


Figure 1.2 Sensitization and IgE-mediated response of mast cells.

Upon initial exposure, the allergen is taken up by the antigen presenting cells (e.g. dendritic cells) and presented to T cells in the draining lymph node. T cells become activated and proliferate to T_H cells. Under the influence of IL-4, T_H cells differentiate to T_H2, which stimulate the differentiation of B cells to plasma cells under the influence of IL-4 and IL-13. These cytokines stimulate isotype class switching to IgE production. IgE binds to FcεRI receptor on mast cells. Upon re-exposure, the allergen binds to IgE- FcεRI complex on mast cells leading to degranulation of mast cells and initiation of allergic response.

1.4.2. Mast cells as major effectors in allergic reactions

Paul Ehrlich first discovered mast cells in the late nineteenth century when he observed "well-fed" cells, or *Mastzellen* in German, in connective tissues. It was the characteristic granular appearance that led to the discovery of mast cells (47). These cells are haematopoietic cells that arise from bone marrow-derived precursors. Mast cell progenitors circulate in the blood before entering various tissues where they develop into mature cells (48). Mast cells are dispersed throughout most tissues but are typically abundant at sites of host-environment interfaces such as skin, lung, gastrointestinal tract and uterus (49). Related to this anatomical distribution, mast cells are considered crucial in the response to environmental insults.

When mast cells settle in the distal tissues, they undergo phenotypic changes that involve alterations in granular content, function and structure. In humans, mast cells exist in two major subtypes based on profile of granular protease content, MC_T and MC_{TC} mast cells. MC_T cells express tryptase whereas MC_{TC} cells express tryptase as well as chymase and CPA3. Schwartz and his colleagues were the first to describe the localization of tryptase to human skin mast cell granules in 1985 (50). Immunohistochemical analysis of tissue sections obtained from the skin, intestine and lung using antibodies directed to tryptase and chymase allowed identification of the presence of tryptase and chymase in variable proportions (51, 52). MC_T cells were found to be present in greatest numbers in the mucosa of the small intestine and alveoli of the lung and MC_{TC} cells were found to predominate in the skin, intestinal submucosa and tonsil. Subsequently, double immunohistochemical staining of human skin, intestine and lung tissues with antibodies specific for tryptase with CPA3 antibodies or for chymase and CPA3 showed the selective localization of CPA3 to MC_{TC} mast cells (53). Therefore, there is heterogeneity of mast cell granular contents in different tissues, but the relationship of these proteases to each other within the mast cell is not clear. In addition, the association of DPPI with the other proteases of human mast cells has not been examined.

Mast cell secretory granules contain a range of preformed compounds. It is important to note that mast cell activation not only leads to the release of preformed mediators, but also triggers *de novo* synthesis of many bioactive substances including cytokines, chemokines and eicosanoids (54). Ultimately, these mediators are released into the extracellular environment and initiate allergic reactions. Table 1.2 lists major mediators of mast cells with their possible pathophysiological roles.

Table 1.2 Major mast cell mediators

Mediator	Type	Key pathophysiologic activities
Histamine	Biogenic amine	Bronchial smooth muscle constrictor, vasodilator, increases vascular permeability, increases mucous secretion, stimulates nociceptive itch nerves, and acts as chemo attractant and stimulus for neutrophils and eosinophils (4).
Tryptase	Serine protease	Growth factor for smooth muscle cells and fibroblasts, recruits neutrophils and eosinophils to the site of inflammation, amplifies mast cell response to environmental insults, cleaves extracellular matrix and fibrin clots, and promotes endothelial cells to form new vessels (55).
Chymase	Serine protease	Alters arterial response to injury, promotes new vessel growth, degrades extracellular matrix protein, alters epithelial cell growth, and increases serous secretion (55).
CPA3	Metallocarboxypeptidase	Protective role against venoms (4)
DPPI	Cysteine protease	Enhance host defense against bacteria via activation of mast cell, neutrophil, and lymphocyte peptidases (tryptase, chymase, neutrophil elastase, granzyme A and B, and cathepsin G;(56))
β-hexosaminidase	Lysosomal enzyme	Lysosomal degradation of glycoproteins (57).
Prostaglandin D2 (PGD2)	Eicosanoid	Smooth muscle contraction and vasodilatation (58).
Leukotrine C4	Eicosanoid	Smooth muscle contraction and increased vascular permeability (58).
Cytokines *	Cytokines	Alter vascular permeability and endothelial adhesiveness (59).

***** Cytokines include tumour necrosis factor (TNFα, β), interleukin (IL)-4, IL-5, IL-6, IL-1β and IL-13

1.4.2.1. Mast cell mediators

1.4.2.1.1. Histamine

Mast cells and basophils are the major cellular sources of histamine (60). Production of histamine from histidine by putrefactive bacteria was first described by Ackermann in 1910 (61)and was later isolated by Sir Henry Dale and his colleagues from the mould ergot (62). They also studied the biological activities of histamine and found that it can stimulate gut and respiratory smooth muscle contraction. In addition, histamine had a stimulant effect on cardiac muscles and caused a shock-like syndrome when injected into guinea pigs (63). The presence of histamine in human tissues was first demonstrated in 1927 by Best et al. (64), and Dale discovered its association with anaphylactic reactions in the following two years by comparing histamine content in the lungs pre and post reaction and showing a marked increase in histamine levels in the blood following anaphylaxis (65). Though it was first identified as a mediator of the anaphylactic reaction in 1932 (66), it took 20 years to establish mast cells as source of histamine (67), and the presence of histamine in basophils was not described until 1972 (68).

Histamine is a diamine synthesized in the Golgi apparatus of mast cells by decarboxylation of histidine and stored in the granules. When mast cells degranulate, preformed histamine is released into the extracellular environment where it is rapidly metabolised by histamine methyl transferase and diamine oxidase (69). Pharmacologically active histamine has a half-life of less than 10 seconds in rats and 20-30 seconds in dogs (70). In human subjects, histamine peak was detected at 5-10 minutes after bee sting-induced anaphylaxis and the marker declined to baseline by 15-30 minutes (71), whereas histamine metabolites in the urine such as methylhistamine were detected up to 24 hours after the allergic reaction (66). Because of its short half-life, the value of histamine as a marker for mast cell activation is limited.

Histamine effects are mediated through H_1, H_2, H_3 and H_4 receptors (66, 72). H_1 receptors mediate different biological responses during allergic reaction including bronchial smooth muscle contraction, vasodilatation, increased vascular permeability, increased glandular secretion, and triggering itch perception. Increased vascular permeability leads to urticaria, which is one of the most common skin manifestations of allergy. Although other mast cell mediators, such as PGD_2, leukotriene C_4, and platelet-activating factor (PAF), can contribute to urticaria, it has been suggested that histamine is the major mediator involved (72). Stimulation of H_2 receptors mediates a physiological role in controlling gastric acid secretion and mediates mucous secretion during allergic reactions (72, 73). H_3 receptors are presynaptic autoreceptors that are involved in controlling histamine synthesis and release from the brain and peripheral tissues (74). H_4

receptors, the last to be discovered is preferentially expressed by peripheral blood leukocytes and through its chemotactic properties can lead to recruitment of mast cells leading to amplification of the immune response (75).

1.4.2.1.2. Tryptase

Tryptase is the major protease stored in mast cell granules (76). Existing as a tetrameric neutral protease of 134 kDa, the enzyme consists of four non-covalently bound subunits of 32-34 kDa (77). Each subunit expresses one active site that faces inward towards the central pore (Figure 1.1). This structural conformation provides protection against macromolecular substrates and enzyme inhibitors (78). Purified skin and lung tryptase preparations in SDS-PAGE showed two diffused bands at 31 and 35 kDa for skin tryptase and one major band at 29 kDa for lung tryptase. However, gel filtration showed molecular weights of approximately 178 and 141 kDa for skin and lung tryptase, respectively (79) consistent with the protease having a tetrameric structure of.

Based on amino acid sequence analysis of human tryptase, two main forms were found, α- and β-tryptase (80). Despite having 90% sequence identity to α- tryptase, β-tryptase appeared to be stored in mast cell granules and not normally secreted unless mast cells were activated (80, 81). Also, its mRNA expression was mainly found in the mast cells extracted from human skin and lung (81). In contrast, α- tryptase is secreted constitutively from mast cells and is predominantly found in the blood of normal subjects (81). However, a recent cohort study included 35 families who had multisystem complaints including flushing and pruritus, joint hypermobility, gastrointestinal irritability and chronic pain has reported that these individuals had duplication in the *TPSAB1* gene encoding α- tryptase in association with elevated baseline levels of tryptase (82).

Within the mast cell granule, processing of β-tryptase precursor involves two proteolytic steps to form the active tetramer. The recombinant precursor form of β-tryptase shows autocatalytic intermolecular cleavage that results in formation of tryptase monomers, which are 50 times less active than the final tetramer. Second, the remaining dipeptide was removed by DPPI at acidic pH to allow mature peptides to form active tetramer spontaneously (83). In both steps, the presence of heparin was found to be essential to stabilize the enzyme (84). Active β-tryptase tetramer is stored under acidic conditions inside the mast cell granule, but upon degranulation it is released in association with heparin to an isotonic media with neutral pH (77). Once dissociated from heparin, the active tetramer forms inactive monomers and this serves as a regulatory mechanism for β-tryptase (77). Experimental studies on purified human lung tryptase showed that the addition of heparin did not restore the tetrameric structure at neutral pH (77). However, activation of tryptase monomers can occur at an acidic pH in the absence of heparin. It was

proposed that this activation occurs in sequential manner involving monomer dimerization followed by the formation and activation of the tetramer.

The biological activities of tryptase, though not well defined, are likely to be mostly proinflammatory (55). The intratracheal administration of a tryptase inhibitor has been found to be able to prevent the development of airway hyperesponsiveness in allergen-challenged guinea pigs and sheep (85). Also, tryptase inhibition has been reported to block airway inflammatory responses in a mouse model of asthma. This effect was evident by the marked reduction in mucous secretion, peribronchial oedema, and tissue eosinophilia as well as in the number of eosinophils in the bronchoalveolar lavage fluid (86). Further support for a proinflammatory role of tryptase has come from mice knockout studies, which have shown that absence of the tryptase mMCP6 is associated with development of less severe experimental colitis (87).

Treatment of a human epithelial cell line with purified lung tryptase has been reported to stimulate increased DNA synthesis which was maximal within 24 hours of treatment. Thus, tryptase appears able to act as a growth factor for the epithelial cells. In addition, tryptase has been found to trigger the release of the granulocyte chemoattractant IL-8 from the epithelial cells (88). Injection of purified lung tryptase into the skin of guinea pigs caused accumulation of eosinophils and neutrophils (89). Similarly, injection of tryptase into the mouse peritoneum stimulated neutrophil accumulation in a dose-dependent manner, while at high doses increased numbers of lymphocytes and macrophages were observed (90). Furthermore, addition of tryptase to human umbilical vein endothelial cells stimulated their IL-8 release and increased mRNA expression for this cytokine and for IL-1β expression (91). Therefore, the protease may be involved in the recruitment of inflammatory cells to the site of mast cell degranulation.

Tryptase has also been found to increase microvascular permeability when purified preparations have been injected into the skin of guinea pigs, an effect likely to be triggered by mast cell activation. Although histamine antagonists abolished the effect, and addition of tryptase to guinea pig skin and lung cells *in vitro* stimulated histamine release (92). Similar findings have been reported in human tonsil mast cells (93) and in synovial mast cells (94). Preincubation of human lung cells with tryptase inhibitors has also been found to prevent IgE-dependent histamine release (95). Therefore, these findings suggest that tryptase can trigger degranulation of neighbouring mast cells leading to amplification of allergen-induced signals.

There is also evidence suggesting that tryptase can degrade fibrinogen and hence may exhibit anticoagulant activity. An experimental study has attributed this activity to tryptase using *in vivo* and *in vitro* models (96), whereas others have found that the anticoagulant activity might be

related purely to the presence of heparin associated with tryptase rather than the intrinsic tryptase activity (97).

During an allergic reaction, β-tryptase is released from activated mast cell in parallel with histamine (98), but it takes a longer time to diffuse away from sites of mast cell activation sites than histamine due to its association with proteoglycan that forms a large size (200-250 kDa) complex (84). During insect sting-induced anaphylaxis, β-tryptase serum levels, unlike histamine serum levels, reached maximum concentrations after 15-120 minutes and declined within 1.5-2.5 hours (71). In clinical practice, this has become significantly useful in cases of anaphylaxis (will be described in Sections 1.6 and 4.1). Serum levels of β-tryptase can be best performed within the first 2 hours of the onset of an allergic reaction and, depending on the reaction severity, β-tryptase can be detectable in the circulation for several hours (71). Tryptase levels of > 10 ng/ml could still be detectable for 6 hours after a peak level of 100 ng/ml and up to 12 hours following a peak of 800 ng/ml.

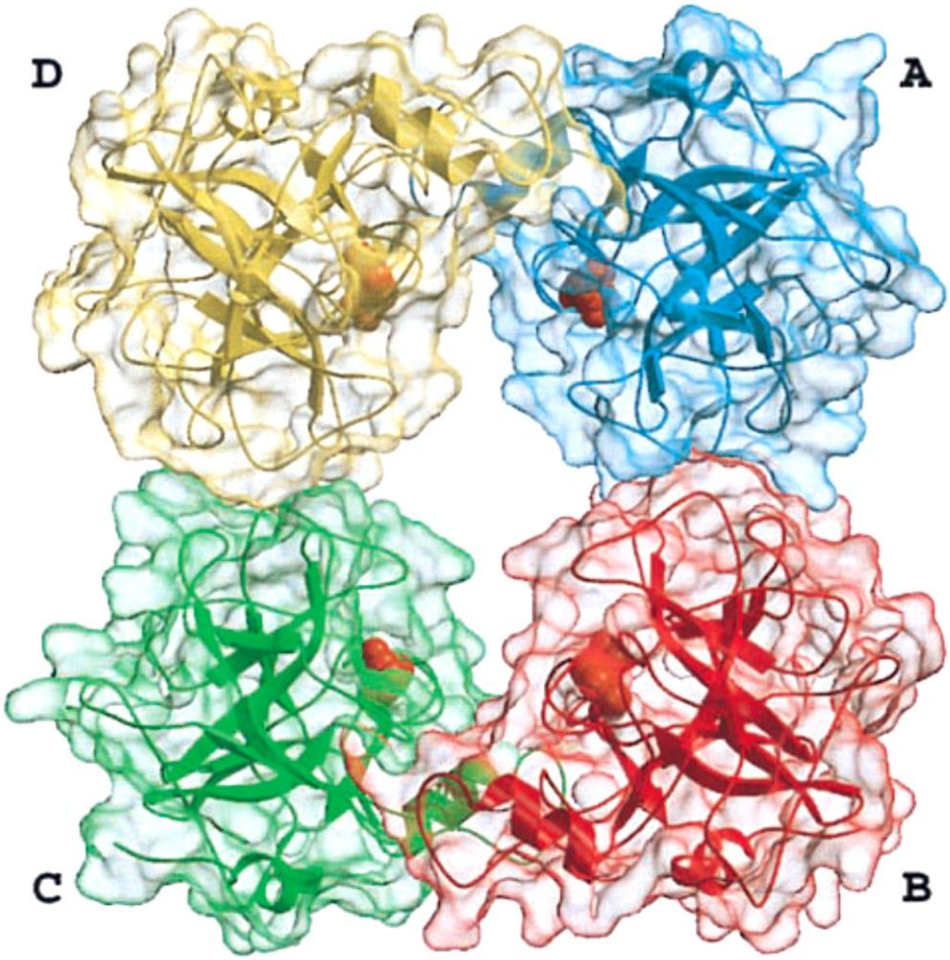


Figure 1.3 Overall tryptase structure.

The four monomers (A-D) are illustrated in blue, red, green and yellow with a globular orange structure at the inner face of each monomer representing the active site (99).

1.4.2.1.3. Chymase

Chymase expression is confined largely to mast cells, which are the only cells to accumulate chymase in secretory granules (100). The chymotrypsin-like serine protease was first reported in rat mast cells in 1959 (101) and was first identified in purified human lung mast cells in 1986 (102). When the cells were challenged with anti-IgE, an angiotensin I-converting enzymatic activity was found, which was correlated with histamine release. Gel filtration demonstrated a monomer with an approximate molecular weight of 30-35 kDa that is distinguishable from tryptase (102). Humans possess a single α-chymase gene while other species including rodents not only have α-chymase gene but also 14 β-chymase genes (103). This diversity in chymase expression among different species contributes to the controversy over its possible biological activity (104). Chymase, like tryptase, needs enzymatic activation before its release from secretory granules. Bone marrow-derived mast cells from DPPI -/- mice contained normal amounts of chymase, but without enzymatic activity. Therefore, DPPI has a significant role in converting chymase to its active form (105).

Chymase activities overlap those for tryptase in some of their targets, though chymase has unique properties. Purified skin chymase is able to convert angiotensin I to angiotensin II, a reaction not mediated by angiotensin converting enzymes as the effect was not blocked by the angiotensin converting enzyme inhibitor captopril (106). Purified skin chymase can also inactivate bradykinin by removing the carboxyl terminal peptide bond (107). Thus, chymase may cause alterations in the arterial caliber in response to injury (108) and this response is critical to compensate volume loss caused by increased vascular permeability during allergic reactions. Therefore, taking angiotensin converting enzyme (ACE) inhibitors may predispose patients to anaphylaxis due to the interference with this protective mechanism (109).

Incubation of cultured bovine airway gland serous cells with purified chymase stimulated their secretion in a dose-dependent fashion and the effect blocked by chymase inhibitors (110). Histochemical study of human bronchi showed that chymase-rich mast cells were located within 20 microns of submucosal glands where secreted chymase can fulfill its potential role as secretagogue (111). In a mouse model of allergic airway inflammation, intratracheal challenge with airway allergen caused elevation of serum chymase MCP-1 within 30 minutes (112). There have been no equivalent studies on the systemic release of chymase in humans, but these findings suggest roles of chymase a potent stimulator of airway hypersecretion and in development of airway allergic inflammation.

Simultaneous intradermal injection of purified dog chymase and histamine into the skin of an allergic dog augmented the size of histamine-induced wheal. Injection of chymase alone into the skin did not cause a wheal formation and pretreatment with chymase inhibitors decreased the allergen-induced wheal size (113). Of note to mention the structural and functional similarity between dog and human chymase thus, chymase can modulate the allergic response working in conjunction with other mast cell inflammatory products. Furthermore, chymase was found to degrade pericellular matrix of cultured human airway smooth muscle and markedly reduce the rate of cellular proliferation (114). Chymase MCP-4 knockout mouse had defective processing of pro-matrix metalloprotease to its active form. This caused accumulation of collagen and fibronectin in the mouse tissues (115). These findings suggest roles of chymase in airway remodeling and connective tissue turnover.

Mice knockout studies have shown that absence of chymase affected mast cell recruitment after *Nippostrongylus brasiliensis* infection (116)and caused defective clearance of *Trichinella spiralis* (117). Chymase also may have a role in regulating bacterial infection as suggested by Orinska *et al* (118). Compared to wildtype mice, IL-15 deficit animals showed better survival rate in sepsis induced by cecal ligation and this effect was largely attributed to chymase suppression by mast cell IL-15 (118). Although these reports have suggested a protective role for chymase in rodents, perhaps it might be hazardous to speculate the same in humans especially with the presence of wide range of chymase genes in rodents compared to humans.

It is of note to mention that chymase is readily cleaved by circulating and extravascular anti-peptidases and hence may have shorter active life after secretion (55). Though chymase has been investigated widely as a marker for allergic reactions in animal models, there have been no available assays to measure chymase levels in human body fluids.

1.4.2.1.4. Carboxypeptidase A3 (CPA3)

CPA3 was first identified in 1987 when a carboxypeptidase activity was detected in human lung and skin mast cells (119). The enzyme was released in correlation with histamine after mast cell stimulation with anti-IgE, suggesting its localization to mast cell granules. Also, skin mast cells had higher content of the enzyme than that of the lung mast cells. CPA3 was later purified from skin extract and was found to have a molecular weight of 34.5 kDa on SDS-PAGE with optimal activity achieved at neutral to alkaline pH (120). The enzymatic content was estimated in neonatal foreskin, adult facial skin and adult foreskin and was found to be 0.5, 5 and 16 µg/10^6 mast cells, respectively (120).

Immunohistochemical study of mast cells in different human tissues showed that CPA3 was abundant in MC_{TC} subclass (53). Gene analysis for human mast cell CPA3 showed the presence of single gene localised to chromosome 3 and the mRNA expression was prominent in dispersed human lung cells but not in other nontransformed cell lines (121). Therefore, CPA3 is restricted to mast cells and can be used as a marker for mast cell degranulation.

CPA3 has a high content of positively charged amino acid residues, which mediate the interactions with the negatively charged serglycin proteoglycans (122). Bone marrow-derived mast cells from heparin-deficient mice showed lack of active CPA3 compared to wildtype. However, the protein expression was intact in both wildtype and heparin-deficient mice (123). In addition, gel filtration and affinity chromatography human skin extract separated tryptase-proteoglycan complex (200-250 kDa) from chymase and CPA3 (400-560 kDa) (124). The incubation of low density lipoproteins (LDL) with chymase and CPA3 resulted in complete degradation of the peptide; and inhibition of either proteases resulted in incomplete proteolytic degradation (125). Taken together, heparin has a vital role in CPA3 storage but not gene expression and it forms a complex with both CPA3 and chymase that is distinct from that of tryptase-heparin complex. Also, chymase and CPA3 act in a cooperative manner once released from mast cell. Chymase cleaves the carboxyl side of the protein leaving a new COOH-terminal for CPA3 to process further (Figure 1.4).

Like tryptase and chymase, CPA3 needs processing to form active protease, though the mechanism of CPA3 activation is not entirely clear. Based on amino acid sequence analysis of the proteases within murine mast cell granules, CPA3 was found as zymogen (pro-CPA3) (126). A mast cell line (KiSV-MC9) identified to produce pro-CPA3 but lack the capacity to process it to the active form despite being able to activate pro-tryptase and pro-chymase. This suggested the presence of a separate mechanism involved in the activation of CPA3. Mast cells derived from cathepsin E-deficient mice displayed accumulation of pro-CPA3, but recombinant cathepsin E processed recombinant pro-CPA3 in an *in vitro* conditions resemble that of mast cell granules (127). This indicates a potential role of cathepsin E in the activation of CPA3. Higher levels of CPA3 protein activity and expression were detected in mast cells derived from DPPI- null mice (128). These findings indicated role of DPPI in regulating the storage of CPA3.

CPA3 has a role in processing angiotensin I to angiotensin II. The addition angiotensin I to mouse peritoneal cells with subsequent activation of mast cells resulted in the formation and degradation of angiotensin II (129). Peritoneal cells derived from heparin-deficient mouse showed marked reduction in angiotensin formation, whereas in chymase-null cells no reduction of angiotensin II was seen. However, inhibition of CPA3 in chymase-null cells resulted in complete

obliteration of angiotensin II formation. Therefore, both mediators have key role in processing angiotensin I independently of angiotensin converting enzymes.

Other roles for CPA3 have been investigated. The intraperitoneal administration of snake venom into mast cell-deficient mice induced death at 5 μg of the venom, whereas in wildtype mice the lethal dose was 50 μg (130). Also, wildtype mice had lower levels of the toxic component (sarafotoxins) than their counterpart. Pre-treatment of the wildtype with CPA3 inhibitor caused increase in the intraperitoneal levels of sarafotoxins and death occurred with one hour. This indicates a protective effect of CPA3 against snake venoms.

1.4.2.1.5. Dipeptidyl peptidase I (DPPI)

DPPI (or cathepsin C) is a cysteine protease found in myelomonocytes, cytotoxic T-cells, neutrophils and mast cells (131). Purified DPPI from dog mastocytoma cells had a molecular weight of approximately 175 kDa and consisted of four subunits (131). In a subsequent study of the structure of human recombinant DPPI a tetrameric structure was also reported with the active sites on four identical subunits exposed to allow catalysis of proteins (132). Mast cells were found to be a major source of DPPI in dog airways, whereas macrophages were the major source in alveoli (133).

DPPI acts intracellularly to cleave amino-terminal dipeptides from variety of targets leading to the activation of some proteases. Inhibition of DPPI activity in a mastocytoma cell line has been reported to be associated with a 90% reduction in the activity of tryptase (134). Treatment of recombinant pro-chymase with DPPI resulted in the formation of enzymatically active chymase (135). It has also been suggested that DPPI has an important role for controlling levels of CPA3 (128). However, the exact localization of DPPI within human mast cells is still not clear. DPPI has been found to be released from dog mastocytoma cells in response to calcium ionophore or substance P stimulation in parallel with tryptase and histamine release (131). In addition, immunohistochemical studies of dog lung tissue with polyclonal antibody suggested that DPPI caused proteolytic cleavage of the extracellular matrix proteins (133). These findings suggest extracellular roles for DPPI, but equivalent studies on human mast cells are still lacking.

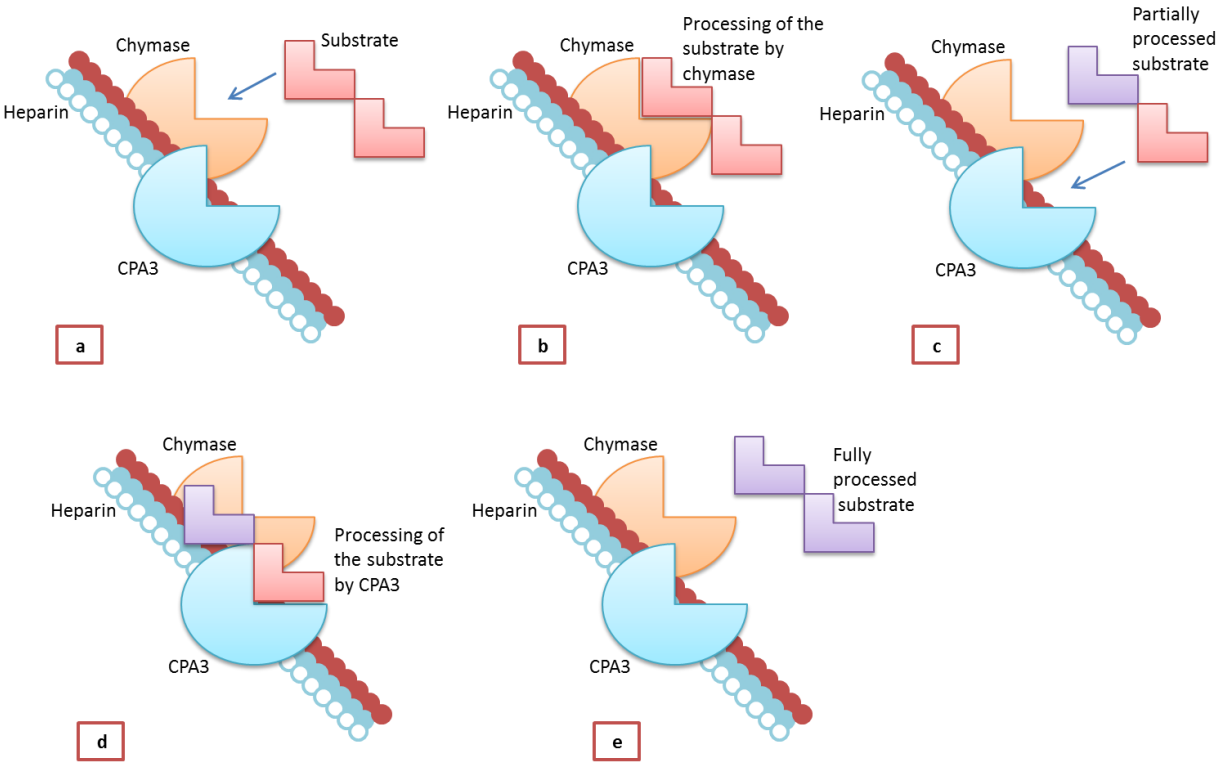


Figure 1.4 Hypothetical model of the cooperative action between CPA3 and chymase complexes for processing of substrates.

Both enzymes are bound to heparin forming a complex. (a-c) Chymase partially process the substrate (or protein) leaving a carboxyl terminal for CPA3 (c-e) to act on leading to full degradation (adapted from Pejler *et al* 2009 (136))

Having a critical role in activating certain peptidases of mast cells, neutrophils, and lymphocytes, DPPI can modulate immune responses against bacteria (137). However, it has been reported that *DPPI-/-* mice have better survival following induction of septic peritonitis (138). This has been attributed to reduced DPPI cleavage of certain protective cytokines, particularly IL-6. A subsequent report has reinforced this theory by demonstrating specific roles for mast cell IL-6 in increasing the survival rate of *DPPI-/-* mice against gram-negative peritonitis and pneumonia by augmenting intracellular neutrophil killing of bacteria, though another protective mechanism in these mice could involve the presence of increased levels of surfactant protein D (139).

Papillon-Lafevre syndrome (PLS) is a rare autosomal recessive disorder caused by complete or partial loss in DPPI function (140). It manifests clinically with palmoplantar hyperkeratosis and periodontitis. Despite impaired activation of neutrophil-derived proteases, lymphocytic counterpart compensation has been reported to help maintain a sufficient immune response (141). Furthermore, the cytokine profile of PLS patients has been reported to not be significantly different from that of normal individuals (142). The studies of DPPI function described must raise the question as to whether PLS patients will be less affected by sepsis or anaphylaxis, and subjects with PLS may represent an intriguing human model in which to explore the consequences of reduced DPPI function on health and disease.

1.5 Cofactors of allergic reactions

Though allergic reactions are solely triggered by exposure to an allergen, there have reports suggesting a role of additional factors, co- or augmentation factors, for eliciting or influencing allergic reactions. These factors include physical exercise, alcohol consumption, co-administration of NSAIDs and infections. Thirty percent of anaphylaxis cases from all causes has been reported to be associated with cofactors (143). It has been estimated that exercise and alcohol consumption were relevant co-factors for up to 22.2% (144) and 15.2% (145) of anaphylactic reactions, respectively. Severe anaphylactic reactions have been reported to be associated with concurrent intake of NSAIDs in 1.2-4.7% of cases (146). There has been controversy regarding the role of infection as a cofactor of allergic reactions. Some reports have suggested that infection was an eliciting factor of anaphylaxis in 2.5% of paediatric cases (145) and in 11% of adult anaphylactic reactions (36). However, there were no details provided on the types of infection. On the other hand, in experimental studies on the effect of infection on mast cell activation and degranulation *in vivo* and *in vitro*, it has been reported that infection can be associated with suppression of IgE-

mediated activation of mast cells (147-152). These studies have employed non-pathogenic bacteria to investigate the release and expression of mast cell mediators.

1.5.1. Exercise

Exercise was first reported to be a cofactor of allergic reactions by Maulitz *et al* in 1979 (153). They have reported a significant association between the ingestion of shellfish and the symptoms experienced following exercise ($p<0.001$). Doing physical exercise or eating shellfish alone was not associated with any allergic symptoms. Subsequently physical exercise has come to be well established as a cofactor in food-induced allergic reactions, a condition known as "food-dependent exercise-induced anaphylaxis". Numerous reports have described the association between exercise and ingestion of foods like pistachio, meat, spinach, wheat and shrimp ingestion (143).

There has been a suggestion that exercise can enhance the bioavailability and distribution of certain allergens. In a study of six patients with wheat-dependent exercise-induced anaphylaxis who ingested wheat, or underwent exercise, or who did both, it was reported that there was an increase in serum levels of gliadins (major wheat allergens) during wheat-exercise challenge. The increase in levels of allergens in the circulation was associated with the onset of allergic symptoms including urticaria, erythema, dyspnea, and/or shock (154). The effect of exercise on the bioavailability of allergens has been related to an increase in the permeability of the small intestinal during intense exercise (155). Exercise has been also shown to influence mast cell responses. Thus in cases of wheat-dependent exercise-induced anaphylaxis it has been reported that enhanced expression of tissue transglutaminase in the intestinal mucosa of patients during exercise could modify wheat proteins leading to formation of large aggregates, which allow more effective cross-linking of IgE-FcεRI complexes on mast cells (156).

1.5.2. Alcohol consumption

Alcohol consumption has been reported to trigger food-induced allergic reactions in about 10% of patients (157). A case report of a 18-year-old woman who suffered several attacks of anaphylaxis has indicated that associated consumption of alcohol and grape allergens induced an anaphylactic reaction, whereas consumption of either did not trigger any allergic reaction despite having a positive skin prick test to grape and alcohol (158). It has been suggested that the tight junctions in

the gastrointestinal epithelium could become relaxed allowing an increase in the intestinal absorption of the allergens (159).

1.5.3. NSAIDs

NSAIDs were first identified as a cofactor for anaphylaxis in 1984 (160). Cant and Gibson have reported that a 14-year-old boy who had peanut allergy and had taken aspirin on several occasions with no harmful effects but had developed life-threatening allergic reaction following ingestion of peanut containing food in combination with aspirin. Non-immunological stimulation of mast cells might contribute to the role of NSAIDs as cofactors of allergic reactions. They inhibit cyclooxygenases (COXs), which mediate the conversion of arachidonic acid into thromboxanes and prostaglandins. This results in metabolism of arachidonic acid towards the 5-lipoxygenase pathway leading to an increase in the release of cysteinyl leukotrienes, which can induce bronchoconstriction, vascular leakage and eosinophilia (161). However, a role for NSAIDs in augmenting allergic reactions has been reported in experimental study in 1976 (162). Flemström *et al* have reported that intragastric administration of dextran to passively sensitized guinea pigs caused no reaction, whereas co-ingestion with aspirin induced anaphylactic reaction. More recent study has also demonstrated that ingestion of aspirin and wheat can provoke the development of allergic symptoms in patients with wheat-dependent exercise-induced anaphylaxis (154). The authors suggested that the absorption of the allergen was upregulated by aspirin administration.

1.5.4. Infection

There have been reports suggesting that infection can augment anaphylaxis. Two case reports in which systemic allergic reactions following respiratory infections have been described (163, 164). In one of these there was the development of repeated episodes of anaphylaxis following coryzal symptoms, and in the other a severe viral infection triggered an anaphylactic reaction in a patient undergoing specific immunotherapy. In a clinical trial of 25 children with food allergic reactions, it was found that 12 patients had a lower threshold for allergic reactions mainly during an infection (165). The underlying mechanism whereby infection may augment allergic reactions is still poorly understood.

Ethical considerations would restrict allergen challenge of patients with a history of severe allergic reactions during an infection, but the effects of infection on allergic reactions in animal models

have permitted investigation of potential underlying mechanisms at the cellular and molecular level (166-168). Thus, it has been reported that mice infected with influenza A virus developed cutaneous anaphylaxis after re-challenge with flu antigen (166). The author has suggested that the structures of pathogens could serve as allergens themselves and elicit an allergic reaction.

On the other hand, an opposite effect of infection on allergic reactions has been suggested, particularly in bacterial infection. Prenatal administration of *Lactobacillus GG* to mothers who had a strong family history of atopic diseases, was associated with a 50% lower frequency of atopic diseases in children compared to those born to mothers who had been treated with placebo rather than the probiotic (169, 170). Similar observations have been reported in mouse model of allergic hyperresponsiveness. Offspring of *Lactobacillus GG*-supplemented mice sensitized to ovalbumin and challenged with allergen had significantly less peribronchial inflammation and goblet cell hyperplasia compared to those derived from non-supplemented mice (148). Though these studies indicate that probiotics suppress the development of allergy by enhancing the maturation of the immune system, there has been another report that probiotics may actually have an impact on allergic reactions in sensitized mice. Daëron and colleagues found that pre-sensitized mice challenged with the allergen had a significant reduction in the symptoms of anaphylaxis when injected with *Lactobacillus casei* compared to the mice injected with phosphate buffered saline (171). When these authors added *Lactobacillus casei* to sensitised mouse bone marrow-derived mast cells prior to allergen challenge, it was found that the release of mast cell mediators including β-hexosaminidase, cytokines and growth factors was reduced, an effect attributed to direct contact between the bacteria and mast cells.

The relationship between bacteria and mast cell activation has been analysed by several studies using different strains of bacteria. Incubation of murine mast cells with non-pathogenic *Escherichia coli* has been reported to downregulate IgE/allergen-induced and calcium ionophore-induced degranulation of mast cells by 70% and 90%, respectively (147). In that study it was also reported that there were no significant differences in the viability of mast cells between infected and non-infected cells. Co-incubation of the cells of human mast cell line LAD3 with non-pathogenic *E. coli* has been reported to cause bacterial internalization and to modulate the expression of genes encoding transcription factors, cell signalling molecules, cell cycle regulators, and proinflammatory cytokines (172). Metcalfe and colleagues have also suggested that the decrease in IgE-mediated degranulation might be due to the downregulation of FcεRI surface expression that has been observed in response to co-incubation of mast cells with *E. coli*. However, recovery of the FcεRI surface expression has been reported after exposure to *Lactobacillus* despite suppression of degranulation of mast cells (152). There have been investigations to study the impact of bacterial infection on mast cell responses via non-FcεRI

activation. It has been suggested that the release of serotonin from mast cells in response to calcium ionophore was inhibited by 50%-70% after incubation with *E. coli* (147). This indicates that the impact of bacterial infection on mast cell responses affects mechanisms downstream of FcεRI engagement.

Staphylococcus aureus is an important human pathogen that causes infections ranging from local skin involvement to systemic sepsis. It has been estimated 30% of the human population is nasal carrier for *S. aureus* (173) and 2-3% of community -acquired pneumonia is caused *S. aureus* (174). It has been demonstrated that *S. aureus* was the most common pathogen identified in biofilms from patients with chronic rhinosinusitis (175). In studies of the interaction between *S. aureus* and mast cells have indicated that incubation of *S. aureus* with murine mast cells can stimulate degranulation and release of antimicrobial compounds resulting in killing of almost all extracellular bacteria (176). Immunofluorescent staining has showed that *S. aureus* could subvert this extracellular eradication by internalizing and surviving within mast cells. This observation has been also demonstrated in a murine model of staphylococcal skin infection. In addition, it has been reported that *S. aureus* manipulate mast cell biology including gene expression of global regulators like *sarA* and *arg* to ensure their intracellular survival (177). Matsui and colleagues have reported that stimulation of mast cells with peptidoglycan derived from *S. aureus* can strongly enhance the production of INF-γ from activated T_H cellsco-cultured with mast cells (178). This effect was suppressed when the cells were treated with the macrolide antibiotic josamycin. That study suggested that *S. aureus* interaction with mast cells could mediate the development of a T_H1 rather than a T_H2 response. However, the author did not include evidence for mast cell degranulation by measurement of β-hexosaminidase or histamine release in response to bacterial infection. That study also did not compare the effect of *S. aureus* on mast cell responses with other IgE/non-IgE derived stimuli. On the other hand, it has been reported that though co-incubation of rat mast cells with peptidoglycan from *S. aureus* failed to induce mast cell degranulation and histamine release, it could stimulate significant *de novo* synthesis and release of many pro-inflammatory cytokines when compared to calcium ionophore A23187-induced generation (179). It is important to note that studies investigating the effect of *S. aureus* on mast cell-drivenimmune responses in the presence of IgE/non-IgE dependent stimulation are limited. Such investigations would reveal whether *S. aureus* can augment or inhibit T_H2 responses and allow a better understanding of the potential effect on the development of an allergic reaction.

1.6 Management of allergic reactions

Diagnosis: The first step in the management of allergic reactions is to establish an accurate diagnosis and that is extensively dependent on detailed medical history. Identification of the agent provoking symptoms, route of entry into the body, the dose in the case of medications, duration, previous contact and time course of the reaction are all important. Physical examination is also critical to detect any skin rashes, facial swelling, or other allergy-related symptoms and vital signs, which is especially important in the case of life-threatening conditions.

Being able to decide which laboratory test is most appropriate for the condition is a challenge by itself and it is largely dependent on the underlying mechanism, which for most instances, is not clear. Moreover, the limited availability of biochemical laboratory tests has added another hurdle in the way of accurate diagnosis of allergic reactions.

Diagnostic tests for allergic reactions are:

Measurement of specific IgE antibodies to food, drugs, and insect stings. Circulating allergen-specific IgE antibodies are determined *in vitro* by radioallergosorbent test (RAST) (180). Though the test has often been described as a sensitive diagnostic test, but there have reports indicating its low specificity. It has been reported that the sensitivity of specific serum IgE to predict hen's egg and cow's milk allergies was 97% and 83%, respectively, whereas the specificity was only 51% for hen's egg and 53% for cow's milk (181). It has been also suggested that a positive test does not necessary correlate with clinical symptoms of the patients. Levels of specific IgE antibodies have been reported to be significantly higher in patients with asymptomatic allergy than those in healthy non-allergic subjects (182). In addition, the test has limited use in drug-induced allergic reactions as most drugs are not completely immunogenic and require a carrier protein, which made RAST less sensitive (183). Antigenic drug metabolites are difficult to be identified and hence a negative RAST does not adequately exclude drug allergy.

Skin prick test is a highly sensitive and specific test (184) in which a small amount of the implicated allergen extract is introduced under the skin. The principle behind skin tests is the introduction of localised cutaneous reactivity when the allergen bound to IgE-FcεRI on mast cells leading to degranulation with the resultant skin reaction. Allergic subjects develop a red flared skin, or a wheal. The test can result in a variety of wheal shapes but taking the average diameter after 15 minutes is commonly performed to characterise the wheal. Saline and histamine are injected as negative and positive controls (185). It is generally accepted that a mean diameter of 3mm greater than the negative control is a positive test (186). It has been reported that skin tests had sensitivity of 80-97% and specificity of 70-95% to diagnose inhaled allergens. Using skin tests

to diagnose food allergy has been reported to have lower sensitivity (30-90%) and specificity (20-60%) (185). This could be related to the type of extract and technique used in the skin tests. The value of skin tests in the diagnosis of drug allergy depends on the drug tested and, in most cases, a negative skin prick test does not necessarily exclude the possibility of future reactions. However, it has been reported that patients with negative skin prick test to penicillin had no reactions when challenged with the drug (187). On the hand, some drugs might cause false-positive skin tests including NSAIDs and opioids due to direct interaction with mast cells rather than IgE-mediated reaction. In addition, some agents can cause skin irritation, which makes skin test interpretation more difficult (for example; chlorhexidine).

Challenge or provocation tests involve the controlled administration of the food or drug to diagnose hypersensitivity. This test is particularly important when skin prick test or specific IgE test results are inconclusive. It is also necessary to test alternative or pharmacologically related compounds for example challenging with COX2 inhibitors in cases with known NSAIDs hypersensitivity (188). Food challenges involve exposing the patient orally to incremental doses of the implicated food whilst monitoring for any signs or symptoms of allergic reactions. Provocation tests for drugs is performed by injecting the allergen into the dermis or administering it orally if appropriate. When no reaction is observed after 20 minutes, the dose of the drug is increased. A positive reaction is composed of raised skin, though systemic reaction may occur. Intradermal tests can also be performed to diagnose insect bite allergic reactions (189). Oral, inhaled, or injected drug challenges involve administration of the drug in small doses and increasing it gradually till a normal dose is given. The observation period is extended for longer time to allow detection of any symptoms. Limitations of such tests are the potential risk of provoking a life-threatening reaction to the allergen administered, and the high costs of providing appropriately trained medical specialists to carry them out with resuscitation facilities being provided (190).

The basophil activation test (BAT) is based on identification of basophil specific markers (CD63 and CD203c) after allergen-induced activation using flow cytometry as a tool of analysis (191, 192). Using only small amount of blood, BAT serves as an indicator of IgE sensitization with high sensitivity and specificity. It may be valuable in situations where there are discrepancies between blood and skin test results. BAT has been validated to diagnose allergic reactions to peanut, egg (193), β-lactam antibiotics (194, 195), NSAIDs (196), muscle relaxants (197), latex (198), inhalants (199), pyrazolones (200), dust mite (201), grass pollen (202) and hymenoptera venom (203). However, using whole blood might reduce basophil recovery rate and the presence of some serum components can interfere with the test sensitivity or can even lead to non-specific activation (204). Platelets aggregates expressing CD63 can also cause inaccurate flow cytometry counting (204).

Histamine release assay is a glass fiber-based fluorometric method based on the *in vitro* activation of basophils by the culprit allergen and quantification of the released histamine fluorometrically. However, it has been suggested that some individuals could be non-releasers and that affected the sensitivity of the test (205). In addition, samples should be processed with 24 hours of collection.

Mast cell proteases can be used as indicators for the occurrence of allergic reaction and mast cell degranulation. National institute of health and care excellence (NICE) guidelines has recommended the diagnostic utility of serum tryptase levels in anaphylaxis (188). Tryptase levels can be detected within 15 minutes of anaphylaxis and last for 2.5 hours hours, depending on the severity of the reaction (71). Therefore, it has been recommended that serial measurements of serum tryptase levels should be made, so as to distinguish anaphylaxis from other conditions with similar clinical course. An acute rise in tryptase level usually correlates with the severity of the reaction and highlights the importance to identify the causative drug and any potential cross-reaction with other related agents to reduce further risk (5). However, a retrospective study has reported a weak correlation between serum tryptase levels and the severity of anaphylaxis (206). In fact, some patients with mild reactions had raised acute tryptase levels (≥14 μg/L) (206). Furthermore, Ordoquie *et al* showed that serum tryptase peak varied considerably among patients with similar clinical reactions ranging from 30 minutes up to 6 hours and was independent of the symptoms severity (207).

Serum measurement of other mast cell proteases like chymase and CPA3 alongside with tryptase might be a better approach to confirm the diagnosis of severe allergic reactions. It has been reported that serum CPA3 levels increased significantly in anaphylaxis cases even when tryptase levels were normal (208). In addition, measurement of baseline levels of CPA3 and its relationship with other biomarkers and with severity of historical allergic reactions have not been investigated so far.

Treatment: Oral antihistamines are given to treat flushing and itching symptoms of allergic reactions. Systemic or topical corticosteroids are potent anti-inflammatory agents that can also be used to relieve allergic symptoms (209). In cases of anaphylaxis, rapid assessment of airway, breathing and circulation is critical. Adrenaline is considered the first line of therapy and delayed administration has been shown to be associated with increased morbidity and mortality (210). It is either administered as intravenous infusion or intramuscular injection (0.01 mg/kg; max 0.3-0.5 mg) repeated 2-3 times at 5-15-minute interval (211). The patients should be kept under observation for at least six hours in the emergency department.

Upon discharge, the patients are provided with epinephrine auto-injector and advised to wear medical identification like bracelet or necklace that indicate their condition. The patient is also advised to avoid exposure to the implicated allergen. Avoidance plans should take into consideration individual factors including age, activity, occupation, access to medical care and patient's mental health. Patients with drug-induced allergic reactions should be informed about possible cross-reactivity between medications. In cases where there is a lack of alternatives and the patient is in serious need for the drug, such as penicillin treatment for meningitis or bacterial endocarditis, then desensitization may be considered (212). Patients who have had allergic reactions to food should be instructed to read food labels for possible hidden allergens. Desensitization has been suggested as an alternative plan of treatment of some food-induced allergic reactions. It has been recently reported that introduction of peanut early in life could significantly reduce the prevalence of peanut allergy at 60 months of age compared to the infants who were avoiding peanut (213). It has been also reported that desensitization for oral milk could be achieved in 36-92% of children (214), though permanent tolerance is unknown. Avoidance measures are also recommended for patients who have had insect sting-induced allergic reactions. These measures include being alert and wearing proper clothing during outdoor activities. It has been suggested that immunotherapy had success rate of 98% of patients (33).

Taken together, management of allergic reactions largely relies on assessment of the condition clinically, by taking detailed history and examination, and immunologically, by applying the available immunological and biochemical tests before appropriate treatment plan can be approached. There is a need for new biomarkers that can help to predict the severity of allergic reactions. The combination of the clinical and immunological features of patients who have allergic reactions may assist to define distinct endotypes that can aid in the diagnosis.

1.7 Hypothesis and aims

Our hypothesis is that mast cell proteases as key mediators involved in the development of allergic signs and symptoms may act as useful biomarkers of disease severity. With new means for detection of mast cell proteases, our aim has been to investigate:

1. Mast cell proteases as potential predictive markers of severe reactions in different forms and categories of allergic reactions.
2. The effect of *S. aureus* infection on mast cell-driven immune responses.
3. The subcellular co-localization of mast cell proteases.

These studies have involved the collection of samples from 313 patients with allergic reactions in two geographically distinct populations, the development, validation and application of assays for mast cell proteases and analyses of large data sets using topological data analysis. Cells of the LAD2 mast cell line were co-incubated with *S. aureus* and the changes in release and expression of mast cell mediators were monitored. Immunolocalization studies were performed to investigate the subcellular localization of mast cell proteases.

Chapter 2: Materials and methods

The materials were purchased from Sigma-Aldrich, Dorset, UK unless stated otherwise.

2.1 Subjects and sample collection

To determine the changes in levels of mast cell mediators during allergic reaction and to investigate whether baseline levels of these mediators may be used as tools to predict severity of a reaction, participants undergoing drug challenges were recruited from the Asthma and Allergy clinic at University Hospital Southampton. Samples collected from previously recruited subjects attending the clinic for drug or food challenges were included in the study. Blood, saliva and urine were collected before and following drug or food challenge (Figure 2.1).

The present study also included participants recruited from outpatient clinic by a separate study team at Queen Medical Hospital in Doha, Qatar. Subjects with allergy-related conditions were consented and their demographic information were reported. Blood, saliva and urine samples were collected at the time of the visit.

Criteria for inclusion in the study were patients with history of (i) drug, food or insect allergic reactions, and (ii) not on any concurrent antihistamine or corticosteroid therapy. Patients on such medications were excluded due to the potential interference with the course of an allergic reaction. Patients with chronic urticaria, drug reaction with eosinophilia and systemic symptoms (DRESS), delayed hypersensitivity reactions were also not included in the study. For the Qatar cohort study, the inclusion criteria were expanded to those with asthma, allergic rhinitis and atopic dermatitis.

Healthy non-atopic subjects were recruited to serve as controls for the study. Subjects with history of allergy to food, drug, or insects were excluded. If the subject agreed to participate in the study, samples including blood, saliva and urine were collected.

The study was approved by the Isle of Wight, Portsmouth and South East Hampshire Region Ethics Committee (registration number 08-H0501-17), and by the University Hospital Southampton NHS Foundation Trust Research and Development Department.

2.1.1 *Southampton cohort study*

Southampton cohort study included subjects undergoing food or drug challenges at the Asthma and Allergy clinic at University Hospital Southampton.

For drug challenge test, samples were collected from patients, aged 18 to 86 years. The testing would be carried out against therapeutic agents if the clinical history and specific IgE were insufficient to establish a diagnosis. The challenge testing included skin prick test (SPT), intradermal test (IDT) and oral challenge.

The patient was first exposed to the suspected agent by doing SPT in which a drop of the agent was placed on the inner forearm and a prick was made through the drop by the tip of a lancet. The skin was marked clearly using marker pen with agents to be tested. Histamine was used as a positive control. SPT involved using two doses for each drug with 15-minute observation. If the wheal was less than 3 mm, then the test would be regarded as negative and IDT would be performed. However, in case the wheal was larger than 3 mm, the challenge would be discontinued and the outcome considered to be a positive.

IDT is performed by injecting a small amount of the index agent into the dermis and observe the reaction for 15-20 minutes. The test starts with the lower concentration of the drug and increases the dose gradually. Typical positive reaction would be a small hive with local redness and swelling. If IDT was positive then no more agents would be introduced. Negative reactions would be either followed by oral challenge or the results would be satisfactory for the agent in question.

Regarding oral drug challenge, there were certain protocols for each group of drugs but generally there was a 30 minutes observation period between each dose, followed by observation period of two hours. A positive reaction varied from skin rashes to systemic reaction; however, the oral doses are optimised to have as minimum systemic symptoms as possible.

For food challenge test, samples were collected previously from participants aged between seven to 40 years old. The challenge is performed when medical history and alternative allergy tests, like SPT or specific IgE, are inconclusive. Oral food challenge involved giving the patient incremental doses of the tested food at 15 minutes intervals with careful assessment of any signs and symptoms of a reaction. The challenge was followed by two-hour observation period in case of a delayed reaction. If the subject reacted to the index food, the challenge was immediately discontinued with administration of appropriate treatment and the outcome reported as positive.

2.1.2 *Qatar cohort study*

Participants aged between two- and 60-years old suffering from allergy-related conditions including recent episode of anaphylaxis, drug or food allergies, atopic dermatitis, hay fever, asthma, or insect bite allergy were recruited from the outpatient clinic. After the diagnosis was

made by the attending doctor and the appropriate medications were prescribed, the subjects or their parents were approached, consented and the samples were collected.

2.1.3 *Sample collection*

For Southampton cohort, samples were collected before the challenge and immediately following a positive reaction or at the end of the challenge in case of a negative outcome. Samples from Qatar cohort were collected at the time patients were seen. Blood was collected through a cannula into tubes with clot activator. Saliva was collected by salivette (Sarstedt, Leicester, UK) and urine was collected by urine containers (Starlab, Milton Keynes, UK).

2.1.4 *Sample processing and storage*

The samples were kept on ice until processed which should be within 30 minutes. The saliva was transferred from the salivette to 1.5 ml microcentrifuge tubes (Alpha laboratories, Eastleigh, UK) in 500 µl aliquots after being centrifuged at 900 g (Falcon 6/300 centrifuge, MSE, London, UK) for 10 minutes at 4 °C. Blood samples were kept at room temperature for 15-30 minutes to allow separation of blood cells from serum then centrifuged at 1500 g for 15 minutes at 4 °C and divided into 500 µl aliquots. All the samples then transferred to -80 °C freezer until analysis time.

The samples collected by our collaborators at Queen medical hospital in Qatar were treated similarly and were shipped to our laboratory in a dry ice package. Upon receiving the samples, they were immediately kept in -80 °C freezer until analysis time.

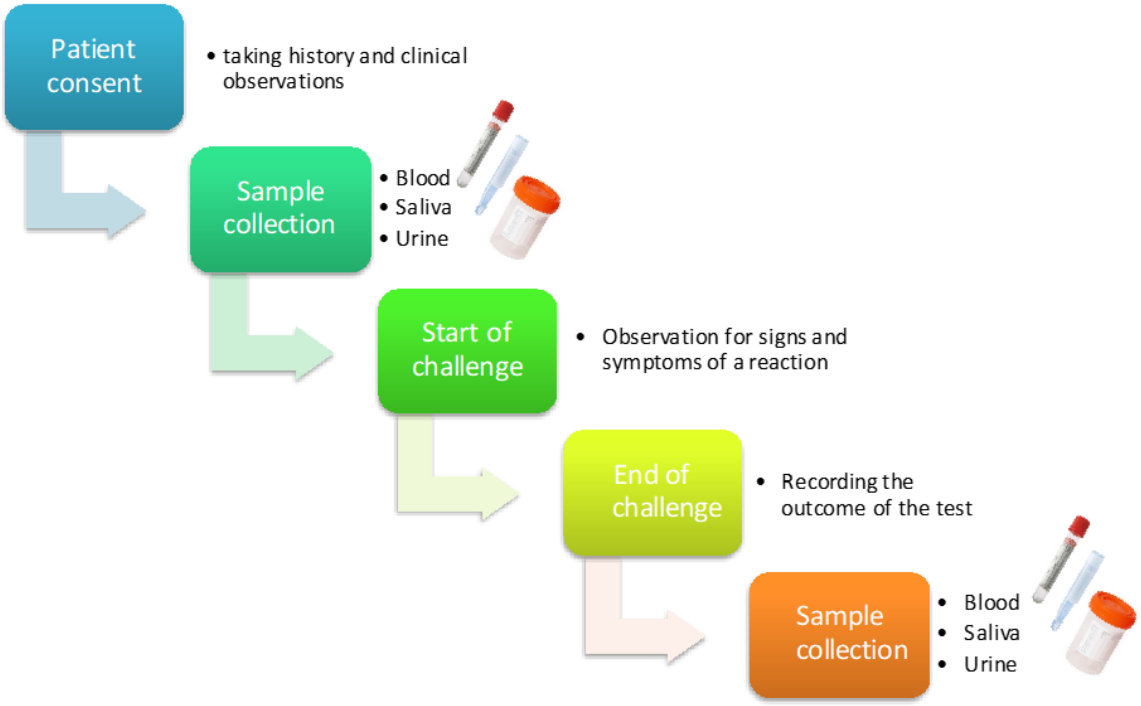


Figure 2.1 Sample collection protocol for Southampton cohort study.

The subjects were first consented and detailed history, including past allergic reactions, and clinical observations were taken. Samples (serum, saliva and urine) were collected before and after the challenge. If at any point the patient developed a positive reaction, the test would be stopped and the patient would be kept under observation with the samples being collected at the end of observation period.

2.2 Assays

Enzyme-linked immunosorbent assay (ELISA) was employed for measurement of mast cell mediators in the samples collected in this study (Figure 2.2). Sandwich ELISAs were employed to determine levels of tryptase, CPA3, chymase and DPPI in human serum using antibodies developed in our laboratory. For screening purposes, indirect ELISA was used to test the immunoreactivity of an antibody against the protein of interest (Figure 2.3).

Human skin collected from consented patients undergoing limb amputation was used for mast cell protein purification which was performed previously in the laboratory (215). The standards for tryptase, CPA3 and chymase were derived from purified human skin protein, whereas DPPI standards were derived from human recombinant protein that were kindly donated from our collaborators in Denmark (Unizyme). Table 2.1 describes the different primary and secondary antibodies used in the assays with their corresponding dilutions.

I. Sandwich ELISA general protocol

Sandwich ELISA was performed by coating a high binding 96-well plate (Fisher Scientific UK limited, Loughborough, UK) with 50µl capture antibody at desired concentration diluted in 50 mM sodium carbonate coating buffer pH 9.6 and incubated overnight at 4°C. The plates were emptied by flipping them into the sink and tapping into paper towel few times, and then washed with phosphate buffer saline containing 0.05% (v/v) tween 20 (PBS-T) three times. Non-specific protein binding was blocked by adding 150 µl 3% (w/v) bovine serum albumin (BSA) dissolved in PBS and incubated at room temperature on microplate shaker (Denley, UK) for 2 hours. This was followed by a washing step and then 50 µl of the standards diluted in 1% (w/v) BSA were loaded into each well. Serum samples were diluted and incubated alongside with the standards at room temperature on shaker for 90 min. After washing with PBS-T, 50 µl/well of the detection antibody was added at the optimised dilution and incubated at room temperature on shaker for 90 min. The plates were washed and 50 µl of HRP conjugated secondary antibody diluted in 1% BSA loaded into each well of and then incubated at room temperature on shaker for 45 min. A three-time washing step was performed before applying the substrate solution. The latter consisted of 1.5% (w/v) sodium acetate trihydrate pH 5.5, 6 mg/ml tetramethylbenzidine (TMB) in dimethyl sulphoxide (DMSO), with 0.01% (v/v) hydrogen peroxide (H_2O_2). The substrate solution was added to each well and the colour reaction was allowed to develop for up to 20 minutes. The reaction was stopped by adding 50 µl of 2M sulphuric acid and the plate was read on microplate reader (thermo-max, Molecular Devices, Berkshire, UK) at 450 nm with 595 nm correction wavelength.

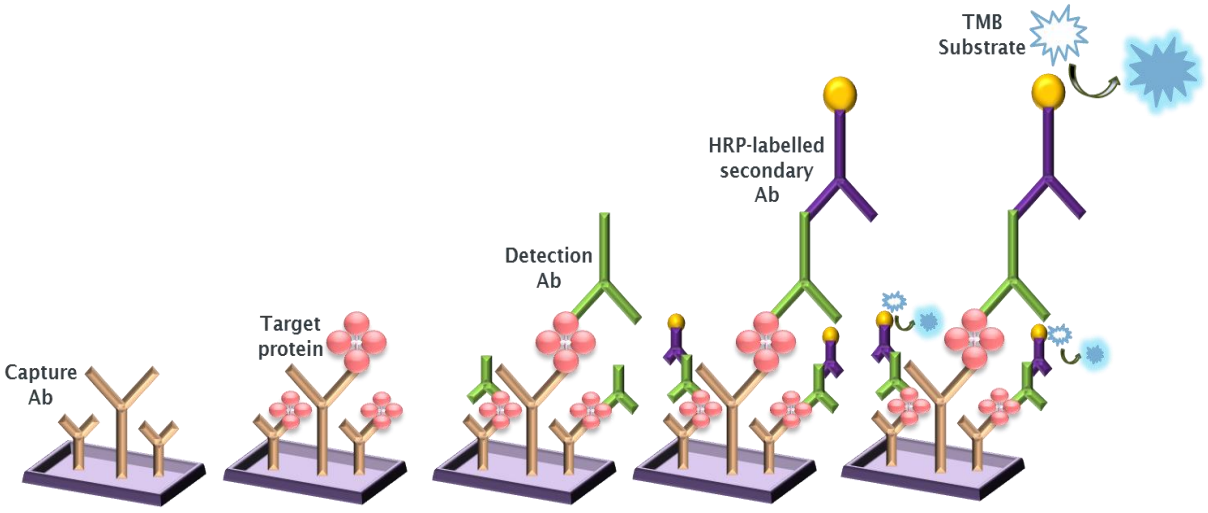


Figure 2.2 Key steps for sandwich enzyme-linked immunosorbent assay (ELISA).
The plate was coated with the appropriate dilution of the capture antibody and after blocking the non-specific binding; the target protein standards and samples were incubated. The detection antibody was applied next, followed by HRP-labelled secondary antibody and the TMB substrate.

II. Indirect ELISA general protocol

The plate was coated with 50µl protein of interest at the desired concentration diluted with 50 mM sodium carbonate coating buffer pH 9.6 and incubated overnight at 4°C. The plates were emptied and washed by same way mentioned earlier in sandwich ELISA. Blocking the non-specific protein binding was performed by adding 150 µl 3% BSA and incubated at room temperature on microplate shaker for 2 hours. The plates were then washed and 50µl of the detection antibody was added at the tested dilution to each well and incubated at room temperature on shaker for 90 min. Another washing step was performed before loading 50 µl/well HRP conjugated secondary antibody. The plate incubated at room temperature on shaker for 45 min after which washed and 50µl/well treated with the TMB substrate solution (TMB preparation is mentioned earlier in the general protocol for sandwich ELISA). The colour reaction was allowed to develop for a maximum of 20 minutes. The reaction was stopped by 2M sulphuric acid and the plate was read at 450/595 nm.

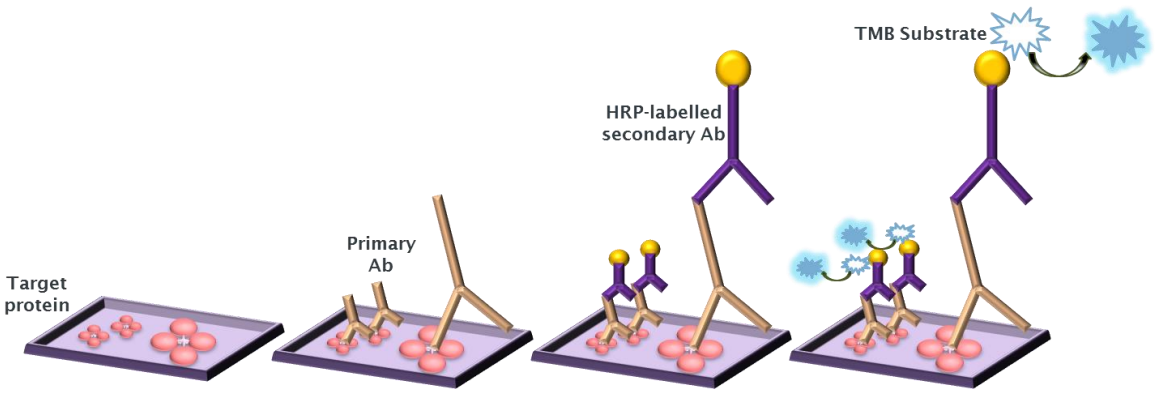


Figure 2.3 Illustration of the key steps of the indirect ELISA used for screening purposes.

The plate was coated with the protein of interest and then incubated with the primary antibody. HRP-labelled secondary antibody was used to detect the antigen-antibody complex and a colour reaction was developed using TMB substrate.

Table 2.1 Dilutions for the antibodies used in the assays

Mast cell protease		Primary antibodies	Species-Class	Dilution	Secondary antibodies	Dilution
1)	**Tryptase**	JJ3	Rabbit-Polyclonal	1/4000	Swine anti-rabbit HRP-conjugated*	1/2000
		AA5	Mouse-Monoclonal-IgG1	1/1000	Rabbit anti-mouse Ig HRP-conjugated*	1/2000
		II3	Rabbit-Polyclonal	1/2000	Swine anti-rabbit Ig HRP-conjugated*	1/2000
		AA8	Mouse-Monoclonal-IgG2	1/500, 1/1000, 1/2000		
		AA9	Mouse-Monoclonal-IgG2	1/500, 1/1000, 1/2000		
2)	**CPA3**	CA2	Mouse-Monoclonal-IgG1	1/4000		
		CA5-b	Mouse-Monoclonal-IgG1	1/2500	Peroxidase-labelled Extravidin**	1/2000
3)	**Chymase**	CC1	Mouse-Monoclonal-IgG1	1/1000	Rabbit anti-mouse Ig HRP-conjugated*	1/2000
		CC4	Mouse-Monoclonal IgM	1/500, 1/1000	Goat anti-mouse IgM HRP-conjugated**	1/2000
		CC5	Mouse-Monoclonal-IgA	1/500, 1/1000	Goat anti-mouse IgA HRP-conjugated**	1/2000
		Rabbit polyclonal anti-chymase	Rabbit-Polyclonal	1/1000	Swine anti-rabbit Ig HRP-conjugated*	1/2000

Table 2.1 Dilutions for the antibodies used in the assays (continued)

Mast cell protease		Primary antibodies	Species-Class	Dilution	Secondary antibodies	Dilution
		Commercial anti-chymase***	Goat-Polyclonal	1/500, 1/1000, 1/2000	Rabbit anti-goat Ig HRP-conjugated****	1/2000
4)	**DPPI**	DD1	Mouse-Monoclonal-IgM	1/500	Goat anti-mouse IgM HRP-conjugated**	1/2000
		Rabbit-Polyclonal anti-DPPI	Rabbit-Polyclonal	1/1000	Swine anti-rabbit HRP-conjugated*	1/2000
		Commercial anti-DPPI*****	Mouse-Monoclonal-IgG2	1/500, 1/1000, 1/2000		

* Dako Agilent pathology solutions, Denmark
** Sigma-Aldrich, Dorset, UK
*** R&D Systems, Minneapolis, MN, USA
**** Life technologies limited, Paisley, UK
***** St. John's laboratories, London, UK

2.3 Bicinchoninic acid (BCA) protein assay

BCA assay was employed to determine the protein concentration in a sample. It is a colorimetric assay based on reduction of copper (Cu^{2+}) to cuprous (Cu^{1+}) by protein in an alkaline medium. BCA reacts with reduced cation forming a coloured reaction that can be detected at a strong linear absorbance. The assay protocol (Thermofisher Scientific) included preparation of working assay buffer, which was consisting of one part of 4% cupric sulphate (BCA reagent B) and 50 parts of BCA (BCA reagent A). A set of BSA standards (Sigma-Aldrich) were prepared to measure the protein concentration of an unknown referring to a standard curve. 25 µl of samples or standards were loaded into 96-well plate (Greiner bio-one, Stonehouse, UK) and diluted with working assay buffer at a ratio of 1:8 (v/v) to minimise the effect of interfering substances. The plate was incubated for 30 minutes at 37ºC and then cooled down to room temperature. The absorbance was measured at 550 nm using microplate reader.

2.4 Dot immunoblotting assay (Dot blot)

Dot blot was used for rapid analysis of our proteins. A simplified diagram of the dot blot protocol is illustrated in (Figure 2.4). The proteins were applied directly into 0.45 µm pore sized nitrocellulose membrane (GE healthcare life sciences, Buckinghamshire, UK) using circular template and then treated with primary and secondary antibodies. A dot blot protocol from Abcam was modified and employed in which 2 µl of protein sample was used and the membrane was allowed to dry. Blocking was performed by soaking the membrane in 5% BSA in TBS-T (20 mM Tris-HCl, 150 mM NaCl pH 7.5, 0.05% (V/V) Tween20) for two hours on rocking platform (Stuart scientific, Staffordshire, UK) at room temperature. This was followed by an overnight incubation for the primary antibody (purified: 1/100, supernatant: neat or 1/10) on the rocking platform at 4ºC. 1% BSA in TBS-T was used to dilute the antibodies. Three times wash was performed with TBS-T for 5 minutes each then the secondary antibody at 1:5000 dilution with 1% BSA in TBS-T was incubated for two hours on the rocking platform at room temperature. After three times wash with TBS-T, the membrane incubated with luminata ™ forte western HRP substrate (Merck Millipore, Hertfordshire, UK) for 1 minute and after discarding the excess substrate, the membrane was covered with transparent film. Serial exposures at 1, 5, 10, and 15 minutes were performed using Syngene image analysis unit (Syngene, Cambridge, UK).

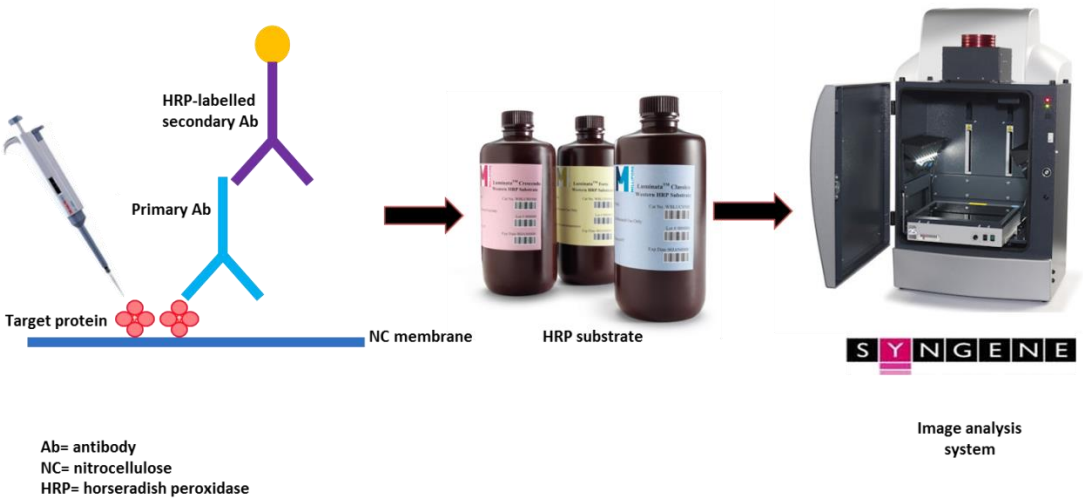


Figure 2.4 A general protocol for dot blot.

The concept of this technique is similar to the indirect ELISA except in dot blot a membrane is used instead of the plate. An image analysis unit is used to capture the chemiluminescent signal at serial exposures.

2.5 Cell culture

Cell culture and preparation of media were performed under sterile conditions in a class II microbiological safety cabinet (Envair Ltd, Lancashire, UK). Media and culture solutions (Thermofisher, Paisley, UK) were pre-warmed to 37ºC in the water bath (Grant, Cambridgeshire, UK) for at least 30 minutes before being used in cell culture. Cells were kept at 37ºC and at 5% CO_2 atmosphere incubator (Triple red Ltd, Buckinghamshire, UK) and were checked regularly under the inverted light microscope (Leica microsystems Ltd, Milton Keynes, UK).

2.5.1 Hybridoma cell culture:

Hybridoma cells were already developed in our laboratory for production of monoclonal antibodies and they were stored in liquid nitrogen. These hybridoma cells were revived occasionally to replace the antibody stock. Initially, the cells were suspended in ten ml of medium which included Dulbecco's modified Eagle's medium (DMEM), 1% (v/v) non-essential amino-acids, 1% (v/v) sodium pyruvate, 1% (v/v) penicillin-streptomycin-glutamine, and 15% (v/v) foetal bovine serum (Life technologies limited, Paisley, UK), then transferred into 15 ml medium and centrifuged at 1100 g for 7 minutes. The supernatant was discarded carefully, the cells were re-suspended with 30 ml of medium with 1X hybridoma cloning supplement (Life technologies limited, Paisley, UK), then maintained in T75 flask (Fisher Scientific UK Ltd, Loughborough, UK). The cells were checked for their growth on a daily basis and were divided when 75% confluency was reached. After 4-5 divisions, the supernatant was collected by centrifuging the cells at 1100 g for 10 minutes and stored at -20ºC.

2.5.2 LAD2 cell culture:

LAD2 cells were supplied from Brooke Laboratory, University of Southampton initially then maintained in our laboratory. LAD2 culture medium consisted of StemPro-34 serum free medium containing 2% (v/v) StemPro-34 supplement, 1% (v/v) penicillin-streptomycin-glutamine, and 100ng/ml stem cell factor. The cells were inspected regularly, counted and fed every week. When the cells reached to 1 x 10^6 cells/ml, they were either divided into two flasks or harvested. When harvested, the cells were centrifuged at 270 g for 10 minutes. This was followed by washing step, in which the cells were resuspended with 10 ml PBS and centrifuged at the same mentioned conditions. The cells were counted and the cell number was adjusted depending on the required

conditions for each protocol. The harvested cells were then either used for immunocytochemistry, fractionation or to perform sensitization studies.

2.6 Immunocytochemistry (ICC)

Immunocytochemistry is a technique used to detect the presence of specific protein or antigen in the cells by the use of a specific antibody. It involves fixation, permeabilisation, blocking, and incubation with primary and secondary antibodies.

In order to preserve the structure of the cell and the protein of interest, a fixation step was performed. Permeabilisation of the cells is a crucial step to allow free access of the antibody to the target protein. Blocking was necessary to reduce the non-specific binding of the antibody to proteins other than the target protein. Primary antibody incubation was followed by a washing step to remove excess antibody. A fluorescent-tagged secondary antibody was then added, which would bind specifically to the protein-antibody complex giving a visible fluorescent signal that can be visualised using fluorescence microscope.

Cell fixation: LAD2 cells were fixed with 2% (w/v) paraformaldehyde. The fixative agent was incubated for 20 minutes at room temperature. The slides then were rinsed with PBS containing 0.02% (w/v) sodium azide.

Permeabilisation: Fixed cells were permeabilised with 1% (v/v) Triton X-100 in PBS for 1 hour at room temperature.

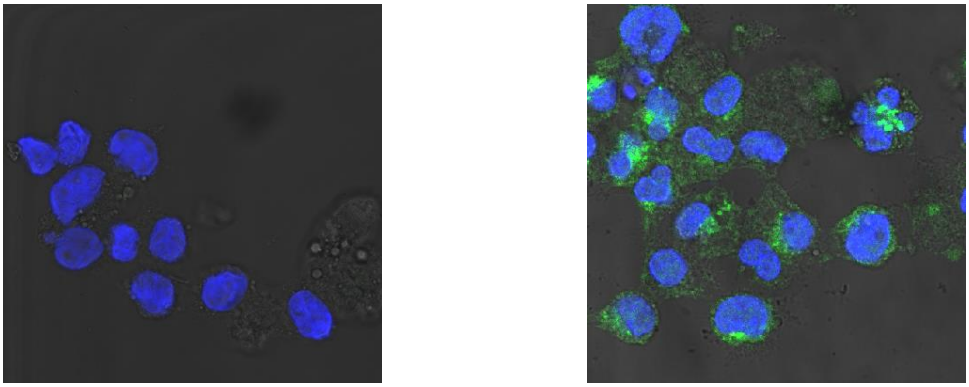
Blocking: The slides were incubated with blocking buffer [5% (w/v) BSA with 0.02% (w/v) sodium azide] for an hour at room temperature. BSA bound into the non-specific proteins thus reducing the affinity of primary and secondary antibodies for non-specific binding.

Antibody incubation: Serial dilutions were performed for both primary and secondary antibodies to determine the optimum concentrations. Primary antibodies including AA5 or JJ3 for tryptase labelling, CC1 for chymase labelling and polyclonal rabbit anti-DPPI to label DPPI were incubated for overnight at 4°C, whereas CA5- Alexa Fluor 488 was incubated for an hour at room

temperature to label CPA3. Following that, the slides were washed for three times for 5 minutes with PBS containing 0.02% (w/v) sodium azide.

Incubation of secondary antibodies [Alexa Fluor 555 donkey anti-mouse (Thermofisher, Paisley, UK) and Alexa Fluor 647 donkey anti-rabbit (Abcam, Cambridge, UK)] was performed for one hour at room temperature, and then the slides were washed in the same way as primary antibodies. Negative controls were carried out for each experiment to rule out false positives in the cell preparations. This involved incubation of the slides with blocking buffer instead of the primary antibodies followed by incubation of secondary antibodies (see Figure 2.5).

Preparation of slides for imaging: The slides were treated with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) nuclear counterstain (supplied from biomedical imaging unit, university of Southampton, UK) and incubated for 15 minutes at room temperature. Three times of 5 minutes washing steps were performed. We used antifade mounting medium (Citifluor limited, London, UK) to preserve the cells until imaging time and to prevent photobleaching. The slides were kept at 4°C for until imaging time.



Negative control Tryptase labelling

Figure 2.5 Tryptase labelling for LAD2 cells using AA5 or JJ3 with their corresponding negative controls.

Additional sets of cells were prepared and incubated with blocking buffer (negative control) instead of the primary antibody. Both sets were processed in the same way afterwards. Negative controls showed nuclear staining (DAPI=blue) only with pale cytoplasm, whereas cells stained with AA5 or JJ3 showed clear cytoplasmic distribution of tryptase (green fluorescence) in addition to DAPI nuclear stain. This indicated the specificity of secondary antibodies.

2.7 Microscopy and imaging methods

Images were captured using Leica TCS-SP8 confocal microscope. The images were taken using x63 glycerol immersion objective lens and were acquired at 1024 X 1024 pixel resolution. Spectra detection windows and laser powers were adjusted to avoid spectral bleed through. Negative controls were included in each experiment to limit detector sensitivity. DAPI was excited at 415nm and fluorescence was detected at 535nm. Fluorescence labelled donkey anti-mouse and anti-rabbit were excited at wavelengths of 555nm and 652nm and the emission was detected at 565nm and 668nm, respectively. Images were analysed using LAS X Core software and Fiji (216).

Chapter 3: Development and optimisation of assays for mast cell proteases

3.1 Introduction

ELISA is considered as a robust method for detecting and quantifying a specific protein. The antigen of interest is "sandwiched" between two antibodies (capture and detection) and detected using a labelled secondary antibody. Successful sandwich ELISA requires a pair of specific antibodies that are mutually compatible i.e. bind to different epitopes expressed on the protein of interest. Optimisation of each component becomes a critical step to improve the performance of the assay. This also involves establishing the appropriate dilution of samples for each assay.

Optimisation was an essential part of the current work since our assays were dependent on reagents developed and prepared in our laboratory. Mast cell proteases (tryptase, CPA3 and chymase) were extracted and purified and specific antibodies (monoclonal and polyclonal) were also prepared. The main objective of the present chapter was to test the binding activity of available antibodies and to optimise assay conditions for each marker prior to applying the assay for measurement of mast cell proteases in body fluids.

3.2 Methods

3.2.1 Tryptase assay optimization

Initial attempts were made to establish a sandwich ELISA for tryptase using newly developed AA8 or AA9 monoclonal capture antibodies instead of the previously prepared rabbit polyclonal (II3) capture antibody with AA5 as a monoclonal detection antibody. The reason for that was the limited availability of II3 antibody. The assay was performed in parallel with the polyclonal antibody-monoclonal antibody sandwich ELISA. The general sandwich ELISA conditions were described in detail in Section 2.2.

Development of rabbit polyclonal antibodies

Because the above method was not successful, we have directed the work for development of new polyclonal antibodies. Recombinant β-tryptase (1.5 mg/ml) isolated from *Pichia pastoris* expression system following protocol described by Niles *et al* (217) was used to immunise three

rabbits for the production of anti-tryptase antisera. Recombinant β-tryptase was dissolved in saline and mixed with an equal volume of Freund's adjuvant. The mixture was passed repeatedly between two syringes connected to each other by a double-ended needle. The process was continued until an emulsion was produced. The rabbits were immunised with Freund's complete adjuvant on Day one and Freund's incomplete adjuvant on Day 21. The immunisation involved two subcutaneous injections of the adjuvant on the back of the neck. On day 56 the first bleed was collected from the marginal ear vein of each rabbit and two weeks later a second bleed was taken followed by booster dose of recombinant tryptase being injected subcutaneously in the sites indicated earlier. Two weeks following the booster dose, the rabbits were sacrificed and bled out. The blood was collected into universal tubes and a wooden stick applied around the edge of each tube to detach the blood clot which was allowed to contract overnight before centrifugation at 3500 g for 15 minutes at 4°C. The serum was then collected and stored in a -20 freezer for screening. The three antisera were named HH, SS and JJ and were numbered according to the number of the bleed (1, 2 and 3).

Screening of antisera (HH, SS and JJ) for anti-tryptase activity

The antisera from three bleeds were tested for their reactivity with tryptase (skin and recombinant forms) by indirect ELISA using a range of dilutions of tryptase. Rabbit polyclonal anti-tryptase antibody (II3) was included in the first two bleeds as a positive control. Normal rabbit serum was included as negative control.

Ammonium sulphate precipitation of antibodies

The most reactive antisera (HH3 and JJ3) were partially purified using an ammonium sulphate precipitation method where 27.7 g ammonium sulphate was added to 100 ml of serum. The mixture was placed on a roller (Denley, UK) for 4 hours at 4°C. This was followed by centrifugation at 14000 g for 30 minutes at 4°C. The supernatant was stored at -20°C freezer for later evaluation of the antibody while the pellet was re-suspended with PBS. Dialysis of the suspension was performed using a 14 kDa cellulose membrane dialysis tubing in PBS overnight at 4°C with gentle mixing. Dialysis was repeated twice for two hours each time. The antibody suspension was aliquoted and stored in a -20°C freezer.

Optimisation of tryptase assay using HH3 and JJ3 precipitated antibodies

According to the general ELISA protocol, plates were coated with three dilutions (1/2000, 1/4000 and 1/8000) of HH3 and JJ3 antibodies. Serial dilutions of tryptase purified from skin (as standards) were prepared with 200 ng/ml as the highest dose. AA5 antibody was used to detect the reaction at a 1/2500 dilution. Successful ELISA was obtained using JJ3 as a capture antibody

and conditions for the new ELISA were optimised. A range of dilutions for each antibody was assessed as well as different concentrations of the blocking and dilution buffers were used. The optimised protocol was carried out five times using a wide range of standard concentrations (0-200 ng/ml) and the mean optical density readings and 95% confidence intervals were calculated for each standard concentration. The lowest standard concentration at which 95% confidence intervals did not overlap with those of the standard below was regarded as the lower limit of detection whereas the upper limit of detection was determined by the concentration of the top standard before a plateau was seen.

Optimisation of sample dilution and recovery studies

Serum samples were diluted serially to assess the optimum dilution to employ in the assay. A range of dilutions were made for nine serum samples. Purified skin tryptase at a concentration of 25 ng/ml was added to equal volumes of serum samples. This gave a final tryptase concentration of 12.5 ng/ml and a final serum dilution of 1/5, 1/10, 1/20 and 1/40. Tryptase levels were measured within the spiked samples and in unspiked control serum samples. The percentage recovery was calculated from the concentration determined for a sample following spiking with purified skin tryptase less the concentration determined in the corresponding unspiked samples, divided by the actual amount of the purified skin tryptase used to spike the sample:

$$\% \text{ Recovery} = \frac{(\text{Spiked sample} - \text{unspiked sample})}{\text{Spike concentration}} \text{ x } 100$$

3.2.2 CPA3 assay

The CPA3 assay was already running in the laboratory and minimum optimisation of assay conditions was required. A protocol similar to the general ELISA protocol was used except for the detection antibody where we used a biotinylated CA5 antibody (CA5-b) and peroxidase-conjugated Extravidin instead of employing a species-specific secondary antibody. The assay was performed five times using CPA3 standard concentrations from 0 to 100 ng/ml. The lower and upper limits of detection were calculated as mentioned above in Section 3.2.1.

As with the assay for tryptase, serum samples were prepared with four serial dilutions to investigate the best dilution for the assay. Purified skin CPA3 at concentration of 25 ng/ml was added to an equal volume of serum samples diluted serially to 1/2.5, 1/5, 1/10 and 1/20. The percentage recovery was calculated according to the formula presented in Section 3.2.1.

3.2.3 Chymase assay

Sandwich ELISA using chymase monoclonal antibodies

There had been purified monoclonal anti-chymase antibodies CC4 and CC5 present in our laboratory and we wanted to use them to establish a sandwich ELISA for chymase. Both antibodies were used as capture and detection antibodies to investigate their compatibility in a sandwich ELISA. Range of dilutions of purified skin chymase was used to prepare the standards for the assay.

Indirect ELISA screening for chymase monoclonal antibodies

Since sandwich ELISA was not successful, indirect ELISA for CC4 and CC5 antibodies was performed using different concentrations of skin chymase for coating the plate and the antibodies for primary detection. At this point we thought of using culture supernatant as well to test the immunoreactivity of chymase antibodies to purified skin chymase. Hybridoma cells for anti-chymase antibodies (CC4 and CC5) were revived from frozen stocks as described in Section 2.5.1 and the supernatants were collected for testing. A monoclonal anti-chymase CC1 antibody that had been prepared previously was included as a positive control (as a supernatant and ammonium sulphate precipitated forms). The same steps mentioned in Section 2.2 for using indirect ELISA in the screening of antibody specificity were followed. Two concentrations of skin chymase preparation were used for coating and different dilutions for each antibody supernatant were used as primary detection antibodies, except for CC1 where we used precipitated antibody in addition to the culture supernatant.

Dot blot for chymase monoclonal antibodies

Dot blot was used as another system for screening the immunoreactivity of the antibodies. It was performed following the protocol described in Section 2.4 by pipetting 2 µl of chymase into the nitrocellulose membrane. Three chymase preparations (A, B and C) at different concentrations were used. The nitrocellulose membrane was then treated with anti-chymase monoclonal antibodies. Dot blot was also performed by using primary anti-chymase antibodies and secondary antibodies alone without the protease. This included adding 2 µl of purified and supernatant preparations of the antibody onto the nitrocellulose membrane and then treating with the secondary antibodies.

Testing Commercial anti-chymase antibody

We had a commercial goat polyclonal anti-chymase antibody (Biorbyt, Cambridge, UK) to test for compatibility with our monoclonal CC1 antibody in a sandwich ELISA. We started by testing serial

dilutions of the antibody in indirect ELISA against the reactivity of CC1-AS and supernatants of CC4 and CC5. Two concentrations of purified skin chymase were used in the screening. Following that, a sandwich ELISA using range of dilutions for the polyclonal goat anti-chymase antibody as capture once and as a detection antibody at another paired with CC1 was performed. Skin chymase was used in different concentrations.

3.2.4 DPPI assay

Sandwich ELISA for DPPI

Sandwich ELISA for DPPI was developed by Dr. Whitworth (215) and initial approaches of the present study were directed at improving the sensitivity of the assay. The reagents used in the sandwich ELISA consisted of purified monoclonal antibody DD1 prepared against recombinant DPPI as capture antibody and rabbit polyclonal anti-DPPI as detecting antibody. Human recombinant DPPI (1 mg/ml preserved in a buffer containing glycerol) was used to prepare standards up to 1000 ng/ml. Foetal bovine serum (FBS) 20% (v/v) in PBS was used for blocking the non-specific binding and 3% FBS was used as a diluent for the assay. Serum samples tested for their DPPI levels were serially diluted to four dilutions. Serum samples were also spiked with 250 ng/ml DPPI and the percentage recovery was calculated. To improve the sensitivity of the assay, various diluents were investigated maintaining the conditions otherwise unchanged. These included 0.5 M sodium chloride in water, commercially available sample diluent (R&D system, Abingdon, UK), and a 3% FBS diluent.

Testing Commercial anti-DPPI antibody

We wanted to improve the sensitivity of DPPI assay by using a commercially available monoclonal anti-DPPI antibody (St. John's laboratories, London, UK) in the sandwich ELISA in combination with our anti-DPPI antibodies. We tested the reactivity of the new antibody (diluted into three dilutions) with recombinant DPPI in indirect ELISA alongside with the other anti-DPPI antibodies.

Testing blocking buffers for DPPI assay

Different blocking buffers were also investigated to prevent the non-specific binding of DPPI to the plate. A commercial protein-free blocking solution (VWR, West Sussex, UK) was tested together with 10% (v/v) glycerol solution in PBS and the previously suggested 20% FBS blocking buffer. PBS was used as a control. The assay included incubation of the plate with 150 μl/well

using any of the three blocking solutions for 2 hours at room temperature on the shaker. Other steps were similar to those described for the DPPI sandwich ELISA.

Screening of the supernatant of DPPI monoclonal antibodies

Because the purified DD1 antibody was not reacting in the above screening for the commercial anti-DPPI antibody, hybridoma cells for two anti-DPPI monoclonal antibodies (DD1 and DD3) were revived from frozen stocks as described in Section 2.5.1 and the supernatants were collected for testing. The supernatants for the two antibodies were screened by indirect ELISA with the rabbit polyclonal anti-DPPI used as positive control. The immunoreactivity of DD1 and DD3 supernatants to DPPI was also tested by dot blot following the general protocol for dot blot described in Section 2.4. Supernatants of DD1 and DD3 were used to treat the nitrocellulose membrane. Rabbit polyclonal anti-DPPI antibody was used as positive control.

3.3 Results

3.3.1 Tryptase assay optimisation

We developed new polyclonal anti-tryptase antisera because our approach for establishing a sandwich ELISA using new monoclonal antibodies AA8 or AA9 for capture and AA5 detecting antibody was unsuccessful. AA8 and AA9 had no capturing effect in the sandwich ELISA; however, a good standard curve was obtained in a sandwich ELISA using II3 as a capture antibody (Figure 3.1).

Screening of the first bleed of the three rabbits after immunization showed reactivity towards native and recombinant forms of tryptase but the signal was less than that obtained by II3 antibody employed as a positive control (Figure 3.2 a and b). The reaction of the second bleed for HH2 and JJ2 antisera was stronger compared to the first bleed reaction and HH2 antiserum showed a higher response with skin tryptase than that of the positive control (Figure 3.2 c and d). The reactivity of SS2 antiserum to tryptase was still low. Levels of anti-tryptase antibodies increased markedly at the final bleed against both forms of tryptase (Figure 3.2 e and f). Normal rabbit serum showed minimum reaction to tryptase. Reaction of SS3 antiserum was associated with high non-specific binding.

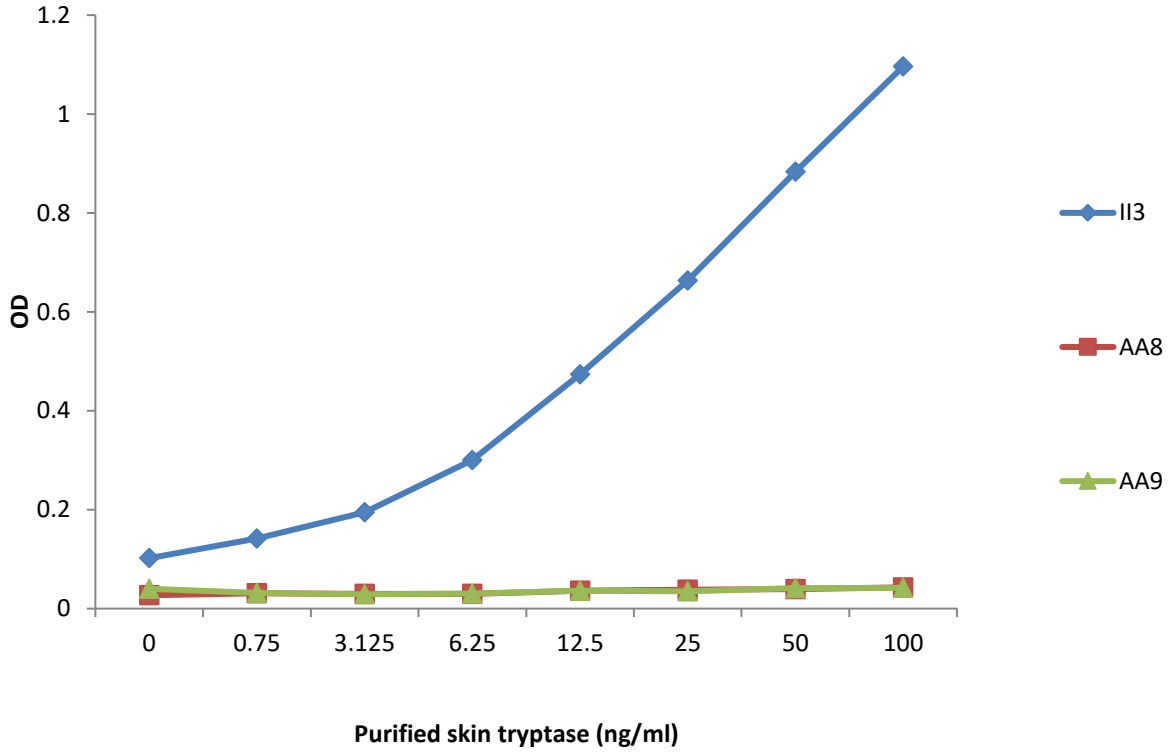


Figure 3.1 Investigation of new monoclonal capture antibodies in sandwich ELISA for tryptase.

Monoclonal antibodies AA8 or AA9 were not reacting as capture antibodies in a sandwich ELISA with AA5 whereas the rabbit polyclonal II3 antibody produced a good standard curve. Purified skin tryptase was used to prepare the standards. Data represent the mean of duplicate determinations. The reaction was read at 450/595 nm.

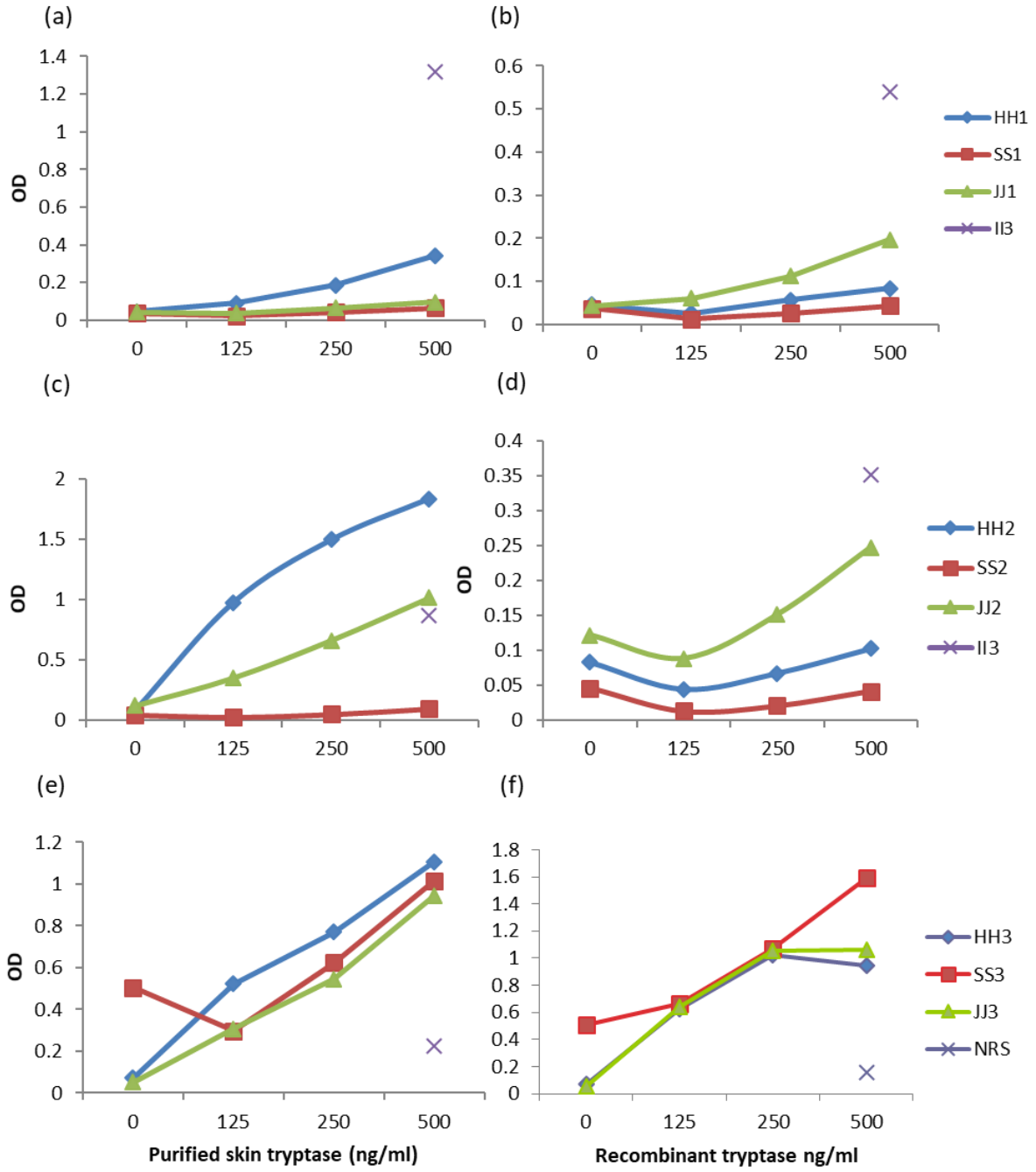


Figure 3.2 Screening of new antisera for binding to tryptase in indirect ELISA.

ELISA results are shown for (a, b) the first, (c, d) second and (e, f) the final bleeds for the rabbits HH, SS and JJ. The reactivity of the antisera was compared to (a, b, c, d) polyclonal II3 antibody as a working antiserum and to (e, f) normal rabbit serum (NRS) as a negative control. The screening was performed against (a, c, e) skin and (b, d, f) recombinant tryptase. Data represent the mean of duplicate determinations. Reactions were read at 450/595 nm.

On the basis of these initial screening results, it was decided to continue working with HH3 and JJ3 only as potential capture antibodies. Sandwich ELISA was successful using ammonium sulphate precipitated JJ3 as coating antibody in serial dilutions (Figure 3.3) when paired with AA5 as the detecting antibody. Using HH3 with AA5 in a sandwich ELISA was not successful (data are not shown). Further optimisation of the antibody dilutions and blocking buffer was performed and a final protocol was developed and used for all assays carried out in the present study (Figure 3.4). Standard curves were generated using both purified skin tryptase and recombinant tryptase as assay standards (Figure 3.5 a and b) but to maintain consistency of the results, all subsequent assays were performed with purified skin tryptase.

Standard concentration of 0.39 ng/ml assay was regarded as the lower limit of detection as it was not overlapping with 95% confidence intervals of the zero-standard concentration. The standard curve showed a plateau after 100 ng/ml and this was regarded as the upper limit of detection.

Tryptase measurement in serum samples (n = 9) diluted into four serial dilutions resulted in a stepwise reduction in tryptase levels which were undetectable in serum diluted to 1/40 (Figure 3.6 a). Calculating tryptase levels in consideration of the dilution factors showed an increase in the tryptase values which was unreliable (Figure 3.6 b). This indicated that at higher dilutions the assay was less sensitive. Levels showed a plateau between 1/5 and 1/10, thus for this assay serum was diluted within this range. The result of spiking serum samples diluted into wide range of dilutions with known concentration of purified skin tryptase indicated that levels determined by ELISA reflected those added by spiking. The spike recovery data is summarized in Table 3.1.

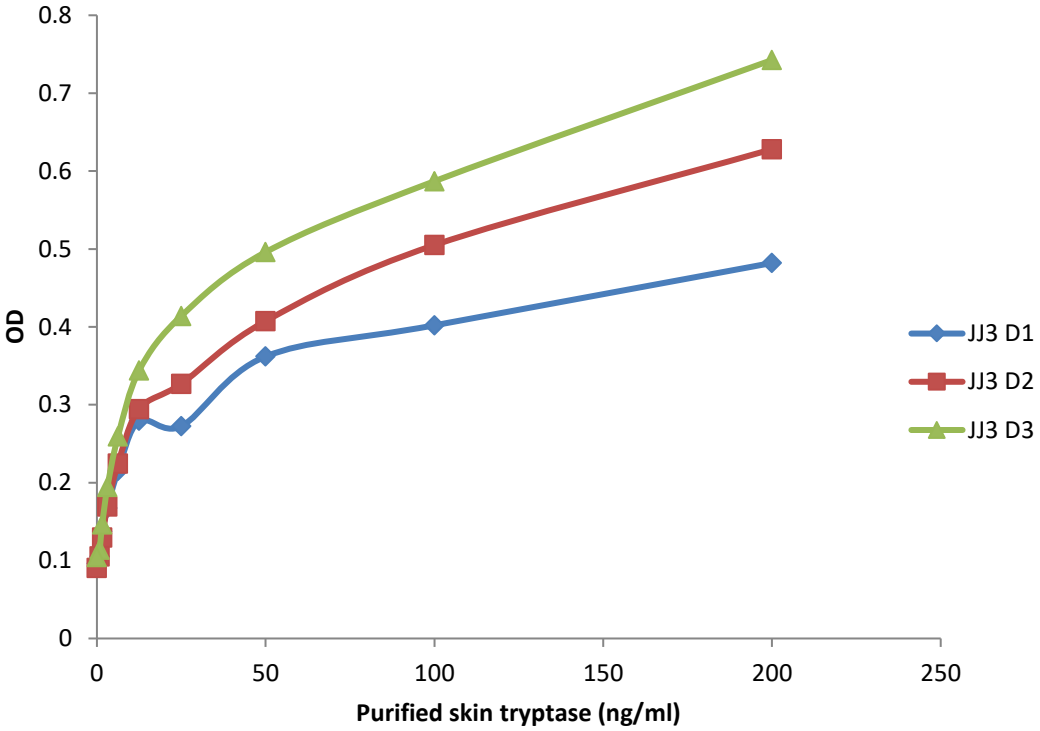


Figure 3.3 Optimisation of JJ3 sandwich ELISA.

JJ3 was used in three dilutions (D1=1/2000, D2=1/4000, D3=1/8000) to optimise tryptase ELISA. Serial dilutions of skin tryptase were used as the antigen and AA5 antibody for detection in the sandwich ELISA. Data represent the mean of duplicate determinations. Reactions were read at 450/595 nm.

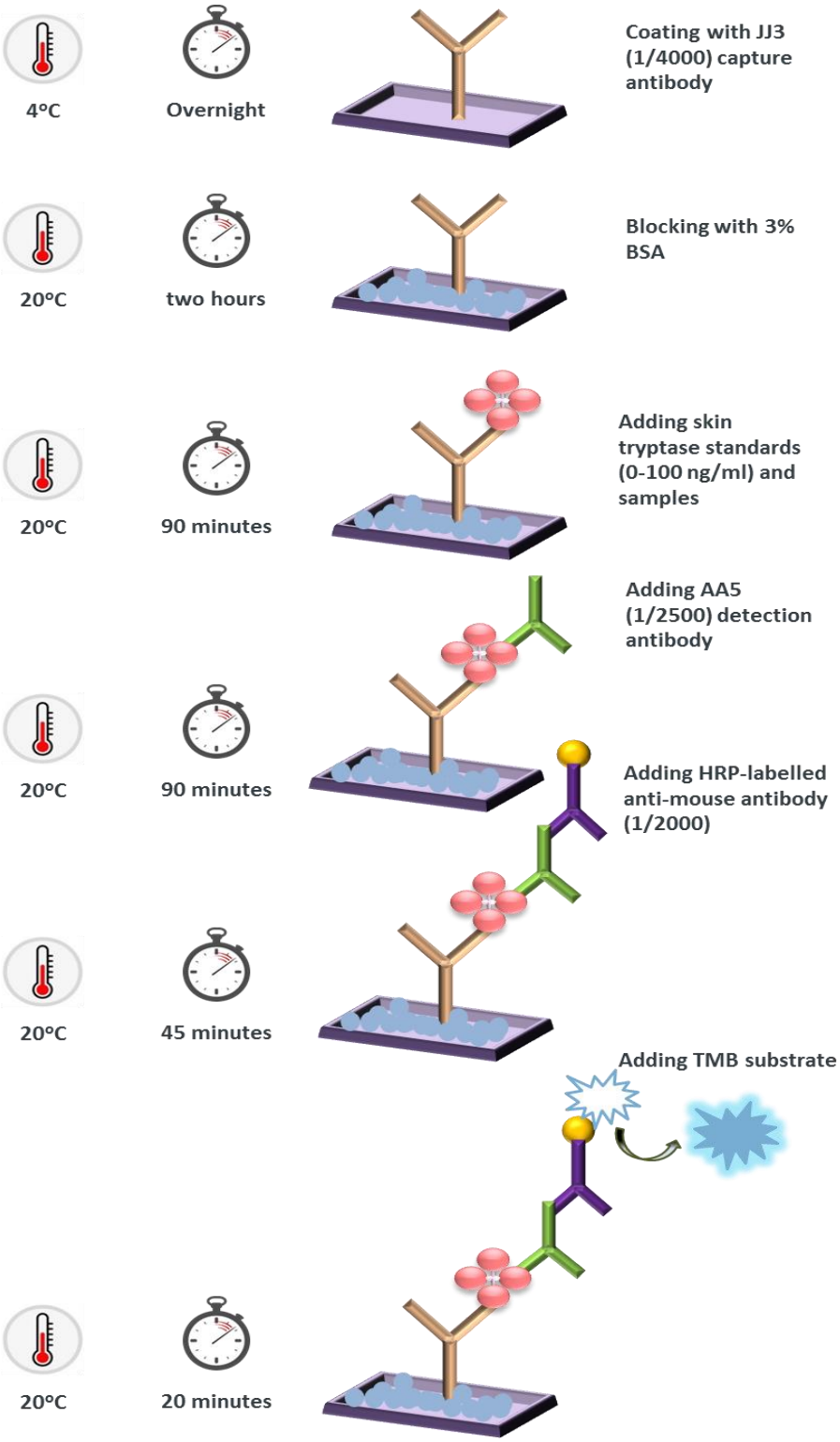


Figure 3.4 Optimised protocol for tryptase sandwich ELISA.

The plate was coated with JJ3 capture antibody for overnight at 4°C. Serial dilutions of purified skin tryptase were incubated for 90 minutes at room temperature. The detecting antibody AA5 was added and incubated for 90 minutes at room temperature. The final step consisted of adding an anti-mouse HRP-labelled secondary antibody and incubating for 45 minutes at room temperature. The TMB substrate was added to allow development of the colour reaction.

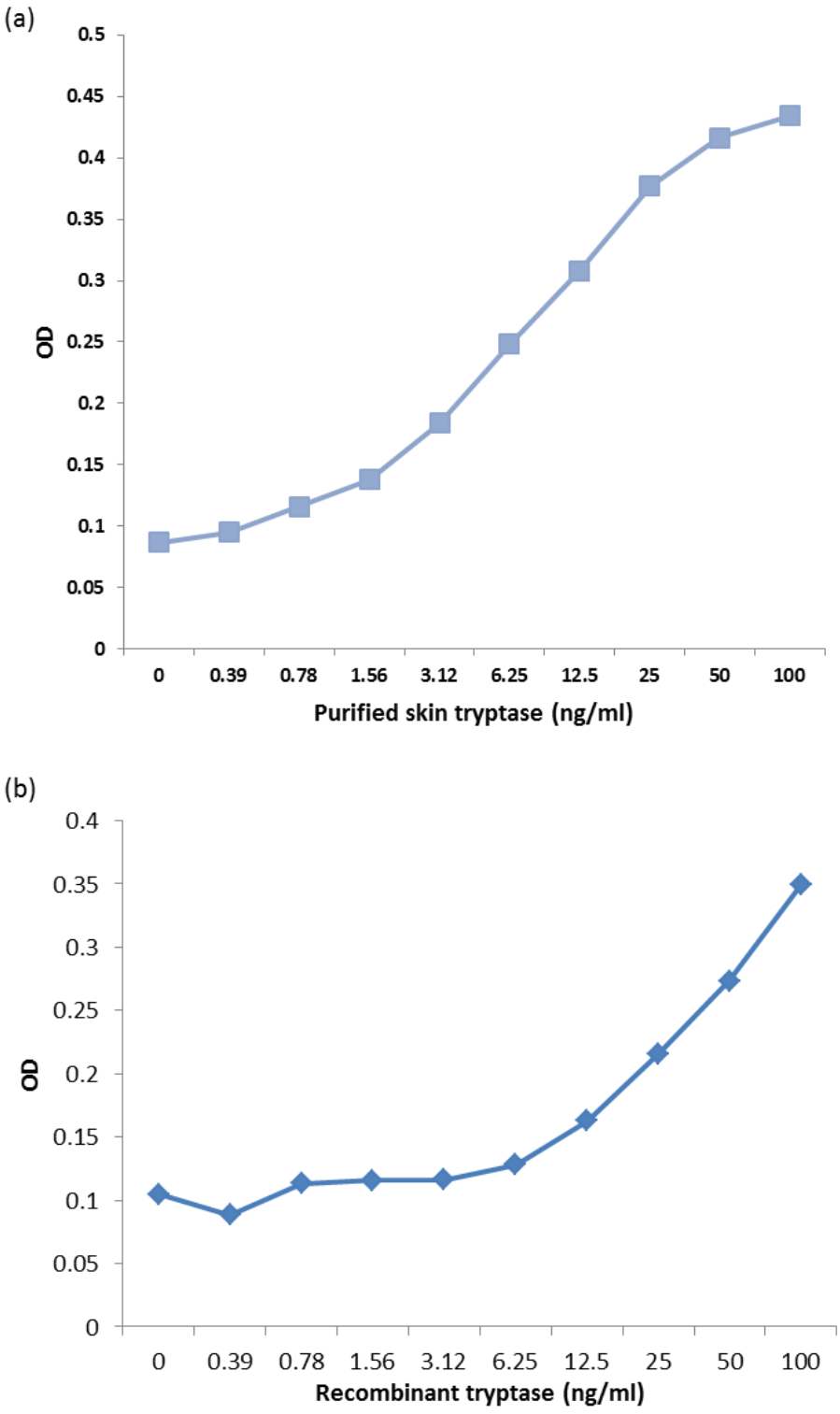


Figure 3.5 Standard curves for optimised tryptase ELISA.

The optimised conditions for tryptase sandwich ELISA were employed standard curve was generated using (a) purified skin tryptase or (b) recombinant tryptase as assay standards. Data represent the mean for duplicate determinations. Reaction was read at 450/595 nm.

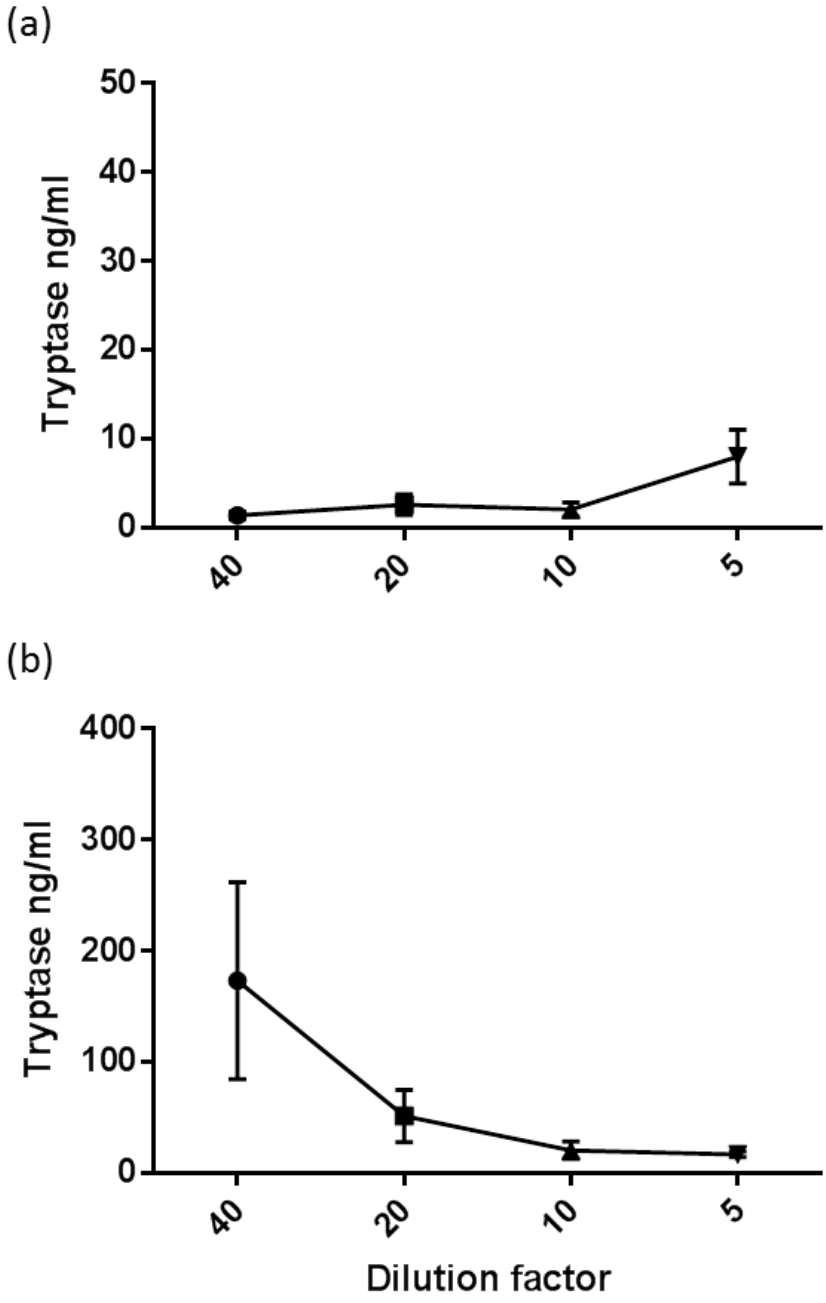


Figure 3.6 Optimisation of serum sample dilutions for tryptase assay.

Tryptase levels were determined in serial dilutions of each serum sample (n=9) at (a) measured concentrations and (b) projected concentrations. Data represent Mean ± SEM.

Table 3.1 Recovery studies for tryptase sandwich ELISA.

Serum samples (n = 9) at various dilutions spiked with 25 ng/ml tryptase.

Dilution factor	Mean % Recovery	Standard deviation
5	75	18
10	62	14
20	70	22
40	97	45

3.3.2 CPA3 assay optimisation

Optimisation of the antibody dilutions and incubation times for the CPA3 ELISA was performed and the finalised protocol was used for determination of levels in serum samples (Figure 3.7). The standard curve was generated using serial dilutions of 50 ng/ml purified skin CPA3 (Figure 3.8). When calculating the 95% confidence intervals for each concentrations of the standard curve, we found the lower limit of detection was 0.23 ng/ml. The upper limit of detection was 50 ng/ml after which levels plateaued.

Serial dilution of a range of serum samples (n = 10) obtained from patients attending the asthma and allergy clinic was performed to choose the most appropriate serum dilution for the assay. Levels of CPA3 were detectable at 1/5 dilution but a gradual decline in CPA3 measurements was observed with further dilutions until levels became undetectable at a 1/40 dilution (Figure 3.9 a). However, as seen with tryptase, multiplication of the concentrations by the dilution factor resulted in a rise in CPA3 levels (Figure 3.9 b) and values were unreliable. Spiking serial dilutions of serum samples with 25 ng/ml purified skin CPA3 and calculating the percentage recovery indicated that there were no interferences affecting the assay. Table 3.2 summarizes the percentage recovery of serum samples in the sandwich ELISA for CPA3.

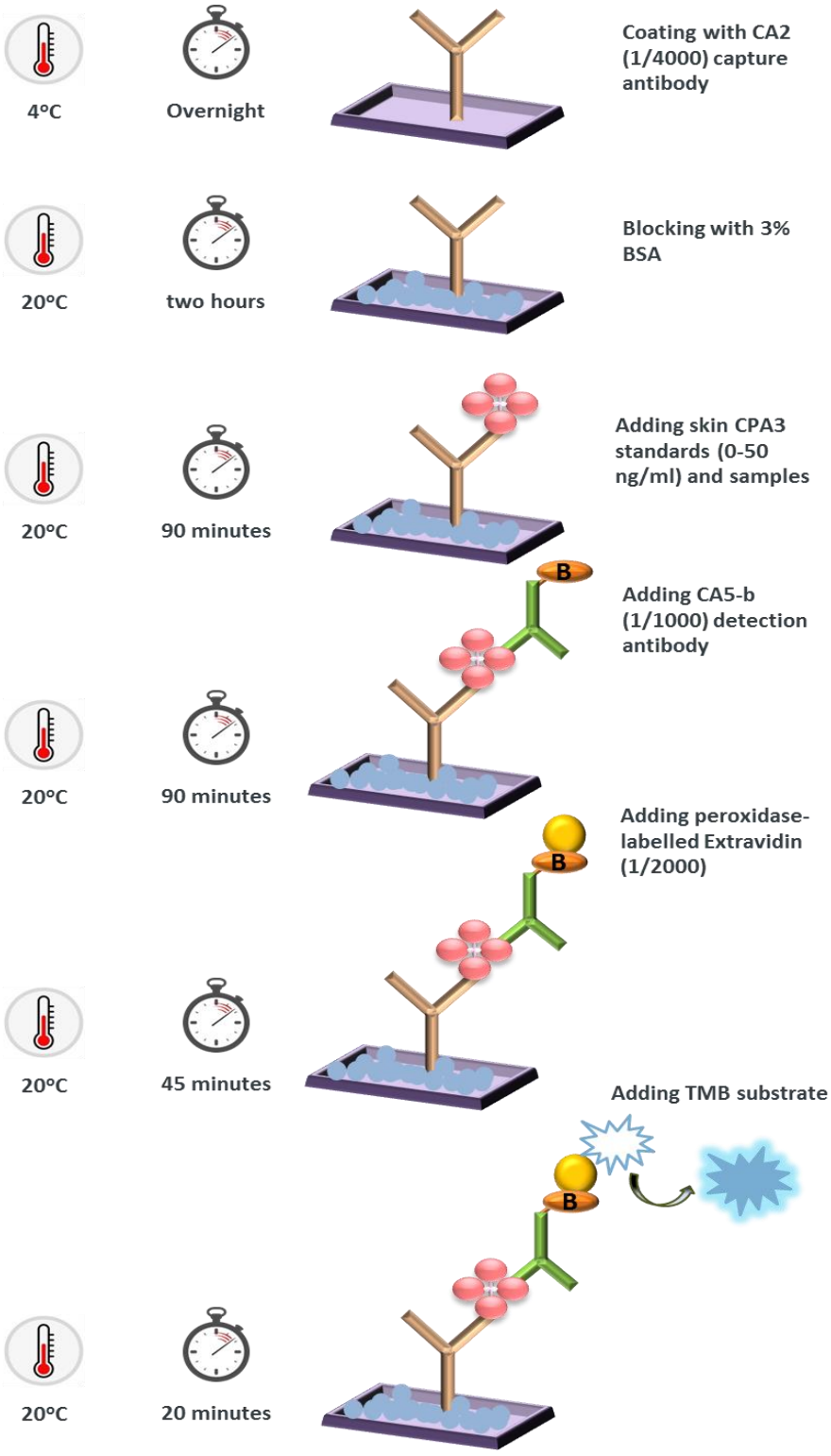


Figure 3.7 Optimised protocol for CPA3 sandwich ELISA.

The plate was coated with CA2 capture antibody for overnight at 4°C. Serial dilutions of purified skin CPA3 was incubated for 90 minutes at room temperature. The detection antibody CA5-b was added next and incubated for 90 minutes at room temperature. The final step consisted of adding a peroxidase-conjugated Extravidin and incubation for 45 minutes at room temperature. The TMB substrate was added to allow development of the colour reaction.

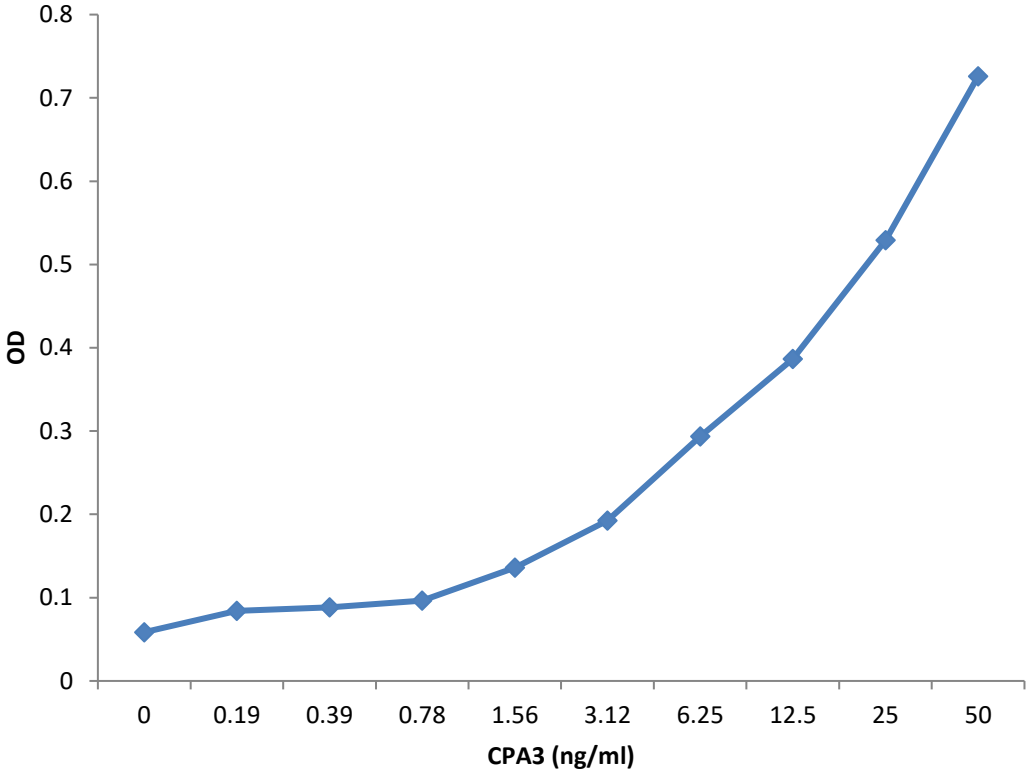


Figure 3.8 CPA3 ELISA standard curve.

The standard curve for CPA3 sandwich ELISA was generated using CA2 capture antibody, purified skin CPA3 for preparation of standards, CA5-b detecting antibody and peroxidase-conjugated Extravidin. Data represent the mean of duplicate determinations. Reaction was read at 450/595 nm.

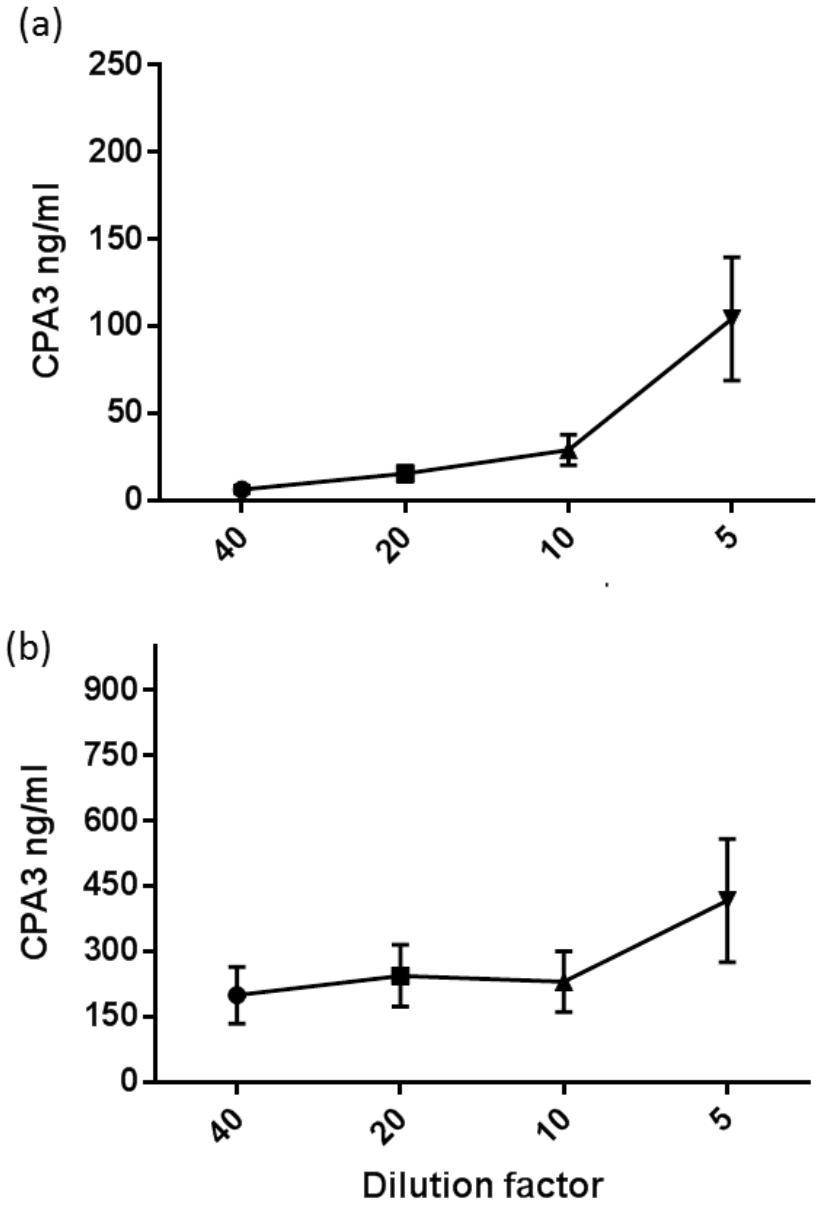


Figure 3.9 Optimisation of serum sample dilutions for CPA3 assay.

CPA3 levels were determined in four serial dilutions of each serum sample (n=10) at (a) measured concentrations and (b) projected concentrations. Data represent Mean ± SEM.

Table 3.2 Recovery studies for CPA3 sandwich ELISA.

Serum samples (n = 10) at various dilutions spiked with 25 ng/ml CPA3.

Dilution factor	Mean % Recovery	Standard deviation
5	77	11
10	73	16
20	74	13
40	121	47

3.3.3 Chymase assay

Sandwich ELISA for chymase using purified CC4 and CC5 antibodies that have been employed previously (215) was unsuccessful (Figure 3.10). Testing the reactivity of these antibodies with chymase by indirect ELISA showed poor signal at all concentrations of chymase and the signal was equivalent to the blank response (Figure 3.11). Even using supernatant forms of CC4 and CC5 in the indirect ELISA did not improve the reaction with chymase (Figure 3.12 a and b) compared to the observed response of CC1 (AS and supernatant) to chymase (Figure 3.12 c). Furthermore, dot blot analysis of CC4 and CC5 supernatants failed to show reactivity with three chymase preparations (Preparations A, B and C) at different concentrations, whereas monoclonal antibody CC1 (ammonium sulphate precipitated and supernatant) reacted well (Figure 3.13). Investigating the binding ability of the secondary antibodies to the primary anti-chymase antibodies (CC4 and CC5) by dot blot analysis showed positive responses for both supernatant and purified forms of the CC4 and CC5 antibodies (Figure 3.14).

At this point, we decided to include a commercially available anti-chymase antibody in the assay. When we screened three dilutions of a commercial polyclonal goat anti-chymase antibody in an indirect ELISA, we found a positive reaction between the polyclonal antibody and chymase (at concentrations of 250 and 500 ng/ml) which was similar to CC1 reactivity. Low reactivity was observed for CC4 and CC5 monoclonal antibodies (Figure 3.15). However, attempts to set up a sandwich ELISA using CC1 and polyclonal anti-chymase (as coating or detection antibodies) were unsuccessful (Figure 3.16).

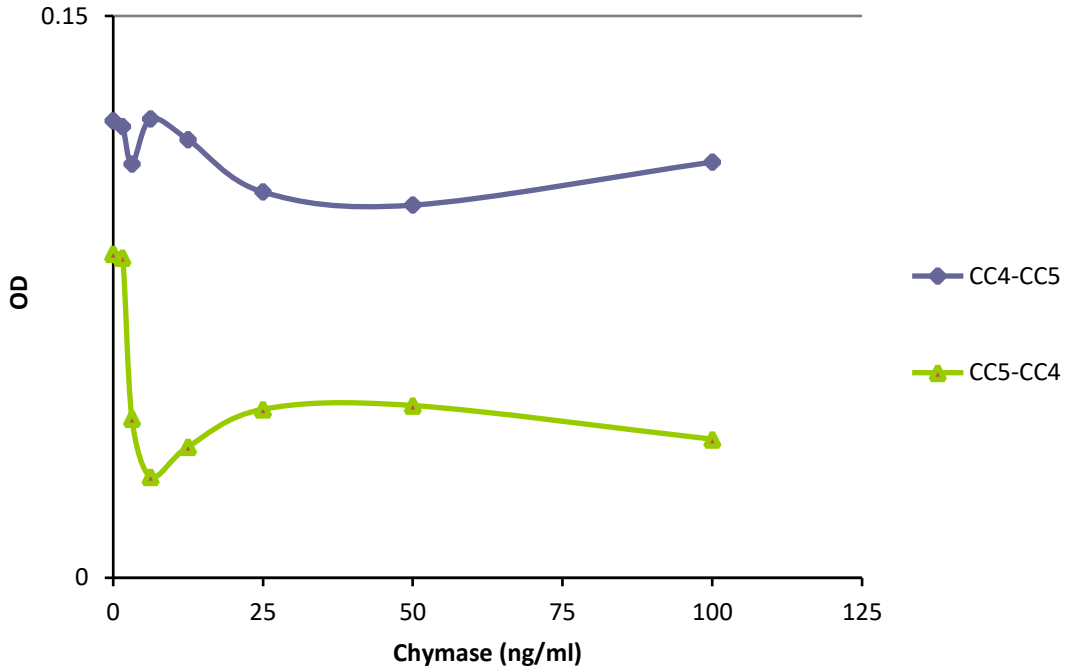


Figure 3.10 Lack of standard curve with sandwich ELISA for chymase.

The two monoclonal anti-chymase CC4 and CC5 antibodies were tested in sandwich ELISA using CC4 as capture antibody and CC5 for detection (CC4-CC5) and vice versa (CC5-CC4). Standards were derived from purified skin chymase. Data represent the mean of duplicate determinations. The reaction was read at 450-595 nm.

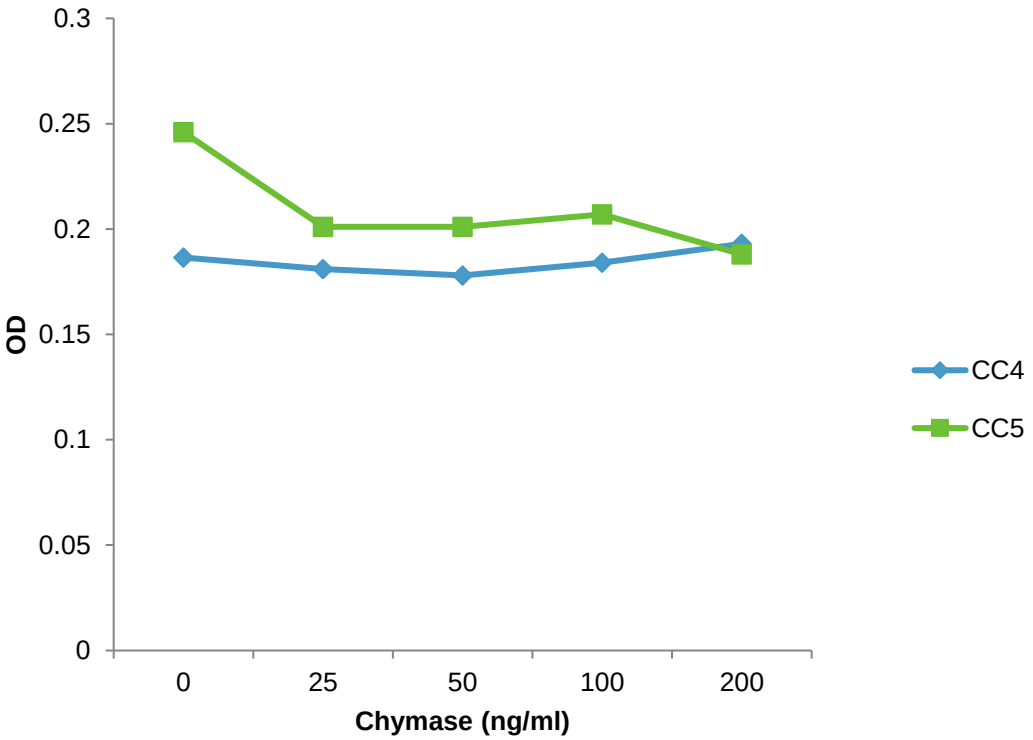


Figure 3.11 Poor reactivity of CC4 and CC5 antibodies in indirect ELISA.

Purified preparations of CC4 and CC5 antibodies were tested for their reactivity with purified skin chymase. Data represent the mean of duplicate determinations. The reaction was read at 450-595 nm.

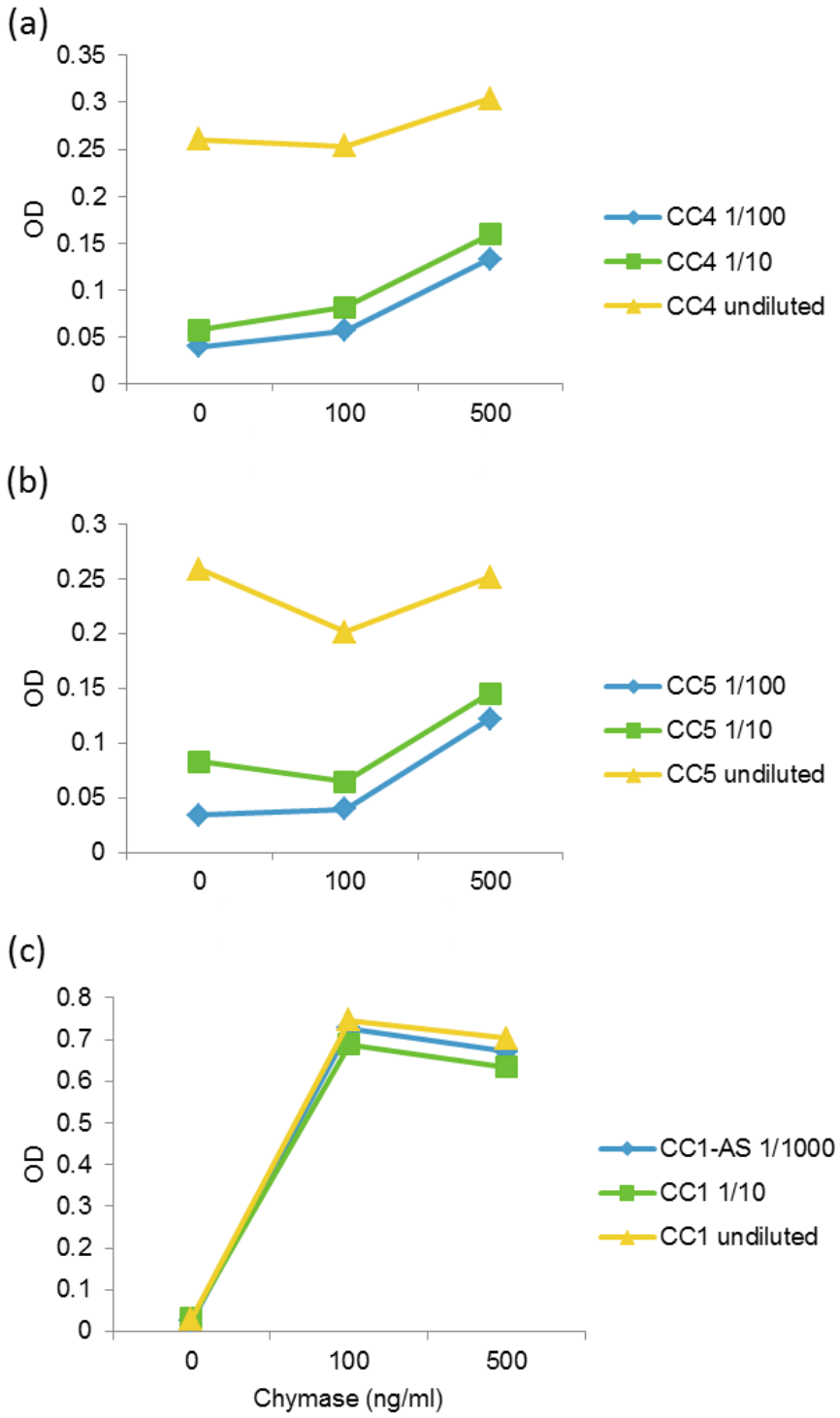


Figure 3.12 Indirect ELISA for supernatants of anti-chymase antibodies.
Screening for immunoreactivity of (a) CC4 and (CC5) diluted (1/100, 1/10) and undiluted supernatants against skin chymase at 100 and 500 ng/ml was performed and compared to (c) CC1 antibody (ammonium sulphate precipitated "AS" and supernatant preparations) response. Data represent the mean of duplicate determinations. The reaction was read at 450/595 nm.

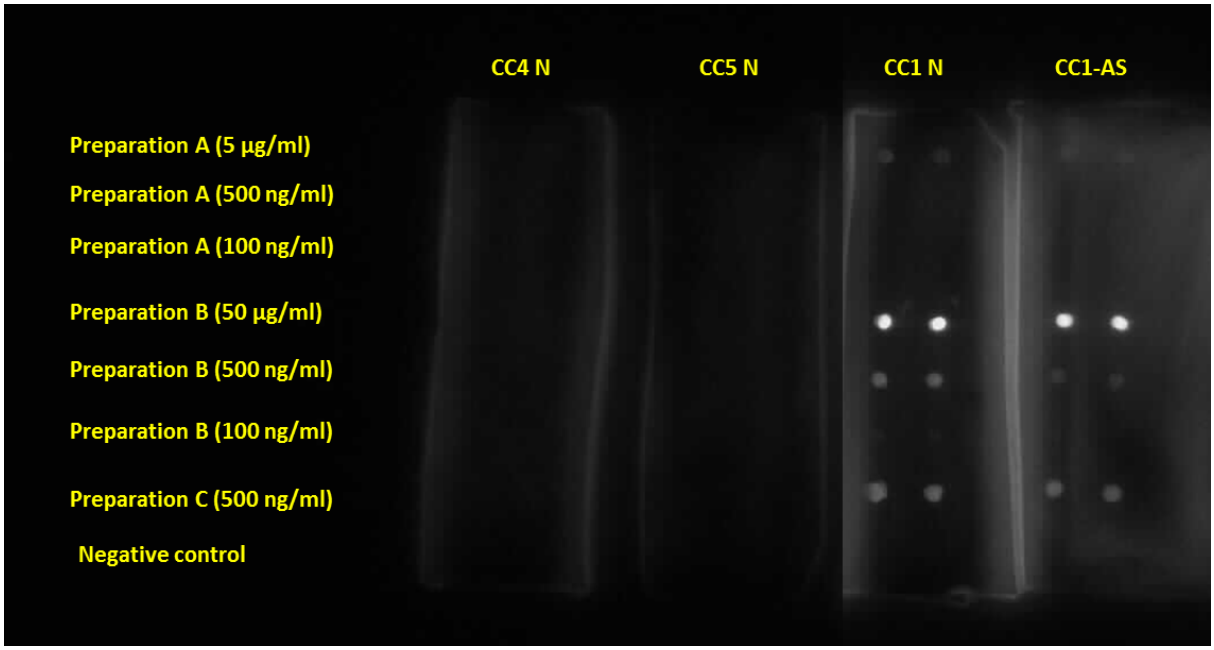


Figure 3.13 Dot blot analysis for chymase antibodies.

Three chymase preparations (A, B and C) at different concentrations were used to investigate the immunoreactivivty of CC4 and CC5 supernatants and compared with CC1 (supernatant and ammonium sulphate precipitated "AS" preparations) reaction. High purity water was used as a negative control. N = Undiluted supernatant.

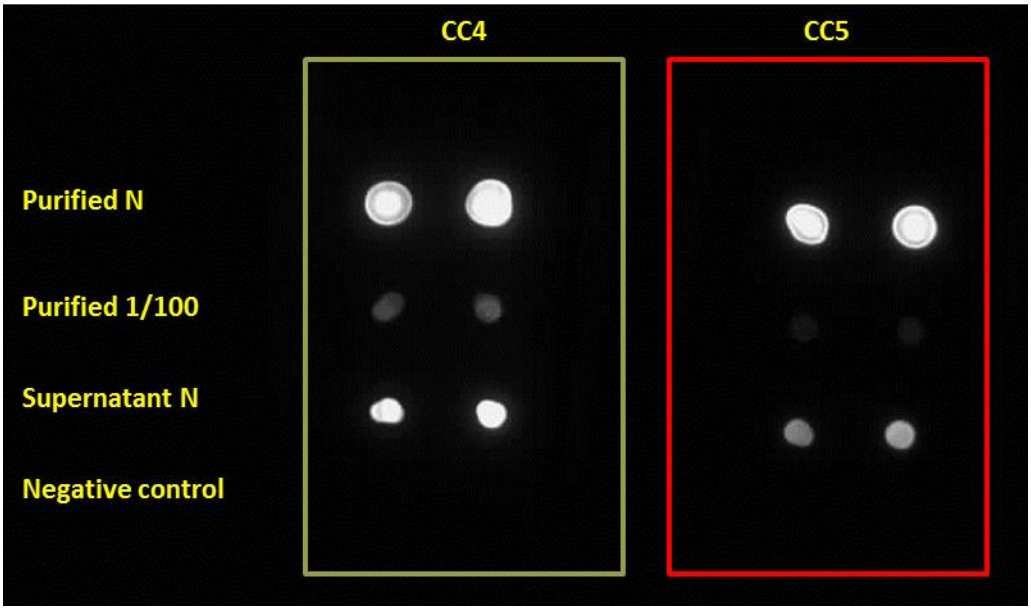


Figure 3.14 Dot blot screening for immunoreactivity of the secondary antibodies to CC4 and CC5 antibodies.

An anti-IgM and anti-IgA mouse secondary antibodies were used against CC4 and CC5 (purified and supernatant preparations), respectively. High purity water was used as a negative control. N = Undiluted.

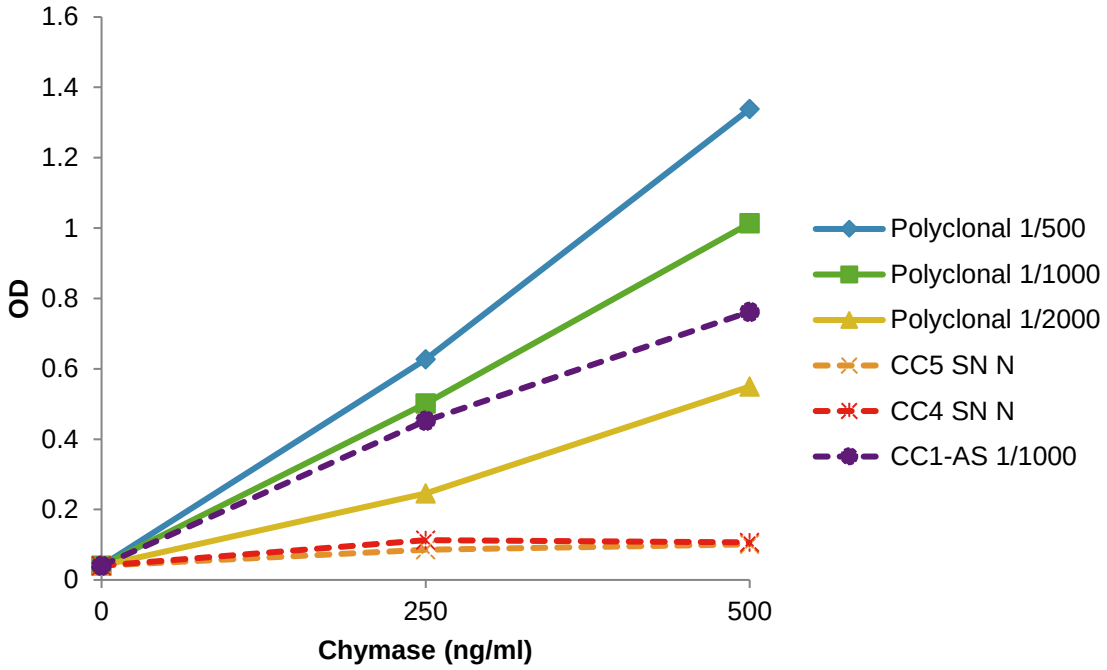


Figure 3.15 Indirect ELISA screening for goat polyclonal anti-chymase antibody. The polyclonal antibody was diluted into 1/500, 1/1000 and 1/2000 dilutions and used in the assay in addition to the precipitated CC1monoclonal antibody (CC1-AS) at 1/1000 dilution. CC4 and CC5 undiluted supernatants (SN N) were included in the assay as well. Purified skin chymase was used at 250 and 500 ng/ml concentrations. Data represent the mean of duplicate determinations. The reaction was read at 450/595 nm.

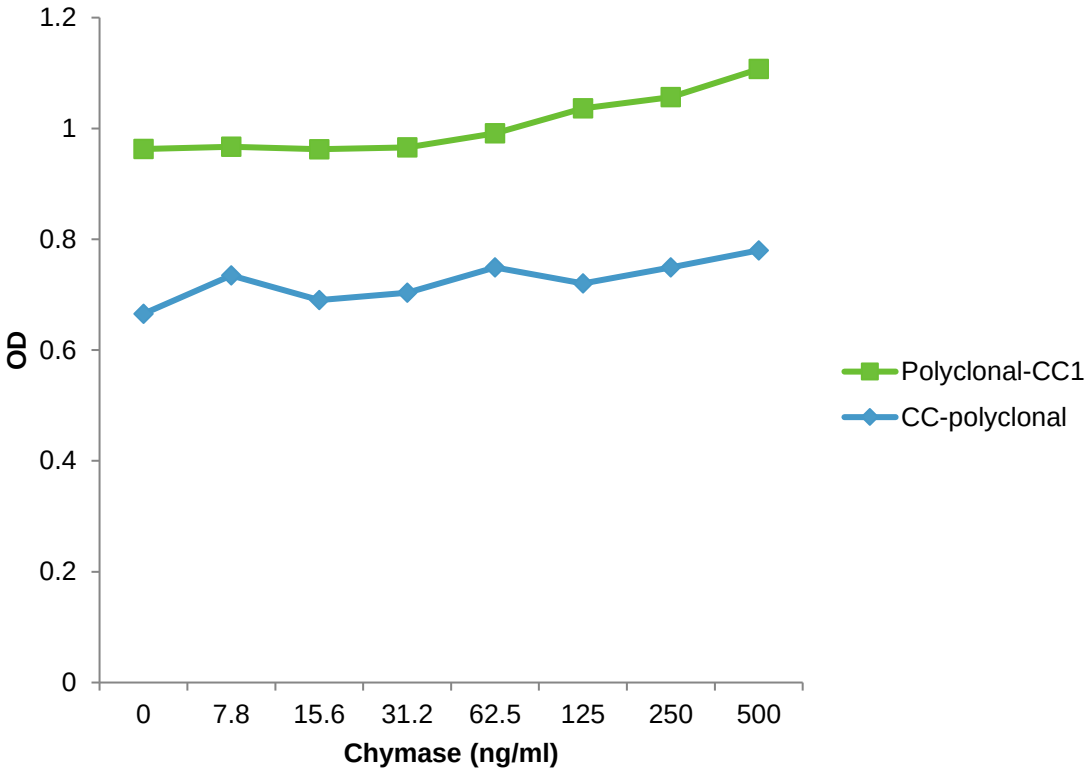


Figure 3.16 Lack of the standard curve in chymase sandwich ELISA using commercially available goat polyclonal anti-chymase antibody and monoclonal CC1 antibody combinations.

The polyclonal antibody was used as capture antibody with CC1 detection side of the assay (polyclonal-CC1) and as detection antibody with CC1 capturing the reaction (CC1-polyclonal). Standards were prepared from purified skin chymase. Data represent the mean of duplicate determinations. The reaction was read at 540/595 nm.

3.3.4 DPPI assay

In the present study, we tried to reproduce DPPI ELISA using the protocol mentioned by Dr. Whitworth in her thesis "Markers of allergic reactions to food based on activation of mast cells and basophils" and a standard curve was generated using DD1 capture antibody and rabbit polyclonal anti-DPPI detecting antibody with serial dilutions of recombinant DPPI as the protein of interest (Figure 3.17). The assay was less sensitive for detection of DPPI levels in serum samples. The signal for most serum samples lay at the lower end of the standard curve and gave unreliable readings whilst the lower four points of the standard curve were at a level equivalent to a blank response. Levels of DPPI in ten serum samples diluted serially from 1/5 up to 1/40 did not show a notable decline in much diluted serum samples (Figure 3.18 a). Dilution factor effect was more pronounced when projected concentrations were calculated resulting in an associated increase in DPPI levels (Figure 3.18 b). The percentage recovery of 250 ng/ml DPPI spiked in various dilutions of ten serum samples was low in the less diluted samples, but was 92% at a 1/40 dilution (Table 3.3).

Using sample diluent (R&D system) or 0.5 M sodium chloride solution, that acts as physiological saline maintaining the correct protein conformation, in the assay for DPPI did not have much effect on the standard curve compared with the one generated using 3% FBS diluent (Figure 3.19). A commercially available monoclonal anti-DPPI antibody (St. John's laboratories) failed to bind to DPPI (Figure 3.20). While screening for St. John's anti-DPPI antibody we found that DD1 was not reacting with DPPI as well and that the rabbit polyclonal anti-DPPI was the only working antibody in the assay (Figure 3.20). We also found that DPPI bound to the plate even after blocking with 20% FBS. After testing various blocking solutions, we found that protein-free blocking buffer prevented DPPI from binding to the plate (Figure 3.21).

In order to have new stocks for anti-DPPI antibodies, two clones of anti-DPPI antibodies (DD1 and DD3) were regenerated. However, we found that freshly prepared supernatants for DD1 and DD3 were poorly responding with DPPI in indirect assay (Figure 3.22). We also screened these antibodies using dot blot analysis but supernatants of monoclonal antibodies DD1 and DD3 failed to bind to DPPI (Figure 3.23).

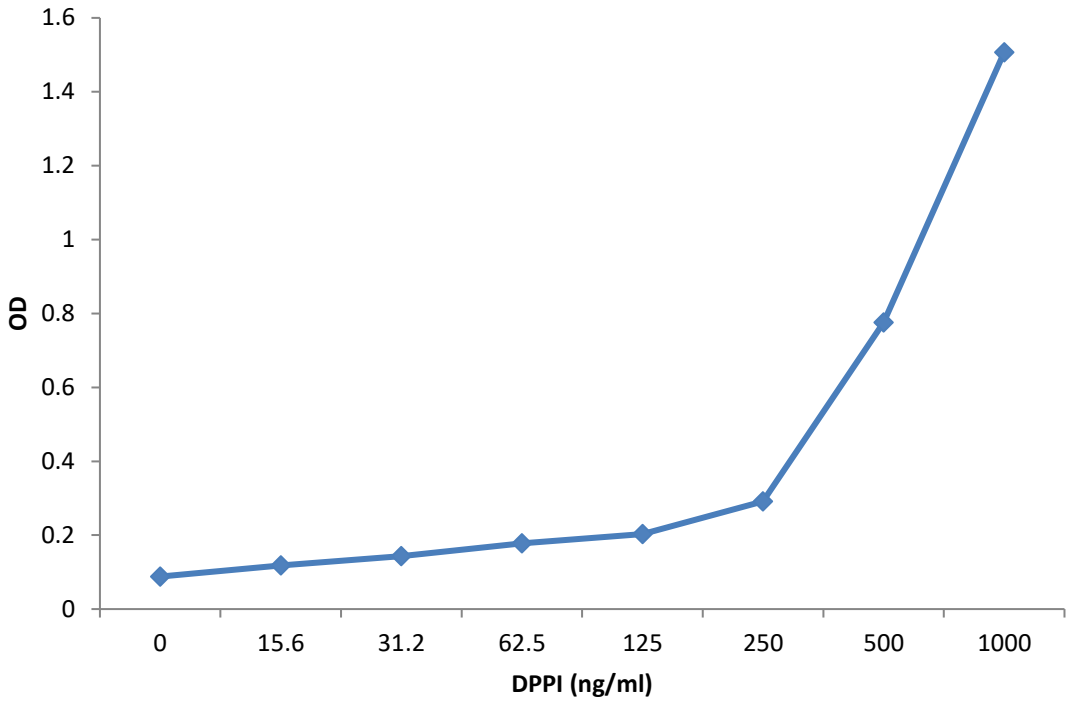


Figure 3.17 DPPI ELISA standard curve.

Previously tested DPPI sandwich ELISA conditions were used and included using DD1 capture antibody, DPPI standards, rabbit polyclonal anti-DPPI antibody and anti-rabbit HRP-conjugated secondary antibody. Data represent the mean of duplicate determinations. The reaction was read at 450/595 nm.

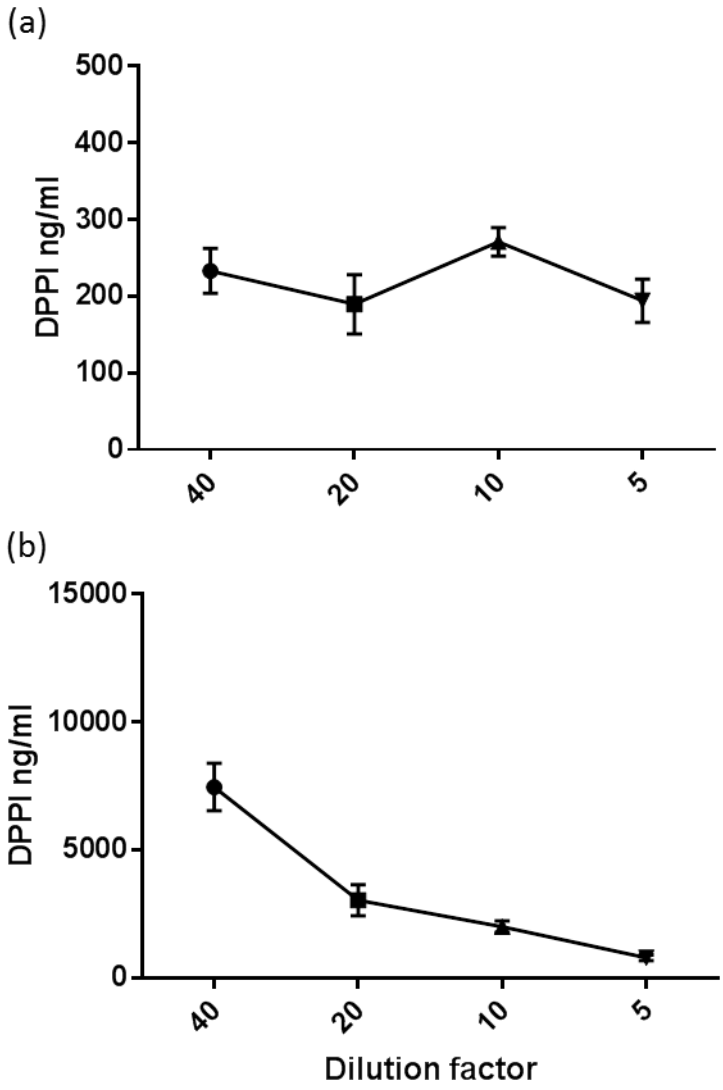


Figure 3.18 Serial dilutions of serum samples for DPPI assay.

DPPI levels were determined in four serial dilutions of each serum sample (n=10) at (a) measured concentrations and (b) projected concentrations. Data represent Mean ± SEM.

Table 3.3 Percentage Recovery for DPPI sandwich ELISA.

Serum samples (n = 10) at various dilutions spiked with 250 ng/ml DPPI.

Dilution factor	Average % Recovery	Standard deviation
5	36	24
10	54	46
20	54	11
40	92	17

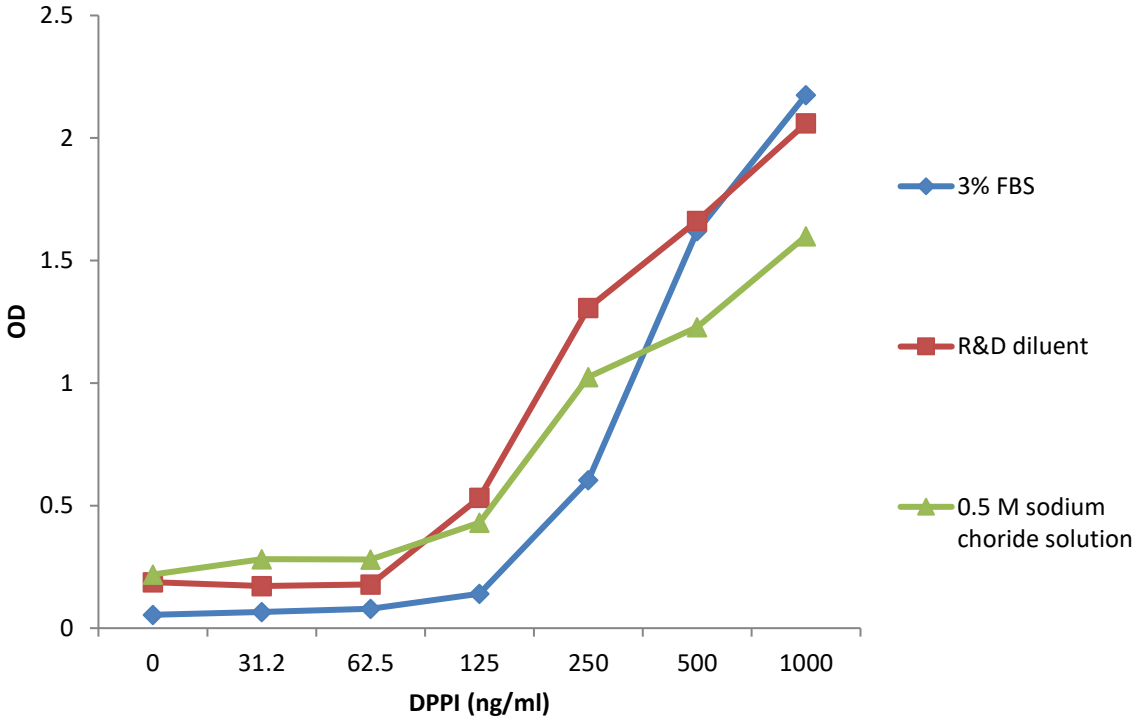


Figure 3.19 Effect of various diluents on the assay of DPPI.

Three standard curves were generated using 3% FBS (blue), R&D system diluent (red) and 0.5 M sodium chloride solution (green). The assay conditions included using 1/500 DD1 as capture antibody, 1/1000 rabbit polyclonal anti-DPPI antibody for detection and the standards were derived from recombinant DPPI. Data represent the mean of duplicate determinations. Reaction was read at 450/595 nm.

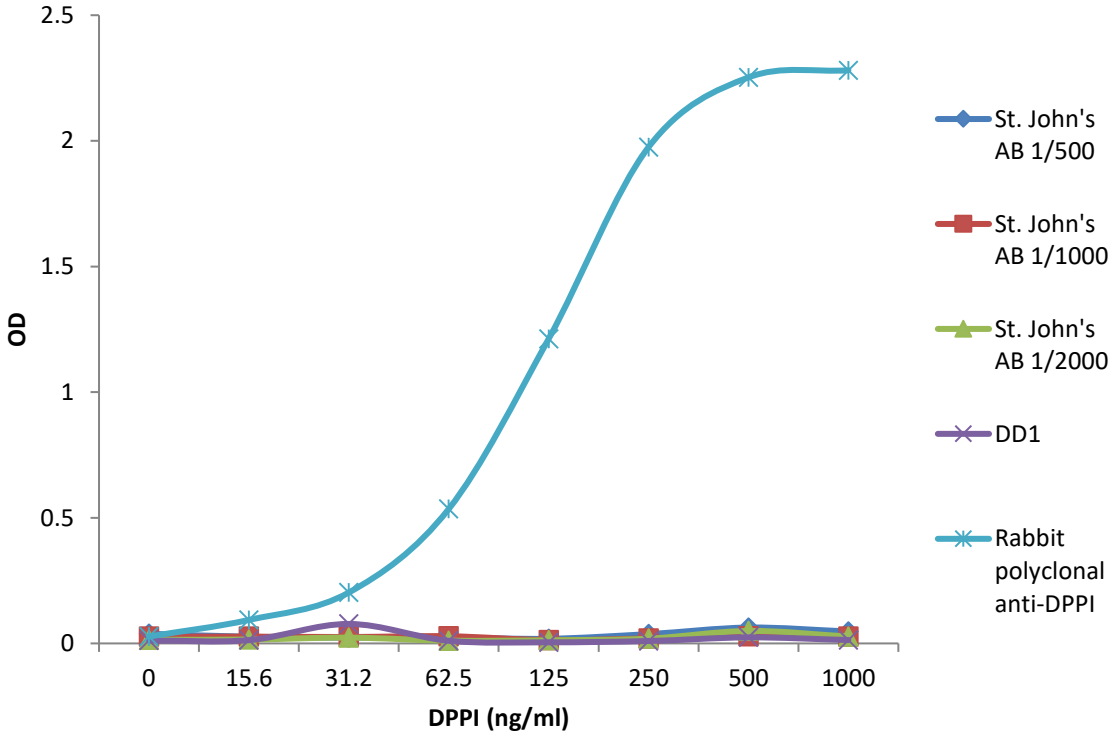


Figure 3.20 Indirect ELISA testing for a commercially available monoclonal anti-DPPI antibody (St John's Laboratories).

Various dilutions for the new monoclonal antibody (St. John's AB) was used in the assay and their reactivity was compared to the reactivity of monoclonal antibody DD1 and rabbit polyclonal anti-DPPI antibody. Standards were derived from recombinant DPPI. Data represent the mean of duplicate determinations. Reaction was read at 450/595.

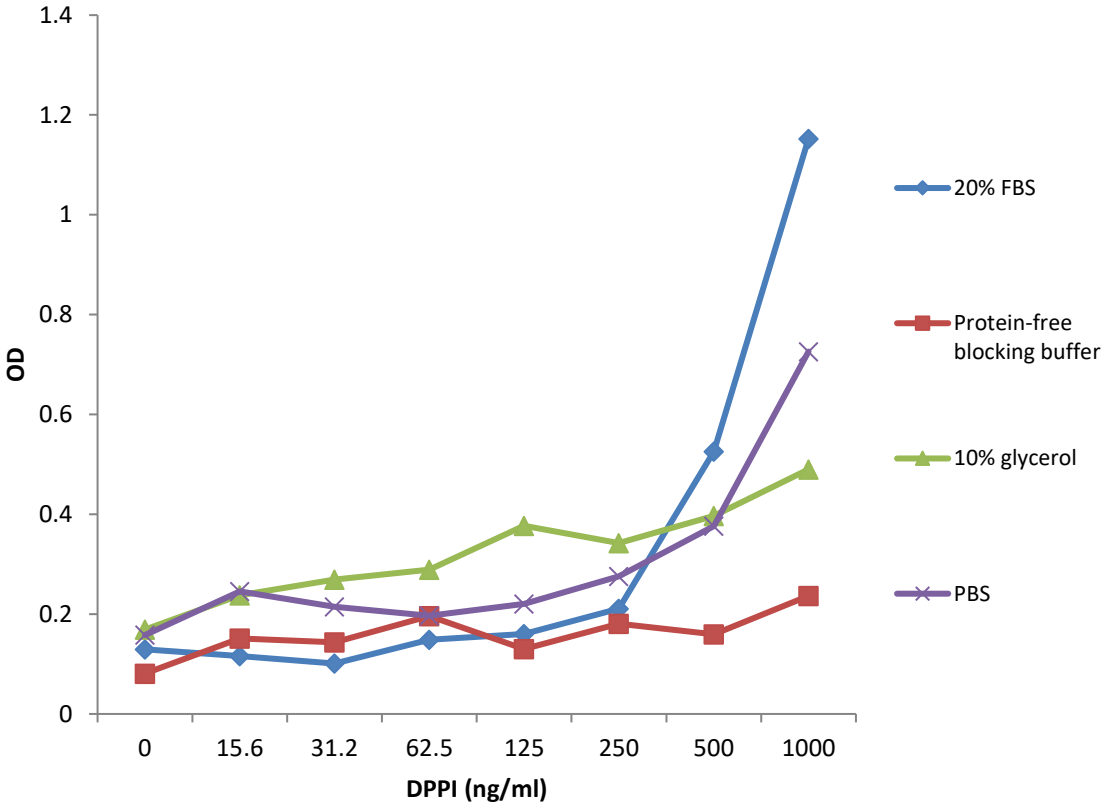


Figure 3.21 The effect of blocking buffers on the assay of DPPI.

The blocking buffer employed previously 20% FBS was used in addition to a protein-free blocking buffer and 10% glycerol solution to prevent DPPI binding to the plate. Rabbit polyclonal antibody was used to detect the reaction. Data represent the mean for duplicate determinations. Reaction was read at 450/595 nm.

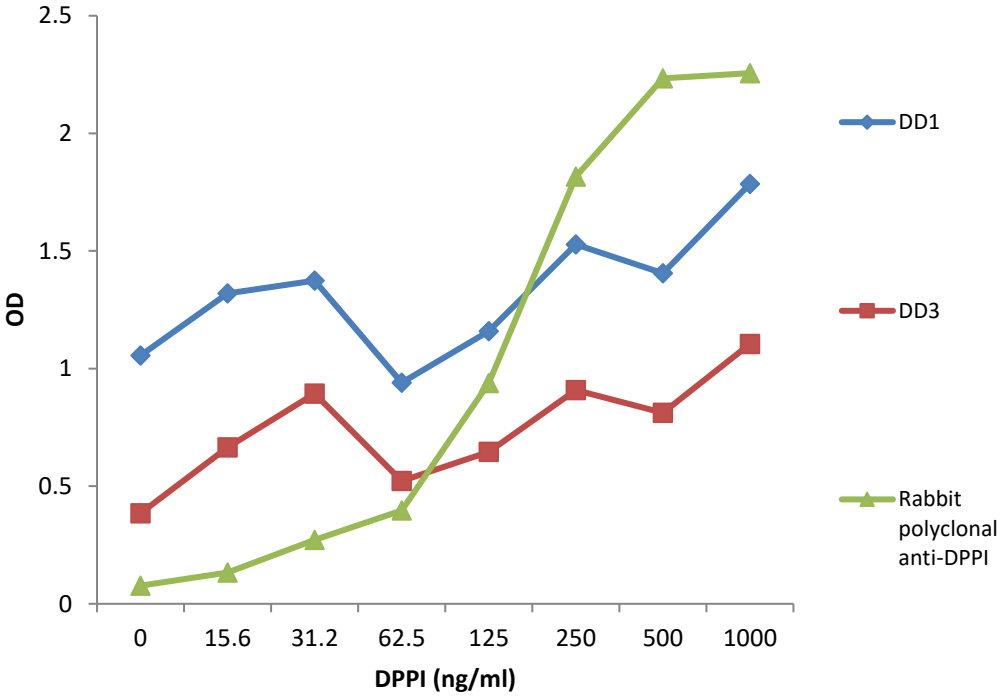


Figure 3.22 Indirect ELISA for DD1 and DD3 supernatants.

DD1 and DD3 supernatants were tested using 1/10 dilution and their reaction was compared to the reaction produced by the rabbit polyclonal anti-DPPI antibody. Standards were derived from recombinant DPPI. Data represent the mean for duplicate determinations. Reaction was read at 450/595 nm.

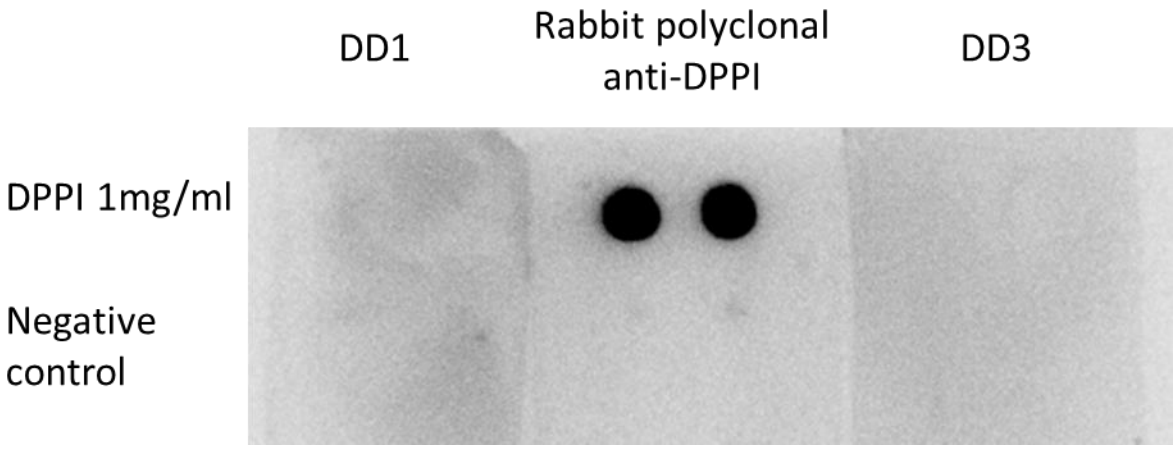


Figure 3.23 Dot blot analysis for DPPI antibodies.

The immunoreactivity of supernatants for DD1 and DD3 to DPPI was screened by dot blot and compared to that for the rabbit polyclonal anti-DPPI antibody. The nitrocellulose membrane was loaded with a concentration of 1 mg/ml DPPI in duplicates. High purity water was used as a negative control.

Chapter 4: Multidimensional endotypes in patients with allergic reactions revealed by topological data analysis of clinical and immunological parameters

4.1 Introduction

Allergic reactions can present with diverse symptoms ranging from a mild skin rash to a life-threatening anaphylaxis. However, little is known of how such clinical heterogeneity can relate to underlying cellular and biochemical events. To date, there have been no distinct subgroups or endotypes of allergic reactions defined by pathophysiological mechanisms that can distinguish those at risk of severe symptoms from the others. There have been several studies on the association of baseline tryptase levels and severity of allergic reactions. In a study performed on children who had previously suffered from food-induced anaphylaxis, it has been reported that baseline tryptase levels higher than the cutoff values of 5.7 and 14.5 ng/ml were associated with 50% and 90% predicted probability, respectively, of moderate to severe allergic reactions (6). The author, however, did not report the sensitivity or specificity of the test. Baseline tryptase levels of more than 4.8 ng/ml have also been reported to be associated with increased risk of severe systemic reactions in children with venom allergy (7). However, at this cutoff value there was a sensitivity of just 45.8% and specificity of 87.1%. The differences in reported cutoff values for tryptase suggest a substantial individual variation in levels of tryptase and with low baseline levels one cannot discount a risk of anaphylaxis in the future. Therefore, defining distinct endotypes and their relationships to unique biomarkers should enable identification of patients with high risk of severe allergic reactions and facilitate proper management.

Topological data analysis (TDA) has been developed by applying geometry and topology to explore and valorise large data sets (218). Three key features have been described for TDA (219). Firstly, the construction of geometric objects does not depend on a chosen coordinate but on the distance function that specifies the shape. Secondly, the analysis is less sensitive to noise and missing values, which means that the topological properties are maintained despite any deformations. Thirdly, the topological network has the ability to compress representation and encodes complex relationships to a simpler form. A study comparing TDA to other conventional analysis like principle component analysis (PCA) using four bacteria strains has shown that the four strains were retrieved with PCA but with strong overlap (219). On the other hand, topological

data analysis extracted distinct clusters for the four strains. Deleting half of the spectra for each bacterial strain has not affected the multidimensional structure of TDA network. The concept of TDA has been employed widely to investigate possible disease endotypes like asthma (220), osteoarthritis (221), cancer (222, 223) and hereditary syndromes (224). As a consequence, TDA can be considered as a robust tool for analysing high-dimensional and complex data sets.

In this chapter, we have investigated serum levels of markers of mast cell activation and a panel of cytokines in patients, who have detailed clinical characterization, with allergic reactions to drugs or food aiming to identify and independently replicate distinct clinico-immunological endotypes using topological data analysis.

4.2 Methods

4.2.1 Southampton cohort (derivation study)

Patients attending the asthma and allergy clinic in University Hospital Southampton for diagnostic drug challenge were recruited from January 2015 to December 2016. Details for subjects and sample collection are described in detail in Sections 2.1. In brief, patients were informed about the project and, if they agreed to take part, given a detailed information sheet (Appendix A) and consent form (Appendix B) to sign. Demographic information, vital signs and details of the drug challenge were recorded (Appendix C). A note was taken also of times of sample collection and the outcome of the challenge were also recorded. In addition, the study also included serum samples that had been collected previously (2009-2012) from patients undergoing drug or food challenge with the same demographic data recorded.

4.2.2 Qatar cohort (validation study)

The validation cohort comprised participants enrolled by a different study team from outpatient clinics at Queen Medical Hospital in Doha, Qatar. Patients with allergy-related conditions including recent anaphylaxis caused by drugs, food or insect bite, drug or food allergies, atopic dermatitis, hay fever, asthma and insect bite allergies were consented, and their demographic information were recorded. Samples were collected at the time of the visit.

4.2.3 Study procedure

For the derivation cohort, a detailed history was taken from the participants recruited regarding the severity of their reactions to drugs or food, and grading was according to the severity grading scheme of Brown *et al* 2006 (24), and the presence of atopic conditions such as atopic dermatitis, hay fever or asthma. Subjects with chronic urticaria, a drug reaction with eosinophilia and systemic symptoms (DRESS), a delayed hypersensitivity reaction or on steroid treatment were not included in the study. Baseline and post-challenge serum levels of tryptase and CPA3 were determined using sandwich ELISAs that had been developed and optimized in the laboratory (described in Chapter 3). Levels of pro-inflammatory mediators in serum before and at the end of the challenge were determined using an electro-chemiluminescence-based V-plex assay (Pro-inflammatory panel 1 kit, Meso Scale Discovery, Rockville, USA).

For the validation cohort, the symptoms and diagnosis of the allergic condition were reported together with the results of any skin tests performed. The severity of allergic reactions were graded according to the grading system of Brown *et al* 2006 (24) to make their characteristics comparable to that of the derivation cohort. As the population of Qatar comprises diverse ethnic groups, the ethnicity for participants was recorded. Serum levels of mediators (tryptase, CPA3 and pro-inflammatory mediators) were determined at one-time point only. Assays for saliva and urine samples were not ready yet to be employed on clinical samples.

Serum samples from healthy subjects who had no history of allergic diseases were analysed for the levels of tryptase, CPA3 and pro-inflammatory mediators to serve as controls for the study.

4.3 Statistical analysis

Data were analysed initially by standard statistical methods. Data were logarithmically transformed because in most cases the distribution was skewed. Comparison of continuous variables across two paired groups was performed using Wilcoxon Signed Ranks test such as when comparing serum levels of mediators at baseline and post-challenge. Comparisons between groups of patients and healthy control subjects were performed using the Kruskal-Wallis test (H), which was followed by the Mann-Whitney U test for multiple comparison. The diagnostic performance of baseline CPA3 levels to predict severity of allergic reactions was determined by receiver operating characteristics (ROC) curve analysis. Sensitivity and specificity were calculated for each selected cutoff points. The degree of association between two continuous variables was

performed by calculation of Spearman's Rank Correlation Coefficient. The association between two or more categorical data sets was performed using the Chi-squared test (χ^2) or its alternative, Fisher's Exact test, if sample size was small. For all analyses, two-tailed *p* values of less than 0.05 were considered significant.

Data from derivation and validation cohorts were analysed individually using topological data analysis (TDA) as an exploratory tool to define multidimensional clusters. TDA was performed using Ayasdi Core (Ayasdi, California, USA) that builds up a three-dimensional network with the parameters entered. Each node within the network represents individuals having similar features. The lines connect nodes that have shared data points. This technique allows visual representation of the data and constructs multidimensional clusters. Metric correlation and two neighbourhood lenses (resolution 30, gain 3) were used as filter functions in TDA settings. The Kolmogorov–Smirnov (K-S) test was used to define the differences between the distribution of one cluster from the others. Data were analysed with SPSS 25 (IBM, Armonk, NY) and Prism 7 (GraphPad software, San Diego, California) software.

4.4 Results

The derivation cohort comprised 194 participants (7-86 years old) undergoing drug (140 [72%]) or food (54 [28%]) challenge. All had a history of allergic symptoms ranging from skin rash to full-blown anaphylaxis. Most patients tested for drug allergic reactions were females, in contrast to those tested for food reactions for whom males formed the majority. Therapeutic agents tested ranged from one to eight per patient, whereas just one type of food was tested on food challenge. Antibiotics were the most frequently tested drugs (employed in a quarter of the patients), followed by local anaesthetics, opioids, general anaesthetics and NSAIDs (Figure 4.1a). Tree nuts and peanuts were employed in two-thirds of the total food challenges (Figure 4.1b).

The challenge was considered positive when the patient showed a response to the agent tested. In general, the responses were mild and included positive skin reactions only. Many of the participants who underwent drug challenge reported mild historical reactions to the agent tested. On the other hand, the majority of patients tested for food reactions gave a history of moderate to severe reactions. Most patients who underwent drug (58%) or food (90%) challenge suffered one or more atopic diseases including atopic dermatitis, hay fever or asthma. There were cases where complete information was not taken or missed. For subsequent analysis, subjects who underwent drug challenges were considered separately from those who underwent food

challenges. Detailed information on the drug and food challenges performed are described in Table 4.1.

The validation cohort comprised 119 participants (2-61 years old) with allergy-related conditions attending the outpatient clinic. The majority of the participants were females and of Middle Eastern ethnic background. Anaphylaxis was the most common condition recorded followed by food allergy, atopic dermatitis, hay fever and drug allergy. It should be mentioned that cases of anaphylaxis, drug or food allergy were seen at least 24 hours after the episode had occurred. Demographic features of the validation cohort are summarised in Table 4.2.

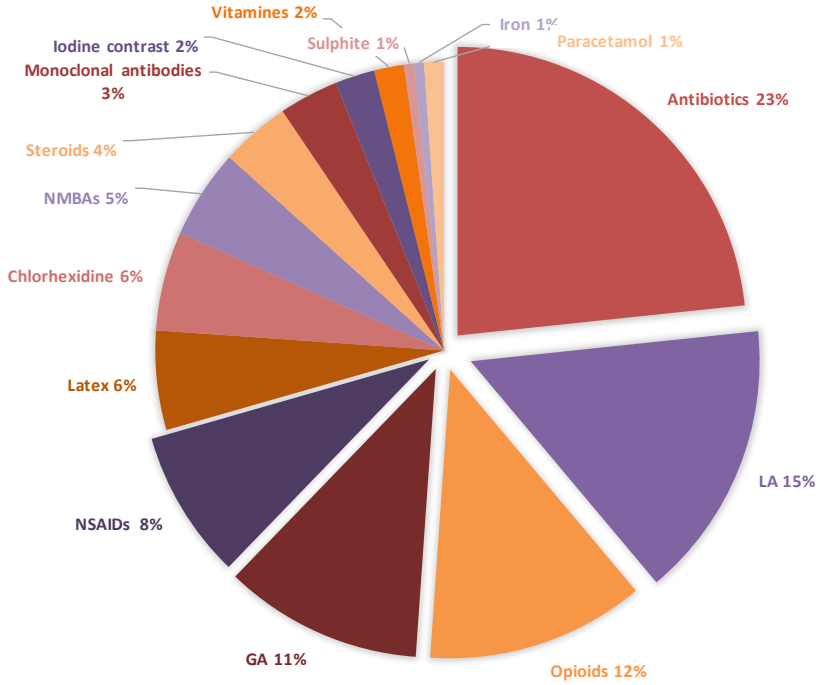
The control subjects included twenty-two (5 males and 17 females) healthy individuals, aged between 22 and 49 years (median = 33), from different ethnic background (Two Caucasian, 8 Middle Eastern, 5 Asian, 5 African and 2 Hispanic) with no history of allergic disease.

4.4.1 The derivation cohort

4.4.1.1 The effect of allergen challenge on serum levels of mast cell proteases and cytokines

When serum levels of tryptase, CPA3 and pro-inflammatory cytokines were determined pre- (baseline level) and post-challenge (n= 179), an increase in levels of IL-13 (p= 0.049) and IL-6 (p< 0.0001) was observed in patients who reacted in the challenge (n= 69). A corresponding significant difference in levels was not seen for tryptase, CPA3 or for the other cytokines (Figure 4.2). When levels of mast cell mediators were determined in serum samples from patients who had negative outcome on challenge (n= 110), no changes in levels were observed except for IL-6 (p< 0.0001), which showed a similar pattern to that seen in participants with positive outcome (Figure 4.3). For 15 of the patients a serum sample collected after the challenge was not available, mainly on account of cannulation errors.

(a)



(b)

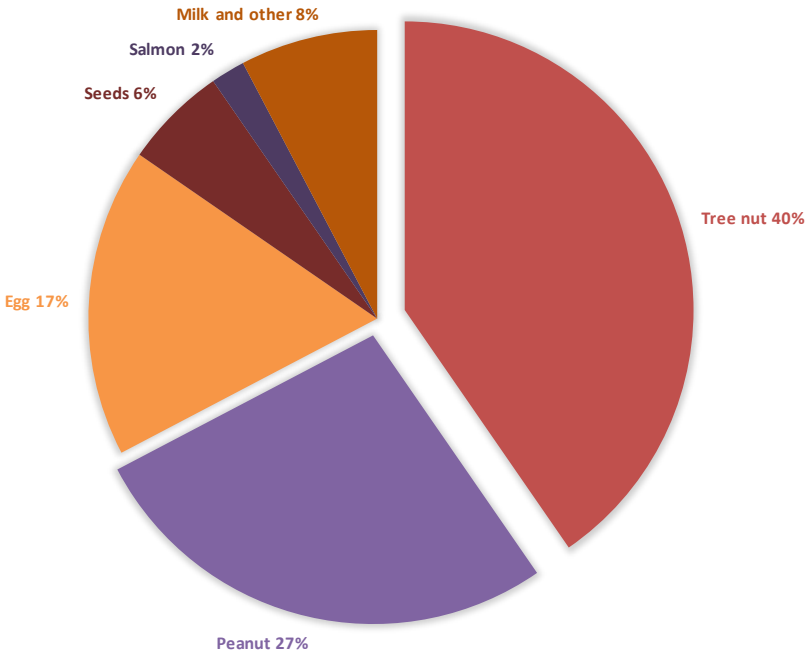


Figure 4.1 (a) Drugs and (b) food tested in allergen challenges of the study participants.

LA = Local anaesthetics, GA = General anaesthetics, NSAIDs = Non-steroidal anti-inflammatory drugs, NMBA = Neuromuscular blocking agen

Table 4.1 Demographics of patients in the derivation cohort

Parameter	Drug challenges	Food challenges
Age (years) **Median (min-max)**	49 (11-86)	14 (7-43)
Gender (male/female) No. (%)	41 (29)/99 (71) *	40 (74)/14 (26)
Challenge outcome **(positive/negative) no. (%)**	53 (38)/87 (62)	16 (30)/38 (70)
Severity of historical reactions **Mild no. (%)**	82 (60) **	7 (14) *
Moderate no. (%)	14 (10)	22 (42)
Severe no. (%)	41 (30)	23 (44)
Atopic conditions **Atopic dermatitis (yes/no) no. (%)**	18 (17)/87 (83) ‡	31 (65)/17 (35) †
Hay fever (yes/no) no. (%)	43 (41)/62 (59) ‡	37 (77)/11 (23) †
Asthma (yes/no) no. (%)	22 (21)/83 (79) ‡	24 (50)/24 (50) †

* missing values (n=2)

**missing values (n=5)

‡ missing values (n=37)

† missing values (n=6)

Table 4.2 Demographics of patients in the validation cohort

Parameter	
Age (years) Median (min-max)	32 (2-61)
Gender (male/female) No. (%)	34 (29)/85 (71)
Ethnicity Caucasian no. (%)	25 (21)
Middle Eastern no. (%)	69 (58)
Asian no. (%)	20 (16)
African no. (%)	2 (2)
Hispanic no. (%)	3 (3)
Diagnosis * Anaphylaxis no. (%)	24 (23)
Drug allergy no. (%)	13 (12)
Food allergy no. (%)	20 (19)
Atopic dermatitis no. (%)	20 (19)
Hay fever no. (%)	15 (14)
Asthma no. (%)	7 (6)
Insect bite no. (%)	8 (7)
Severity of historical reactions Mild no. (%)	12 (19)
Moderate no. (%)	26 (42)
Severe no. (%)	24 (39)

* missing values (n=12)

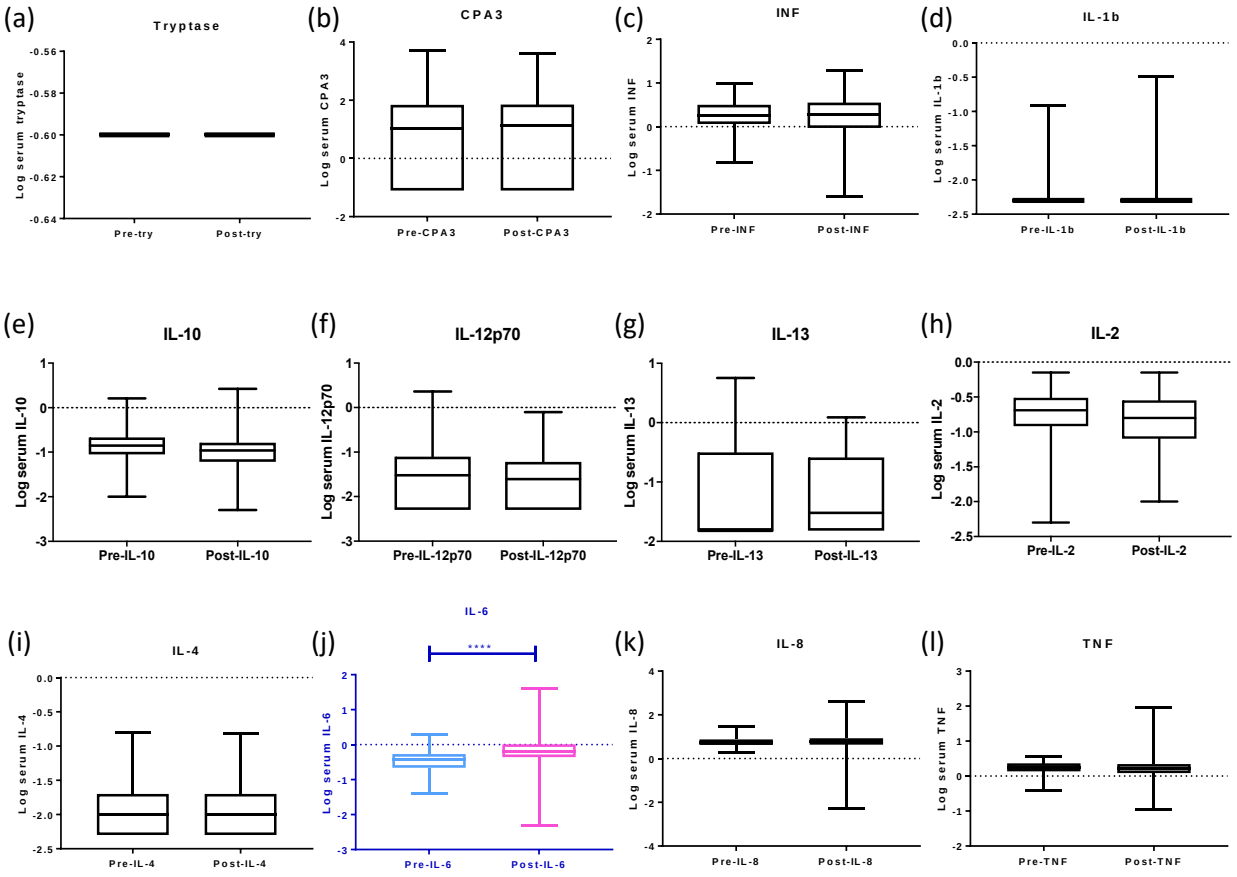


Figure 4.2 Serum levels of mediators at baseline and post-challenge for the patients with positive outcome.

Levels for (a) tryptase, (b) CPA3, (c) INF, (d) IL-1β, (e) IL-10, (f) IL-12p70, (g) IL-13, (h) IL-2, (i) IL-4, (j) IL-6, (k) IL-8 and (l) TNFα are shown. Statistical analysis was determined by Wilcoxon Signed Ranks test on log-transformed data (****p<0.0001) (Refer to Appendix D for a larger size graphs).

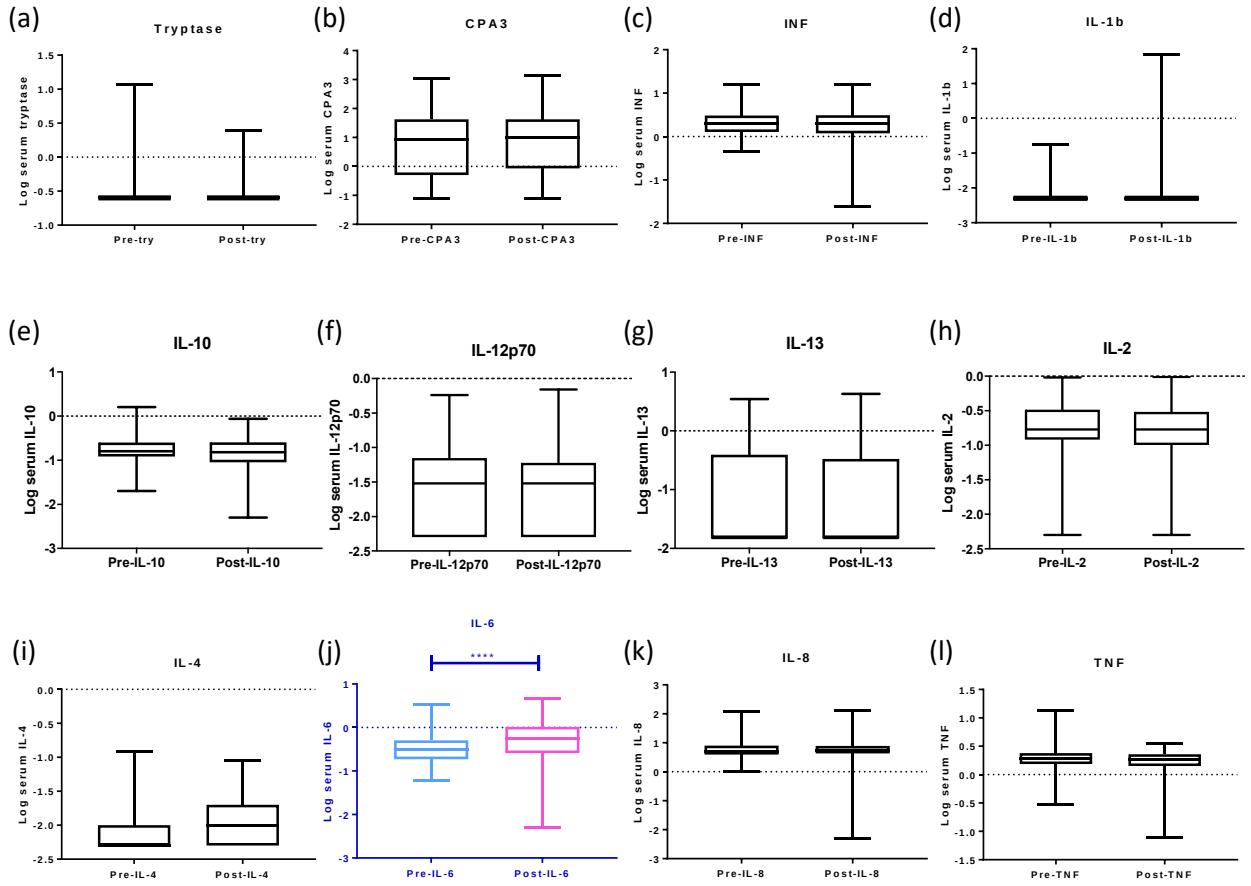


Figure 4.3 Serum levels of mast cell mediators at baseline and post-challenge for the patients with negative outcome.

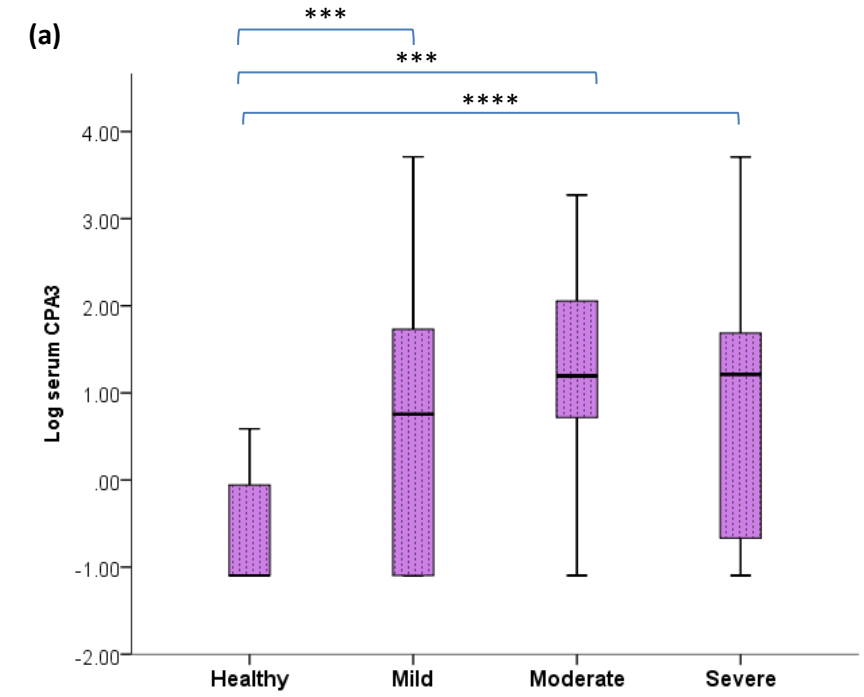
Levels for (a) tryptase, (b) CPA3, (c) INF, (d) IL-1β, (e) IL-10, (f) IL-12p70, (g) IL-13, (h) IL-2, (i) IL-4, (j) IL-6, (k) IL-8 and (l) TNFα are shown. Statistical analysis was determined by Wilcoxon Signed Ranks test on log-transformed data (****p<0.0001) (Refer to Appendix D for larger size graphs).

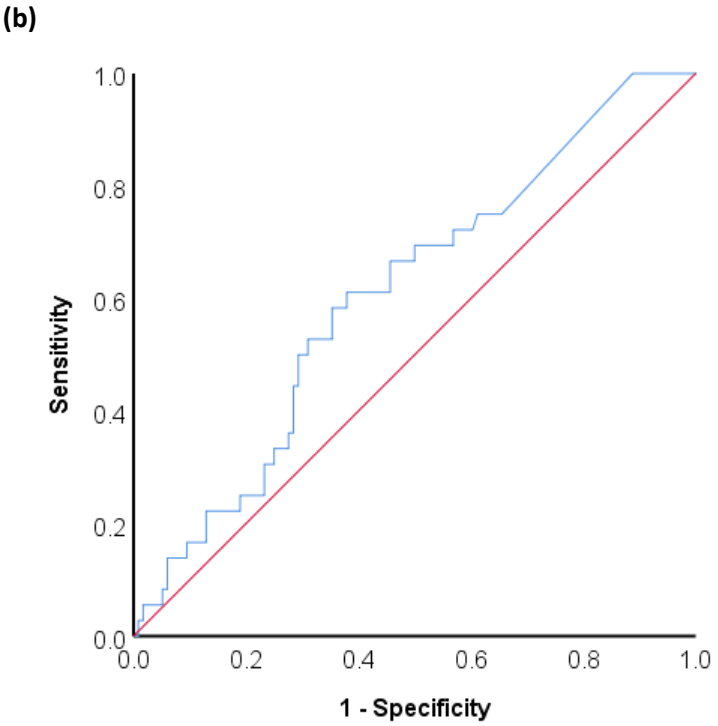
4.4.1.2 The association between baseline levels of mast cell mediators in serum and severity of historical reactions

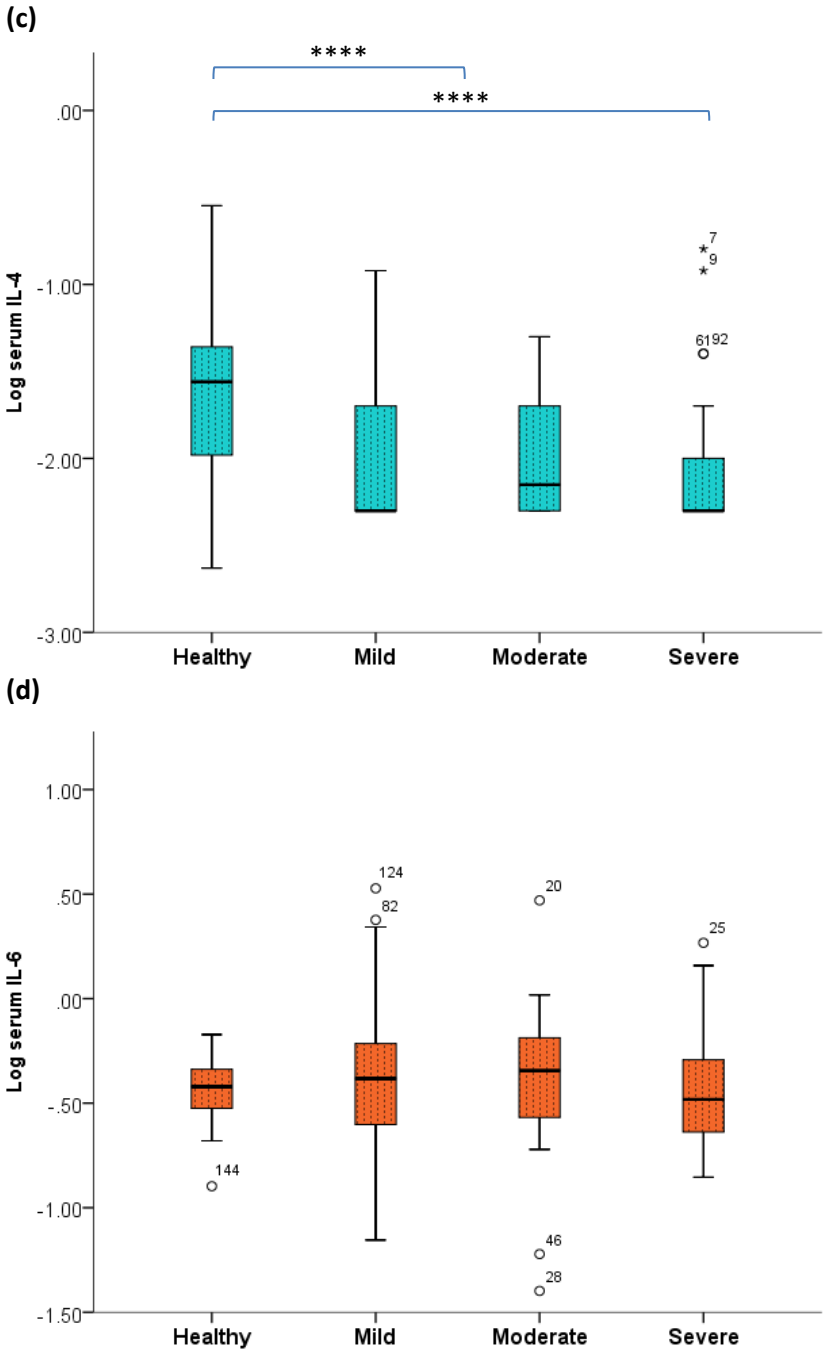
CPA3 serum levels were significantly higher in patients who had experienced mild, moderate or severe allergic reactions compared to those of healthy individuals (H (3) = 32, $p< 0.0001$) with a mean rank of 80, 97, and 89 for patients with mild, moderate and severe reactions, respectively, and a mean rank of 29 for healthy subjects (Figure 4.4a). ROC curve analysis identified a potential role for baseline CPA3 levels in predicting the severity of allergic reactions (AUC: 0.61, 95% CI: 0.51-0.71, $p= 0.048$; Figure 4.4b). When the coordinates of the curve were analysed for best cutoff value with the highest sensitivity and specificity, a cutoff value of 6.46 ng/ml for CAP3 baseline levels was obtained with a sensitivity and a specificity of 61% and 60%, respectively.

Higher levels of IL-4 (Figure 4.4c) and IL-8 (Figure 4.4e) were present in those who had had milder historical reactions than in those with more severe symptoms; and levels were significantly higher in healthy subjects compared to the patients (H (3) = 24, $p< 0.0001$ for IL-4 and H (3) = 9, $p= 0.03$ for IL-8) with mean ranks for IL-4 and IL-8 of 75 and 79 for mild reactions, 78 and 83 for moderate reactions, 68 for severe reactions, and 121 and 103 for healthy subjects, respectively. Comparison of serum levels of IL-6 in patients with drug challenge with history of allergic reactions of varying severity to those of healthy subjects showed no significant differences (Figure 4.4d).

When the relationship between baseline serum levels of mediators and severity of previous allergic reactions was analysed for the patients who underwent food challenges, the differences in levels of CPA3, IL-4 and IL-8 between patients and healthy subjects mirrored those seen with patients underwent drug challenge (H (3) = 24, $p< 0.0001$ for CPA3, H (3) = 11, $p= 0.01$ for IL-4, H (3) = 33.5, $p< 0.0001$ for IL-8) with mean ranks for CPA3, IL-4 and IL-8 of 28, 36, 30 for mild reactions, 41, 33, 27 for moderate reactions, 46, 31, 29 for severe reactions, and 19, 50, 60 for healthy individuals, respectively (Figure 4.5a, c, and e). As seen with drug challenges, ROC curve analysis illustrates the predictive role of baseline CPA3 levels for the severity of allergic reactions (AUC: 0.74, 95% CI: 0.62-0.86, $p= 0.002$; Figure 4.5b) with a cutoff value of 3 ng/ml that had a sensitivity and a specificity of 68% and 70%, respectively. In contrast to the findings in patients who underwent drug challenge, serum levels of IL-6 were significantly lower in patients with food challenge compared to levels in healthy control participants (H (3) = 16, $p= 0.001$) with a mean rank of 33 for mild reactions, 32 for moderate reactions, 30 for severe reactions, and 53 for healthy individuals (Figure 4.5e).


(a)
***
***
****
4.00
3.00
2.00
1.00
.00
-1.00
-2.00
Log serum CPA3
Healthy
Mild
Moderate
Severe


(b)
1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity


(c)
****
****
.00
-1.00
-2.00
-3.00
Log serum IL-4
7
9
6192
Healthy
Mild
Moderate
Severe
(d)
1.00
.50
.00
-.50
-1.00
-1.50
Log serum IL-6
124
82
20
25
144
46
28
Healthy
Mild
Moderate
Severe

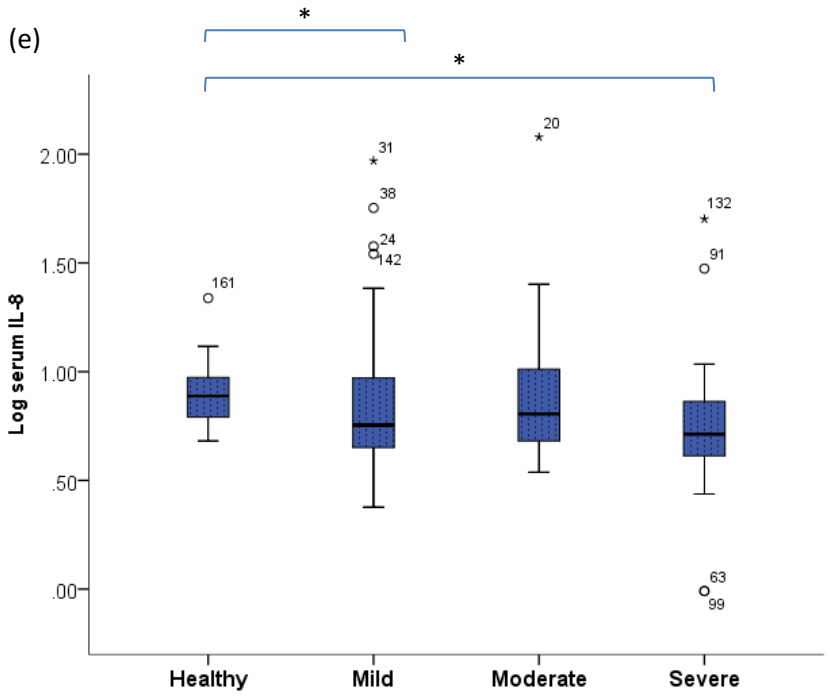
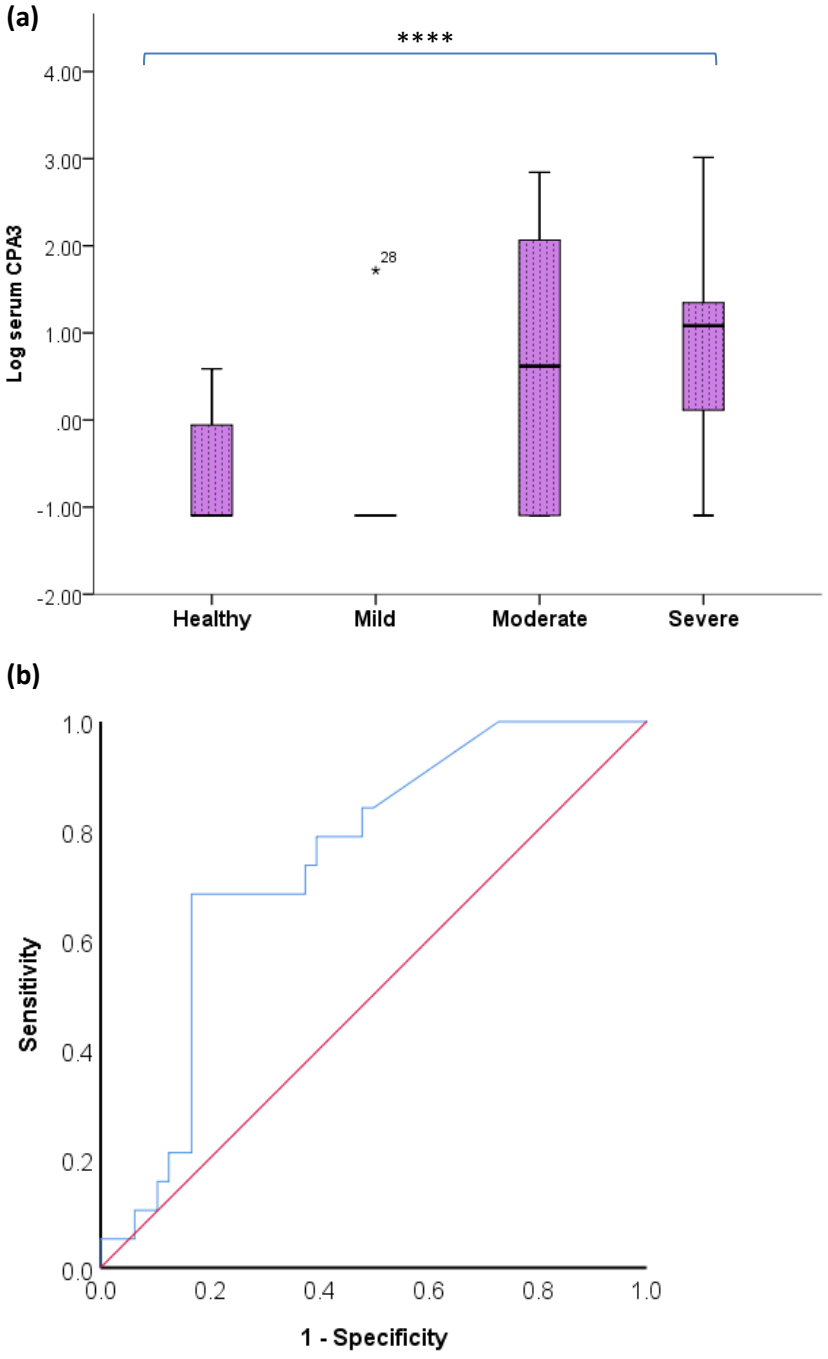
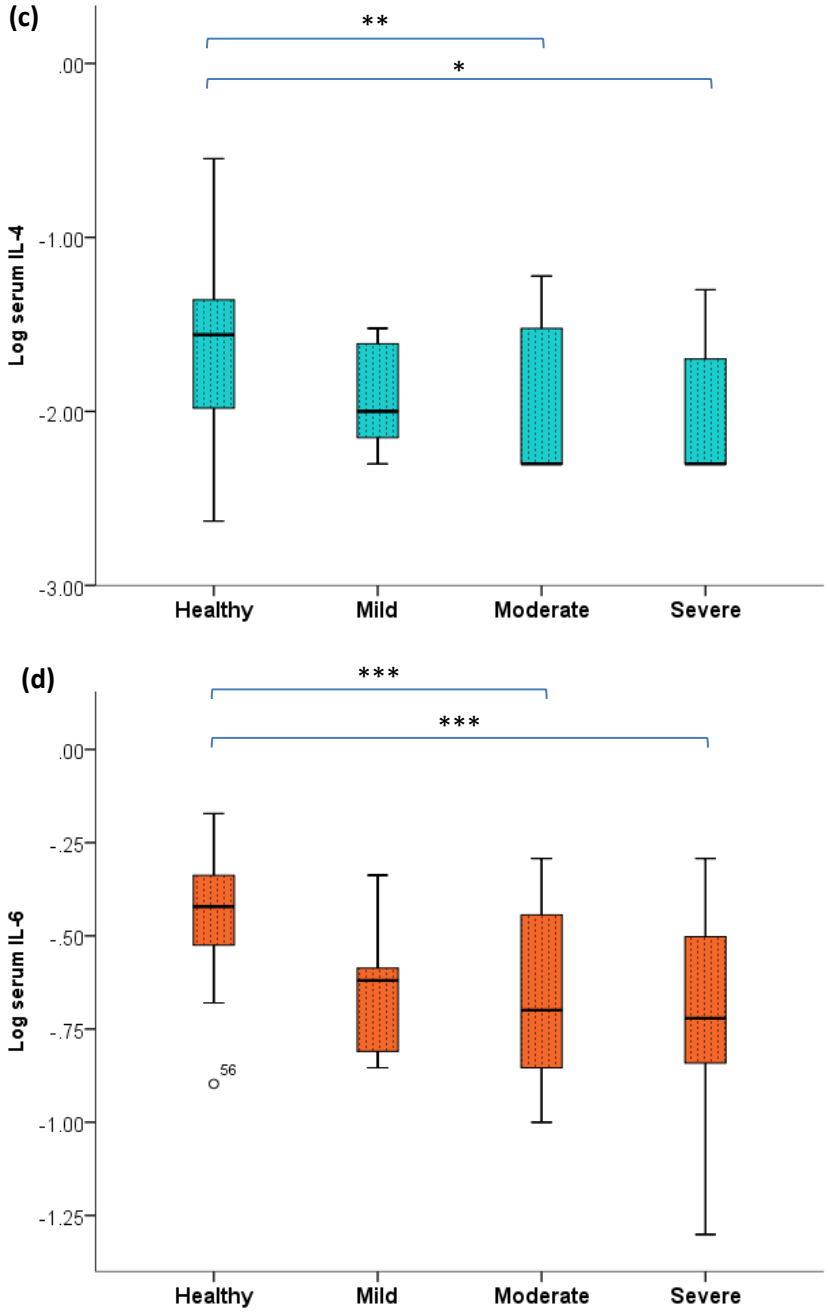


Figure 4.4 Levels of (a) CPA3, (c) IL-4, (d) IL-6 and (e) IL-8 in patients who underwent drug challenges and had mild, moderate or severe historical allergic reactions compared to those in healthy control subjects.

The role of baseline levels of CPA3 in prediction of severity of allergic reactions is illustrated in (b) ROC curve analysis. Statistical analysis was determined by Kruskal-Wallis test followed by Mann-Whitney U test on log-transformed data (* p<0.05, *** p<0.001, ****p<0.0001).


(a)
****
4.00
3.00
2.00
1.00
.00
-1.00
-2.00
Log serum CPA3
28
Healthy
Mild
Moderate
Severe
(b)
1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity


(c)
**
*
.00
-1.00
-2.00
-3.00
Log serum IL-4
Healthy
Mild
Moderate
Severe
(d)
***
***
.00
-.25
-.50
-.75
-1.00
-1.25
Log serum IL-6
56
Healthy
Mild
Moderate
Severe

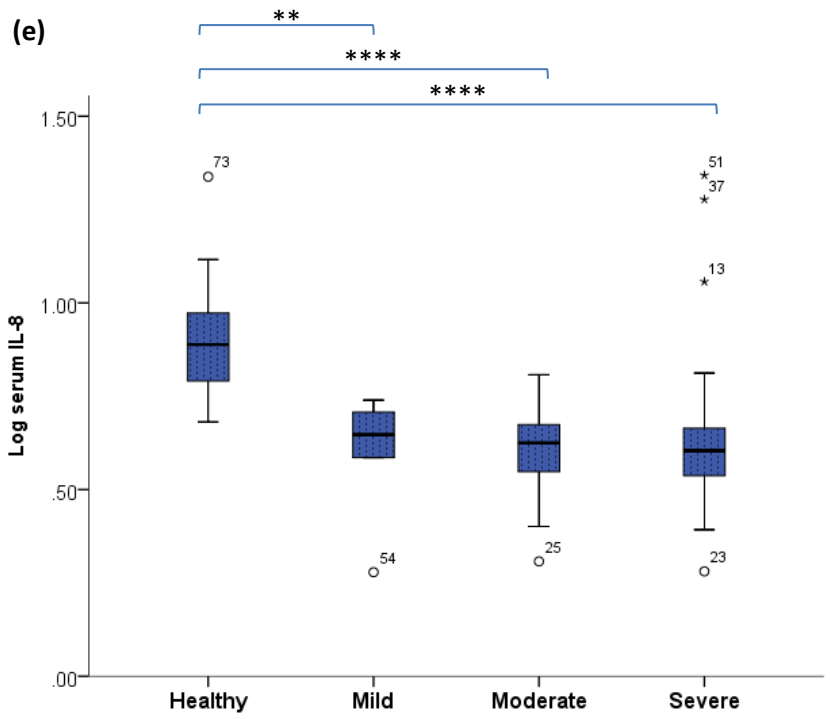


Figure 4.5 Levels of (a) CPA3, (c) IL-4, (d) IL-6 and (e) IL-8 in patients who underwent food challenges and had historical allergic reactions of varying severity compared to those in healthy control subjects.

The role of baseline levels of CPA3 in prediction of severity of allergic reactions is illustrated in (b) ROC curve analysis. Statistical analysis was determined by Kruskal-Wallis test followed by Mann-Whitney U test on log-transformed data (* p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001).

4.4.1.3 The association between serum levels of mast cell mediators and atopic diseases

When baseline levels of mast cell mediators in patients with atopic disease (atopic dermatitis, hay fever, or asthma) who underwent drug challenge were analysed, higher levels of CPA3 were seen in patients with one or more of the atopic conditions compared to the levels measured in healthy individuals ($H(2) = 26.7$, $p < 0.0001$) with a mean rank for CPA3 levels of 72 for patients with atopic conditions, 66 for patients with no associated atopic conditions, and 27 for healthy subjects (Figure 4.6a). The differences between the levels of IL-4 in patients who suffered one or more atopic diseases and those in healthy subjects contrasted with the variances seen with levels of CPA3 ($H(2) = 22.3$, $p < 0.0001$) with mean rank IL-4 level of 60 for patients with atopic diseases, 54 for patients who had not experienced atopic illnesses, and 95 for healthy control subjects (Figure 4.6b). Levels of IL-6 and IL-8 showed a similar trend to the levels of IL-4 but the differences between the studies groups did not reach a statistical significance ($H(2) = 1.4$, $p = 0.5$ for IL-6 and $H(2) = 6$, $p = 0.05$ for IL-8; Figure 4.6c and d, respectively).

When baseline levels of mediators measured in patients undergoing food challenge and having atopic dermatitis, hay fever, or asthma were compared with those of healthy individuals, it was found that there were higher levels of CPA3 in the patients with one or more atopic illnesses ($H(2) = 21.5$, $p < 0.0001$) with a mean rank for CPA3 levels of 41 for patients with atopic conditions, 36 for patients without concomitant atopic conditions, and 18 for healthy subjects (Figure 4.7a). As seen with the drug challenge group of patients, lower levels of IL-4, IL-6 and IL-8 were detected in the patients who had associated atopic diseases than in the control subjects, and this reached statistical significance for the three biomarkers ($H(2) = 11$, $p = 0.004$ for IL-4, $H(2) = 16$, $p < 0.0001$ for IL-6, and $H(2) = 36$, $p < 0.0001$ for IL-8) with mean ranks for IL-4, IL-6 and IL-8 levels of 29, 28, 24 for patients who suffered atopic diseases, 39, 39, 44 for patients without associated atopic illnesses, and 47, 50, 56 for healthy individuals; respectively (Figure 4.7 b, c and d).

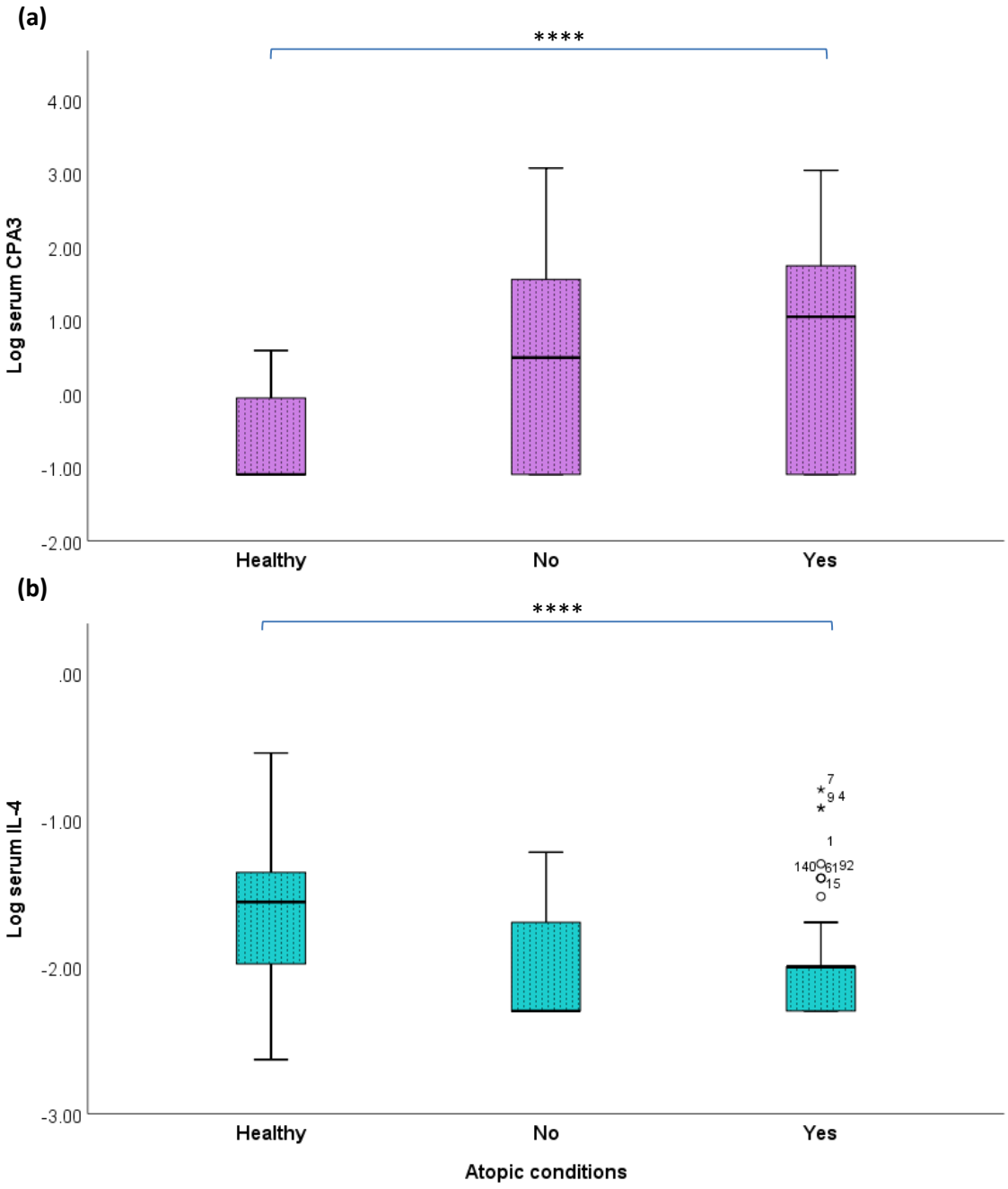
4.4.1.4 The effect of age and gender on levels of mediators

When the association between age of participants undergoing drug or food challenge and serum levels of mast cell mediators at baseline was analysed, levels of IL-6 and IL-8 showed a positive correlation with age (Figure 4.8). Levels of CPA3, IL-4 and the other biomarkers did not show any association with the age (Table 4.3).

Levels of IL-13 were significantly higher in females than in males when the analysis performed on a group of patients with comparable clinical characteristics ($p< 0.0001$; Figure 4.9a). The other biomarkers measured were not influenced by the gender of participants (Figure 4.9b-l).

4.4.1.5 The association of concomitant atopic diseases and severity of historical reactions

Analysis of the relation between concomitant atopic illnesses and severity of previous allergic reactions experienced among patients undergoing drug challenge indicated that subjects having one or more atopic illnesses had more severe reactions compared to those who had no atopic conditions (χ^2 (2) = 11.4, $p< 0.003$; Figure 4.9a and b). Likewise, most patients undergoing food challenge who had one or more atopic illnesses had moderate or severe historical allergic reactions in contrast to those without concomitant atopic diseases who had mild reactions (Fisher's Exact test = 12.4, $p< 0.001$; Figure 4.9 c and d).


(a)
****
4.00
3.00
2.00
1.00
.00
-1.00
-2.00
Log serum CPA3
Healthy
No
Yes
(b)
****
.00
-1.00
-2.00
-3.00
Log serum IL-4
7
9 4
1
140 61 92
15
Healthy
No
Yes
Atopic conditions

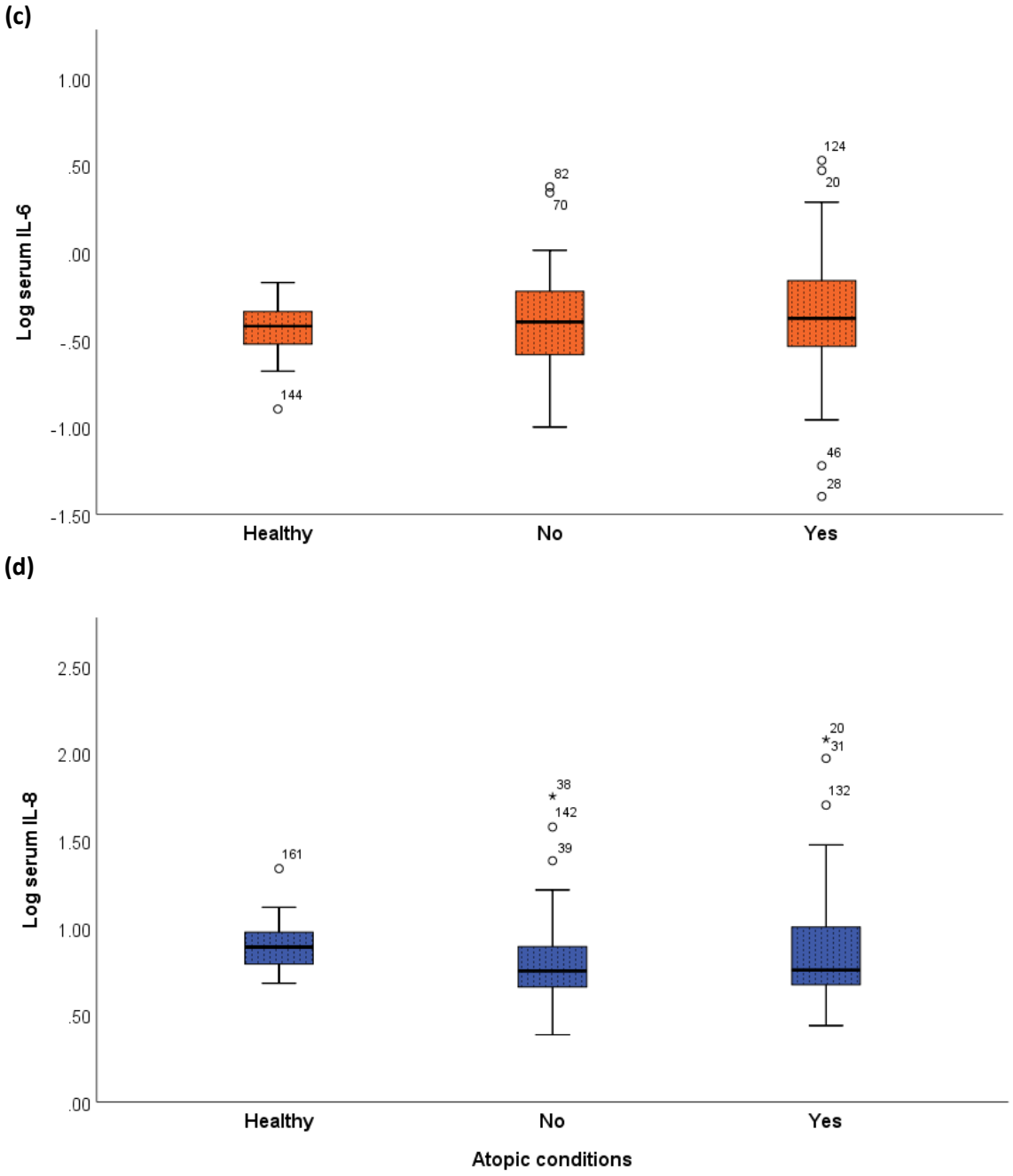
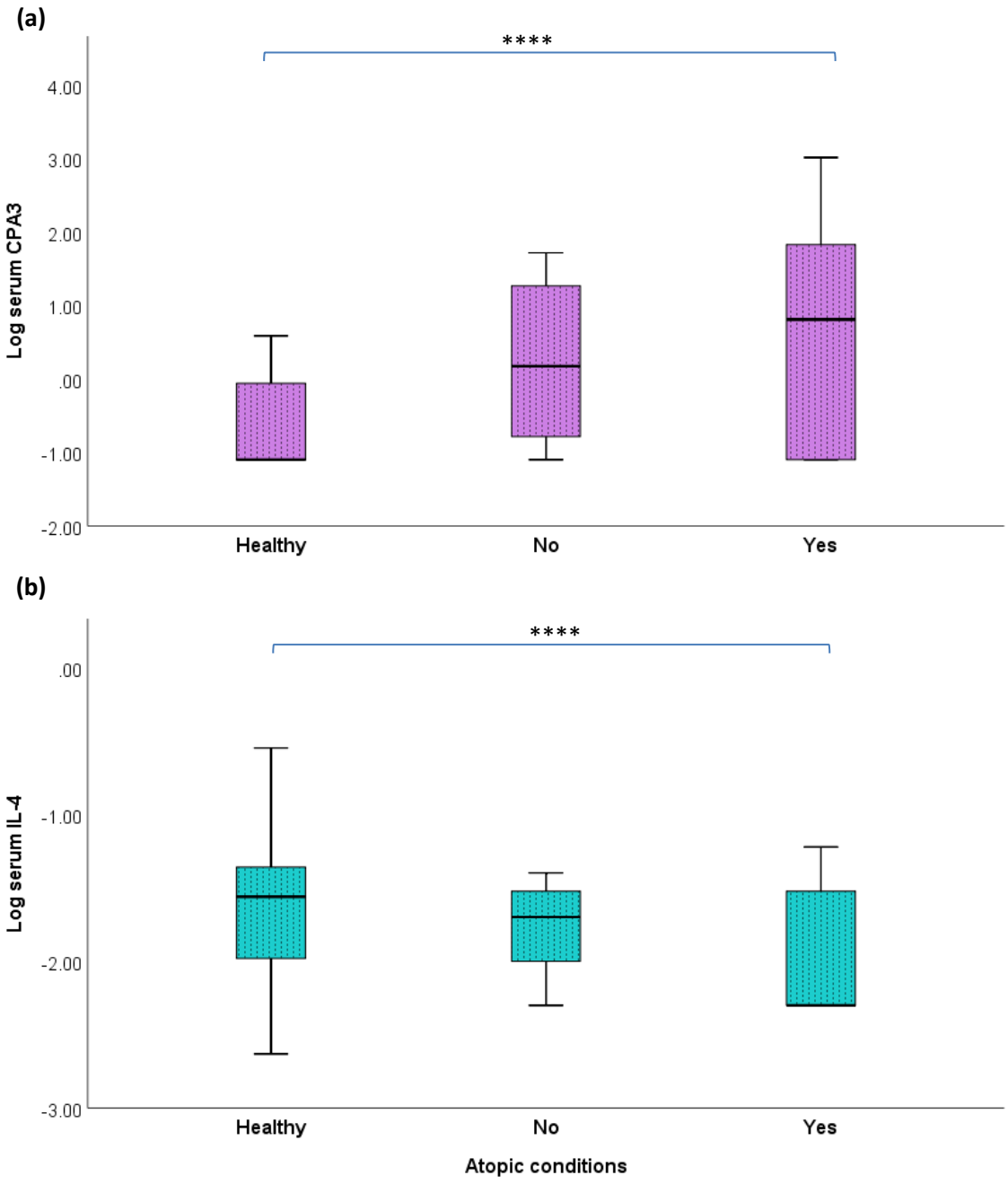


Figure 4.6 The association between levels of mediators and the presence of concomitant atopic conditions in patients undergoing drug allergy testing. Levels of (a) CPA3, (b) IL-4, (c) IL-6 and (d) IL-8 in patients with or without atopic illnesses were compared to those in healthy participants. Statistical analysis was determined by the Kruskal-Wallis test followed by Mann-Whitney U test on log-transformed data (****p<0.0001).


(a)
****
4.00
3.00
2.00
1.00
.00
-1.00
-2.00
Log serum CPA3
Healthy
No
Yes
(b)
****
.00
-1.00
-2.00
-3.00
Log serum IL-4
Healthy
No
Yes
Atopic conditions

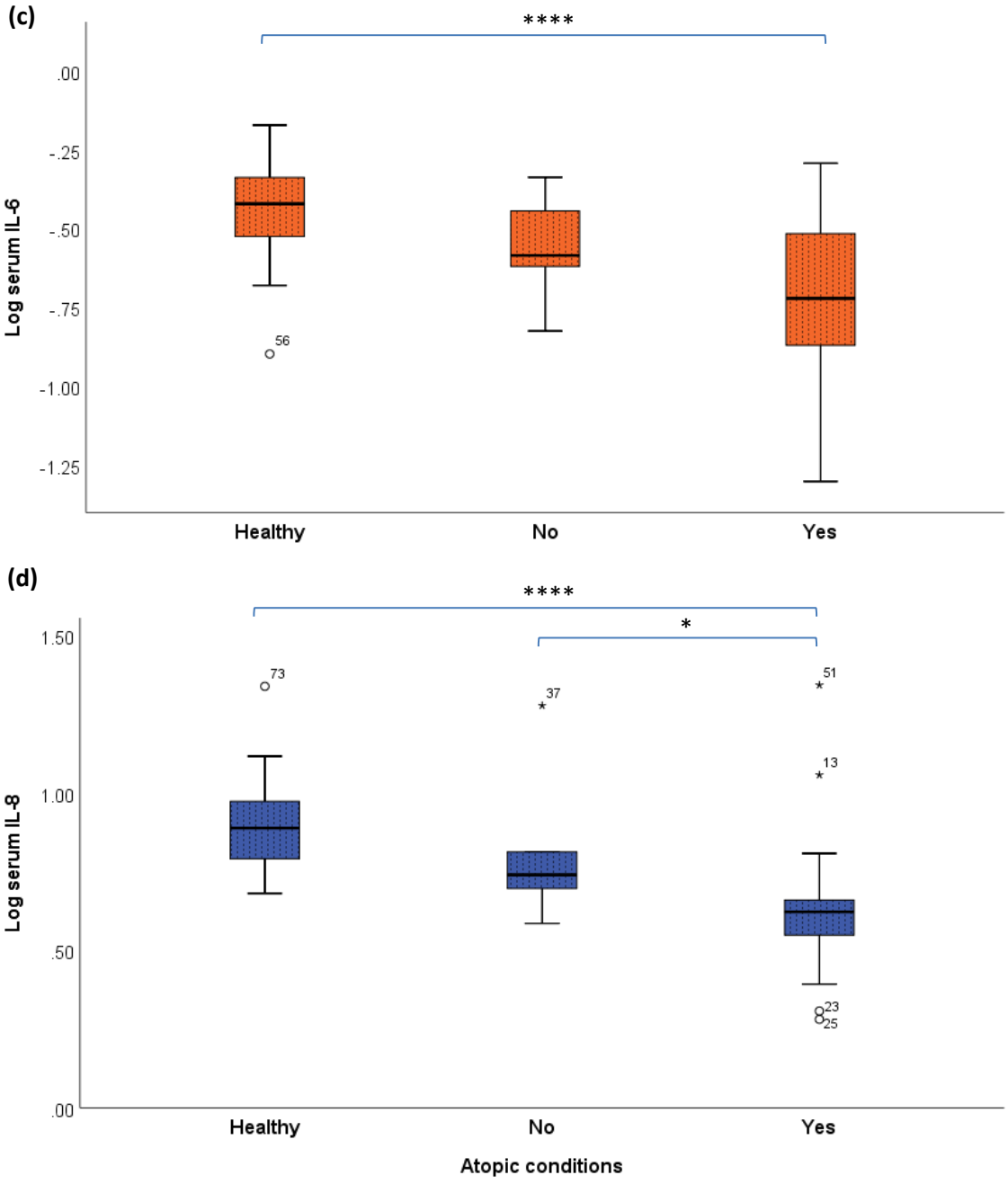


Figure 4.7 The association between levels of mediators and the presence of atopic conditions in patients undergoing food challenge.

Levels of (a) CPA3, (b) IL-4, (c) IL-6 and (d) IL-8 in patients with or without atopic illnesses were compared to those in healthy participants. Statistical analysis was determined by the Kruskal-Wallis test followed by Mann-Whitney U test on log-transformed data (* p<0.05, ****p<0.0001).

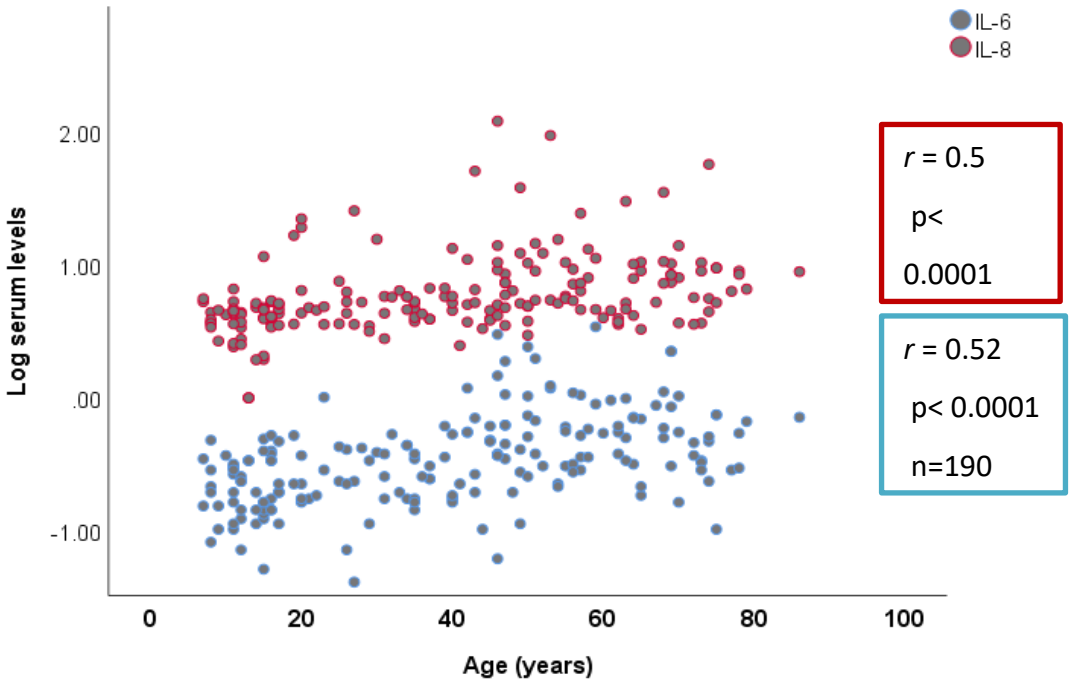
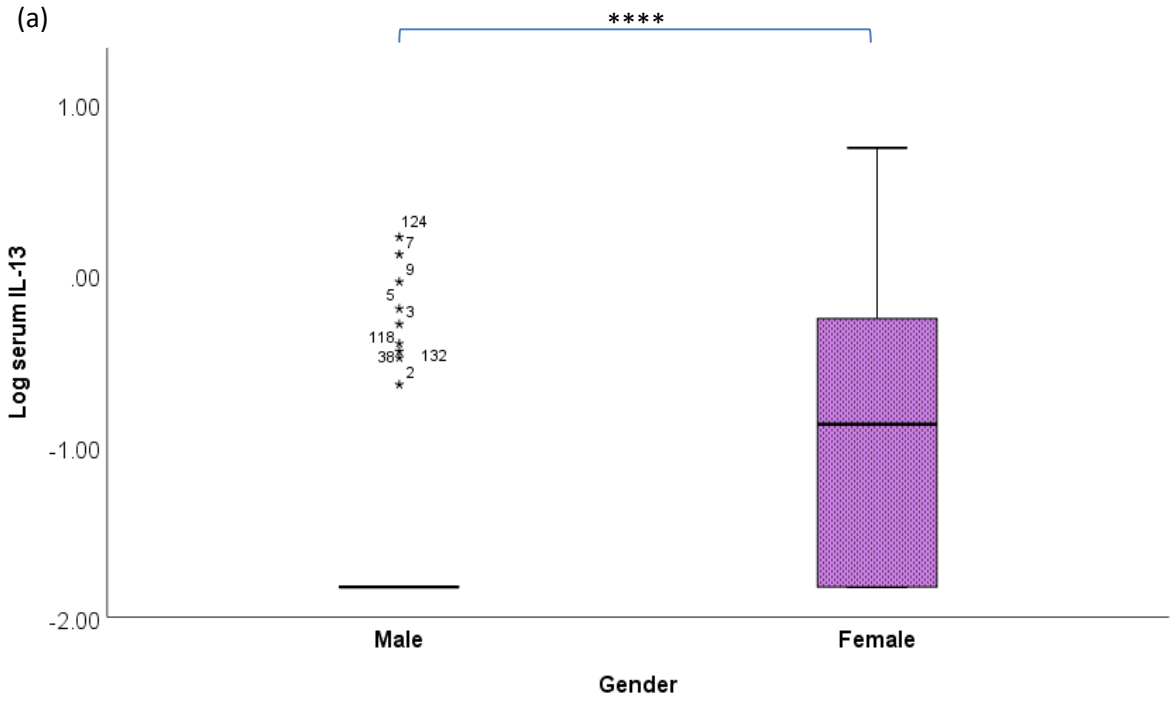


Figure 4.8 The association between serum levels of IL-6 and IL-8 with the age of participants.

Values for Spearman coefficient of rank correlation (*r*), *p*, and number (n) of patients are shown for analysis of log-transformed data.

Table 4.3 Spearman's correlation analysis of biomarkers and age of participants in the derivation cohort

Biomarker	*r*	*p*	n
Tryptase	0.1	0.15	190
CPA3	0.02	0.83	176
INF	0.13	0.06	190
IL-1β	-0.08	0.4	122
IL-2	0.11	0.22	122
IL-4	-0.09	0.18	190
IL-10	-0.12	0.12	190
IL-12p70	-0.06	0.44	190
IL-13	0.05	0.52	190
TNF	-0.05	0.52	190


(a)
****
1.00
.00
-1.00
-2.00
Log serum IL-13
124
7
9
5
3
118
38
132
2
Male
Female
Gender

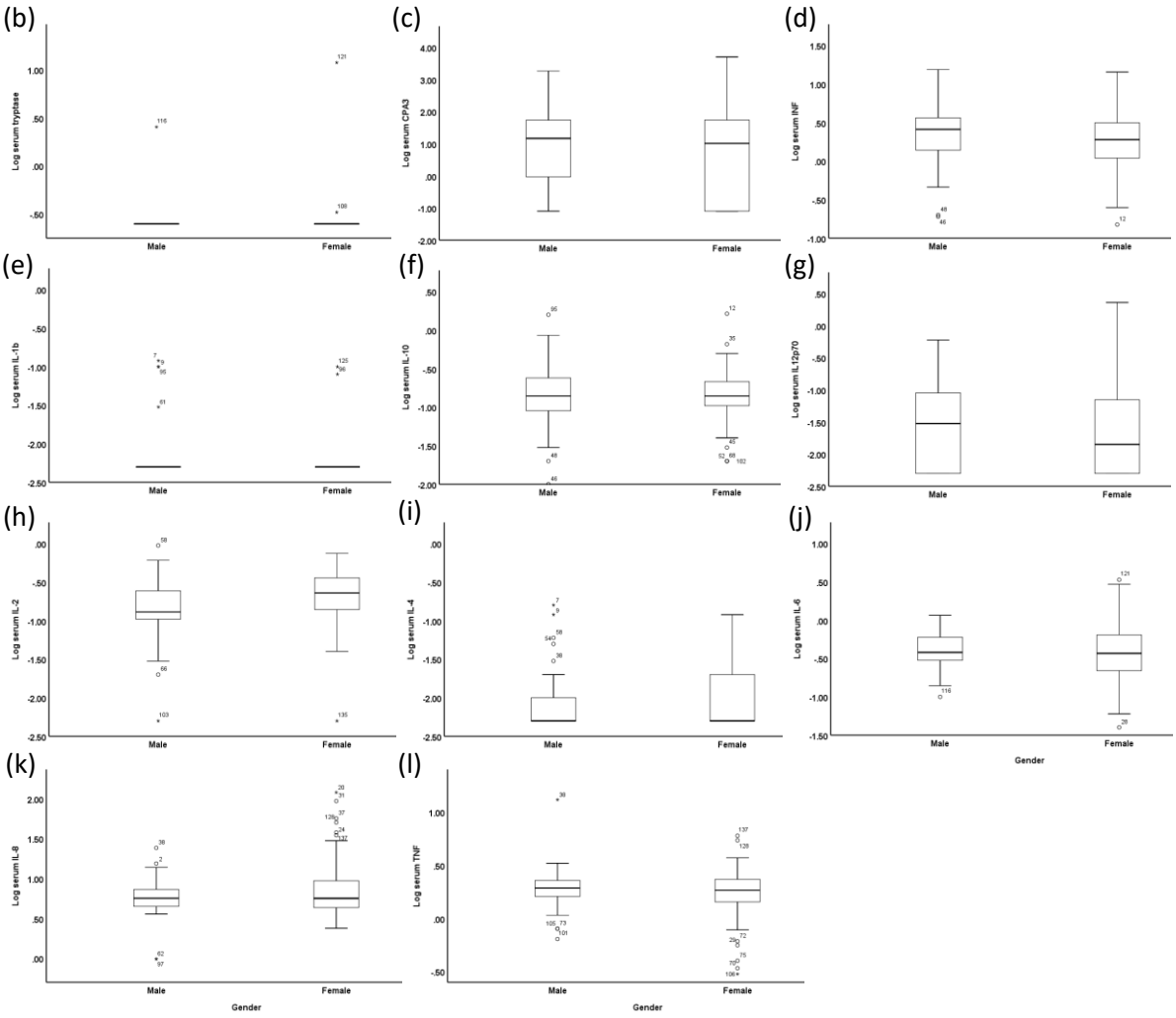


Figure 4.9 Gender-associated effects on the levels of mast cell proteases and pro-inflammatory cytokines in the derivation cohort.

Levels for (a) IL-13, (b) tryptase, (c) CPA3, (d) INF, (e) IL-1β, (f) IL-10, (g) IL-12p70, (h) IL-2, (i) IL-4, (j) IL-6, (k) IL-8, and (l) TNFα were analysed in patients aged 35-55 years who underwent drug challenges and had had mild historical allergic reactions (n=80). Statistical analysis was determined by the Mann-Whitney U test on log-transformed data (****p<0.0001).

Figure 4.10 The association between the presence of concomitant atopic conditions and the severity of historical reactions in patients undergoing (a, b) drug challenge or (c, d) food challenge.

Percentages of mild, moderate or severe reactions among (b, d) patients who have associated atopic illnesses and (a, c) those without associated illnesses are described. Statistical analysis was performed using Chi-squared test or Fisher's exact test on log-transformed data.

4.4.2 The validation cohort

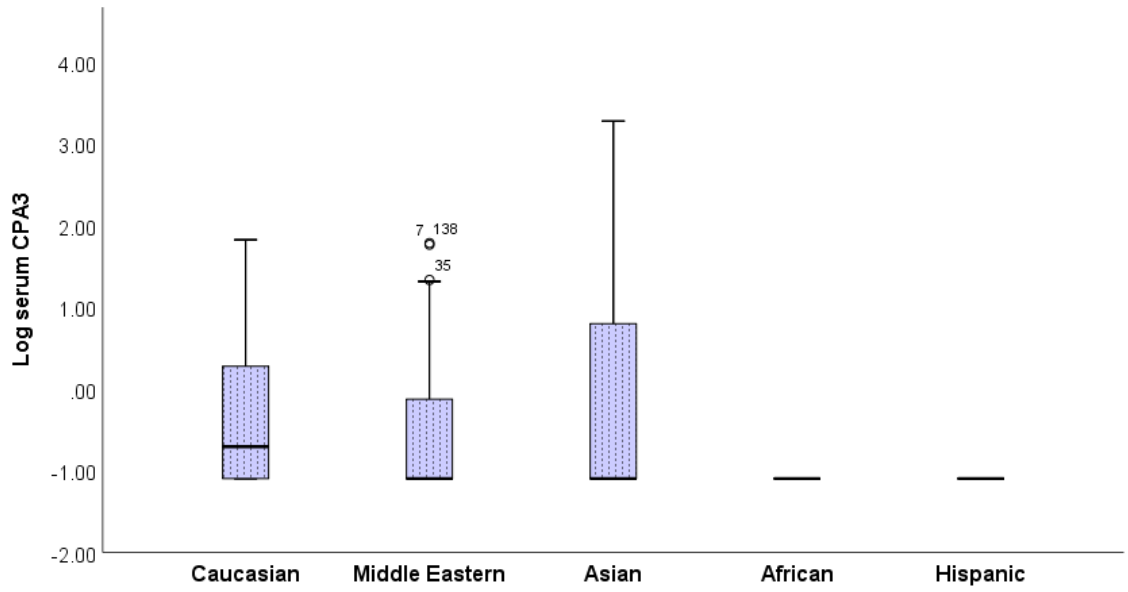
When levels of CPA3 and pro-inflammatory cytokines were compared between different ethnic groups, no significant differences were observed (illustrated for levels of CPA3, IL-4, IL-6 and IL-8 in Figure 4.11). Analysis of the variation in the levels of biomarkers among an age-, gender- and clinically-matched group of patients in relation to ethnicity indicated lack of effect of ethnicity on serum levels of measured mediators (See Appendix D). Levels of tryptase, IL-1β and IL-2 could not be measured in serum samples from the validation cohort.

No significant differences were observed between serum levels of mast cell proteases or pro-inflammatory cytokines in patients with allergic conditions and those in healthy non-atopic participants. This is illustrated in Figure 4.12 for levels for CPA3, IL-4, IL-6 and IL-8 and data is representative of that for the other biomarkers measured.

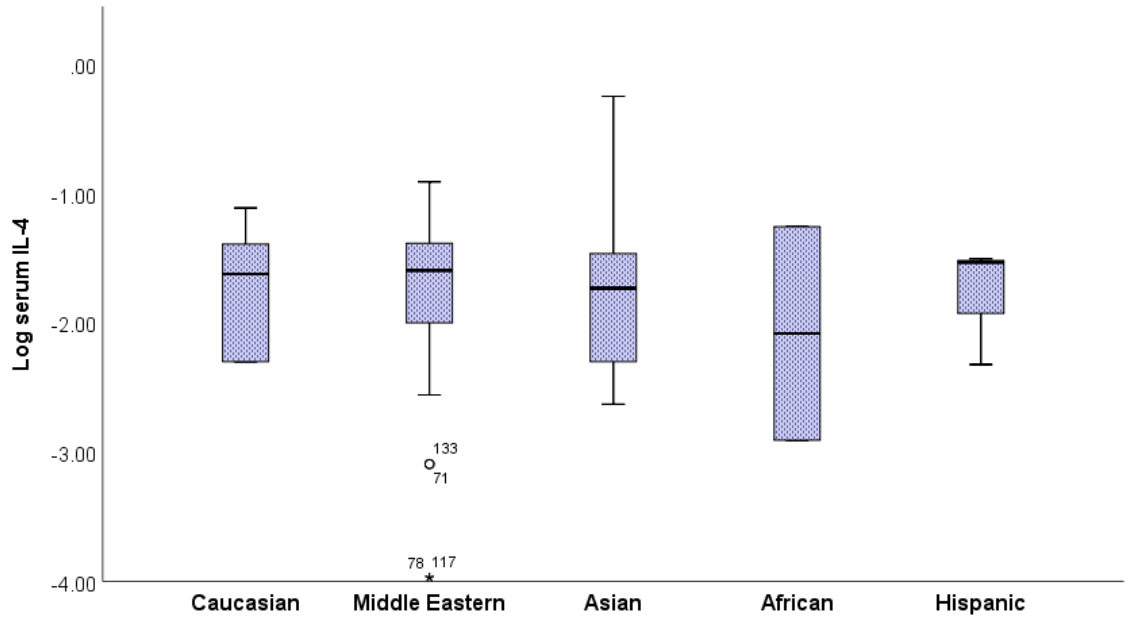
When the severity of symptoms experienced by patients with anaphylaxis, drug, food or insect bite allergic reactions were graded according to the scheme of Brown *et al* (24), serum levels of CPA3, IL-4, IL-6, and IL-8 in patients with mild, moderate or severe allergic symptoms did not differ significantly from the levels in control subjects. However, a similar trend for levels of CPA3 and IL-4 in relation to severity of reactions to that seen in the derivation cohort was observed (Figure 4.13). Comparisons involving the other mediators was not associated with a significant difference.

As performed for the derivation cohort, the association between serum levels of CPA3 and pro-inflammatory cytokines with age was analysed. Serum levels of IL-6 showed a significant correlation with the age of the participants, unlike serum levels of IL-8, in which a lack of significant correlation was observed (Figure 4.14). As seen with the derivation cohort, females showed higher levels of IL-13 compared to males in an age- and clinically matched group of patients, though the differences did not reach a statistical significance (Figure 4.15a). The gender of participants did not influence the levels of other measured biomarkers (Figure 4.15b-i).

(a)


Log serum CPA3
4.00
3.00
2.00
1.00
.00
-1.00
-2.00
7
138
35
Caucasian
Middle Eastern
Asian
African
Hispanic

(b)


Log serum IL-4
.00
-1.00
-2.00
-3.00
-4.00
133
71
78
117
Caucasian
Middle Eastern
Asian
African
Hispanic

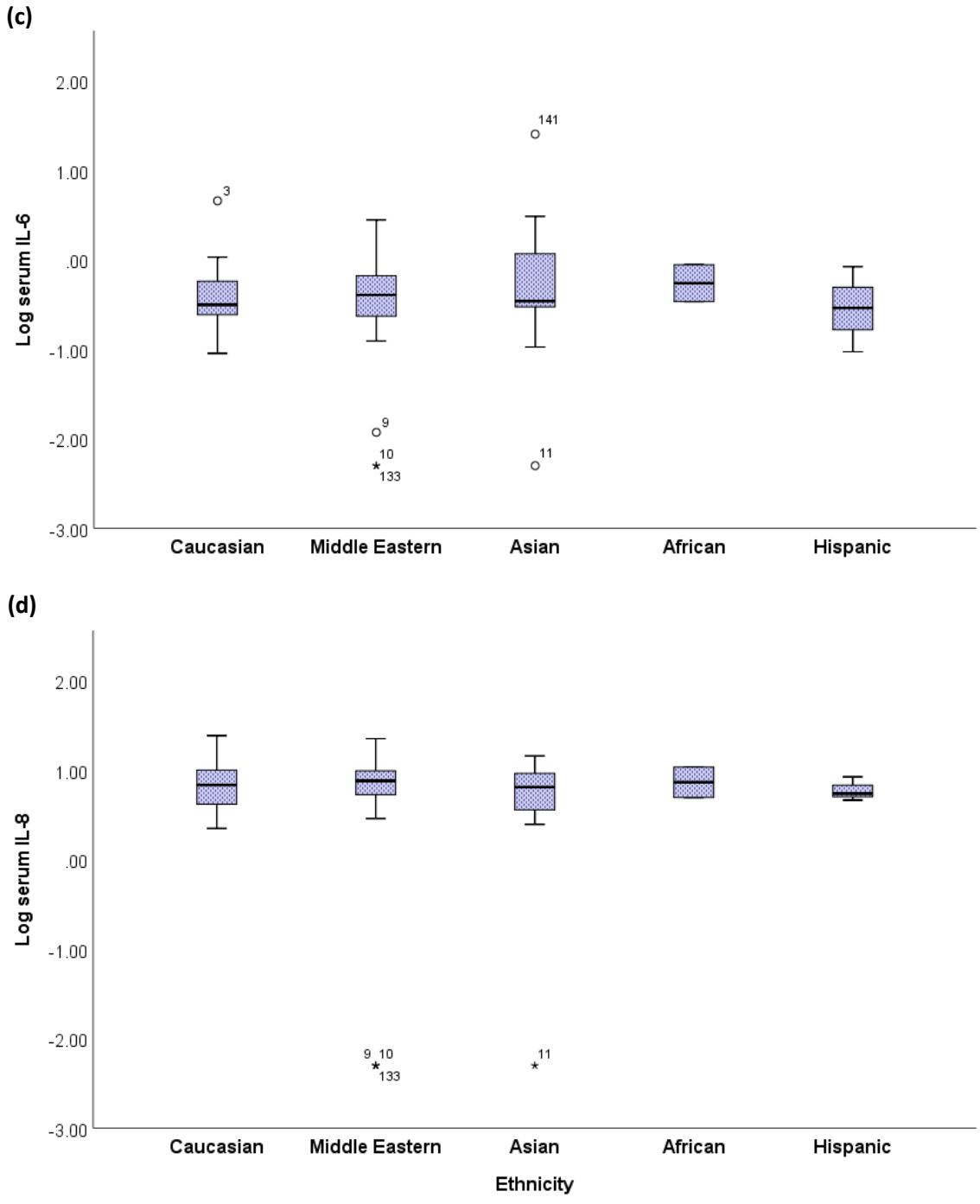
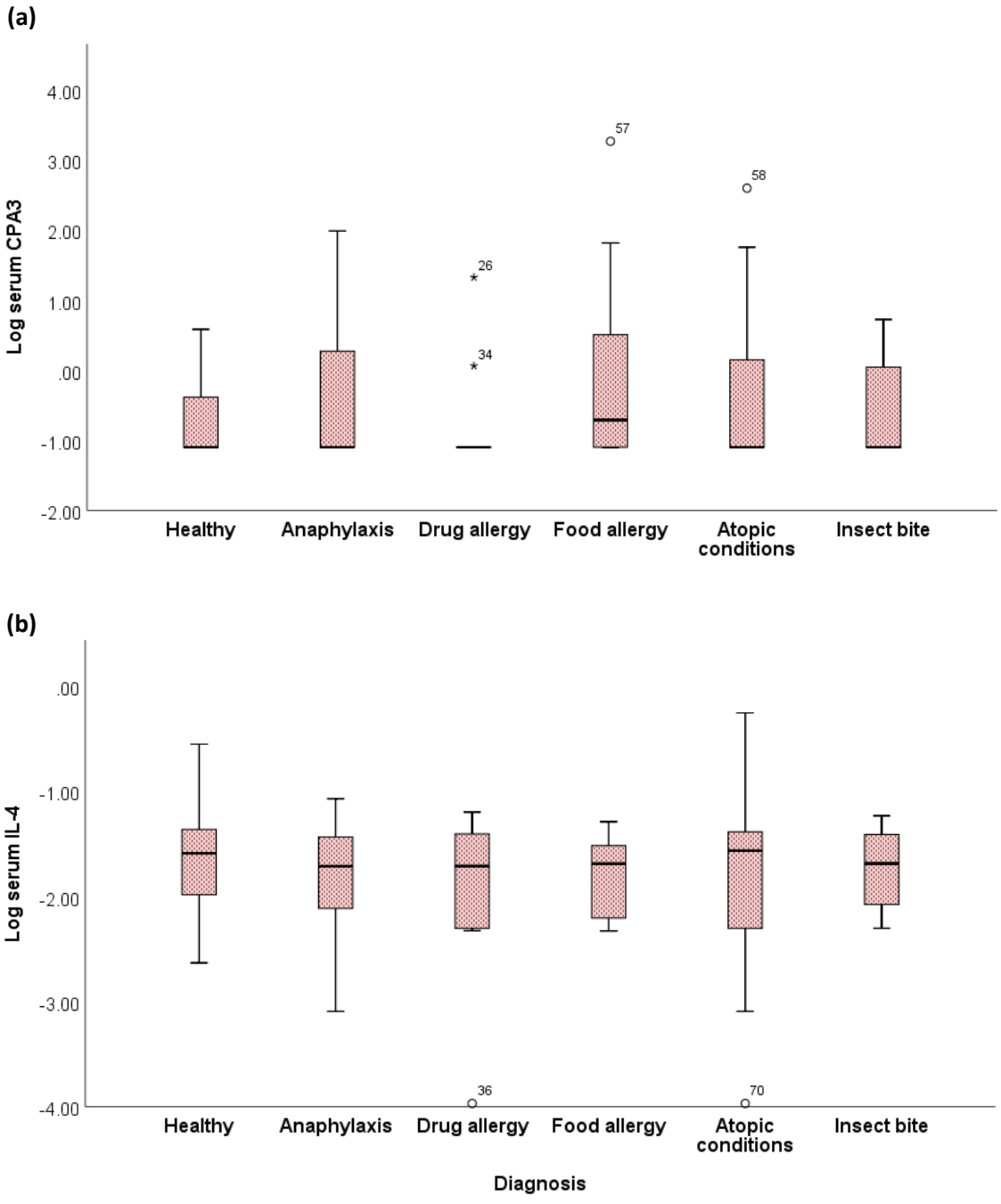


Figure 4.11 Lack of effect of ethnicity on levels of (a) CPA3, (b) IL-4, (c) IL-6 and (d) IL-8.

Levels were compared in patients with different ethnic backgrounds. Statistical analysis was determined by the Kruskal-Wallis test on log-transformed data.


(a)
Log serum CPA3
4.00
3.00
2.00
1.00
.00
-1.00
-2.00
57
58
26
34
Healthy
Anaphylaxis
Drug allergy
Food allergy
Atopic conditions
Insect bite
(b)
Log serum IL-4
.00
-1.00
-2.00
-3.00
-4.00
36
70
Healthy
Anaphylaxis
Drug allergy
Food allergy
Atopic conditions
Insect bite
Diagnosis

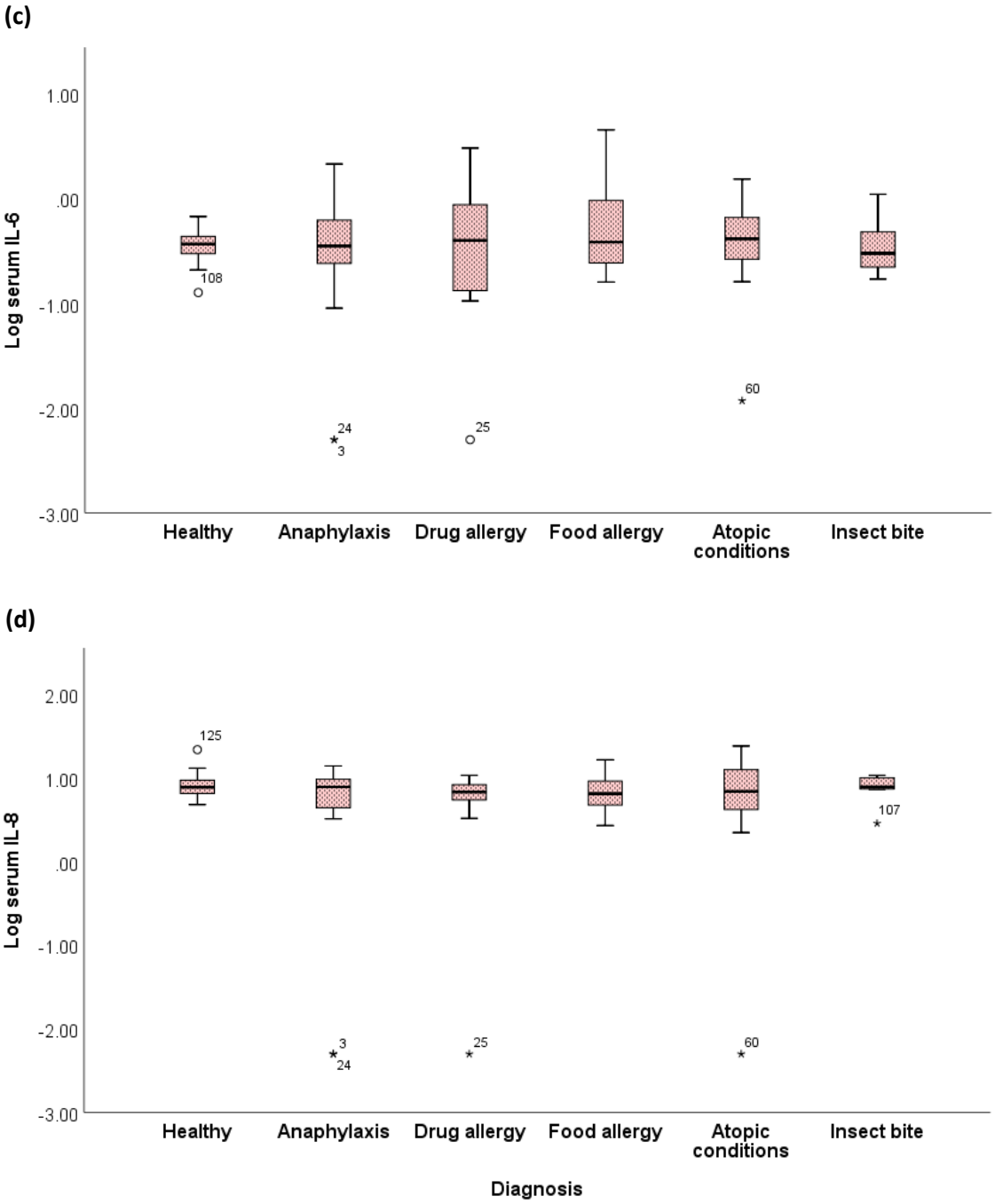
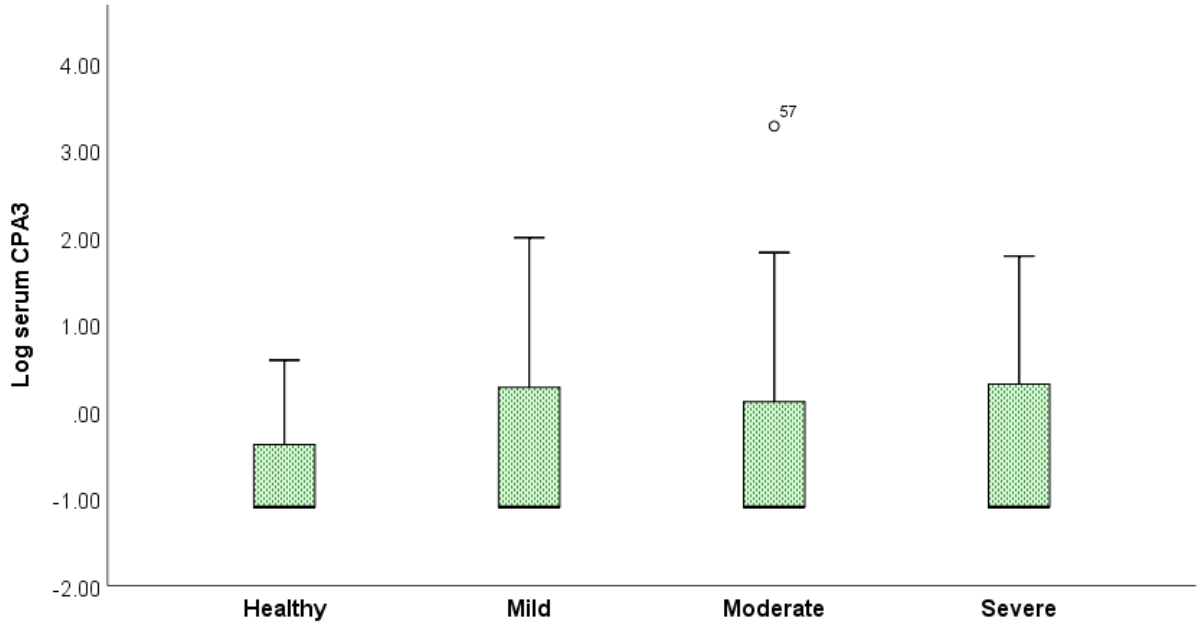


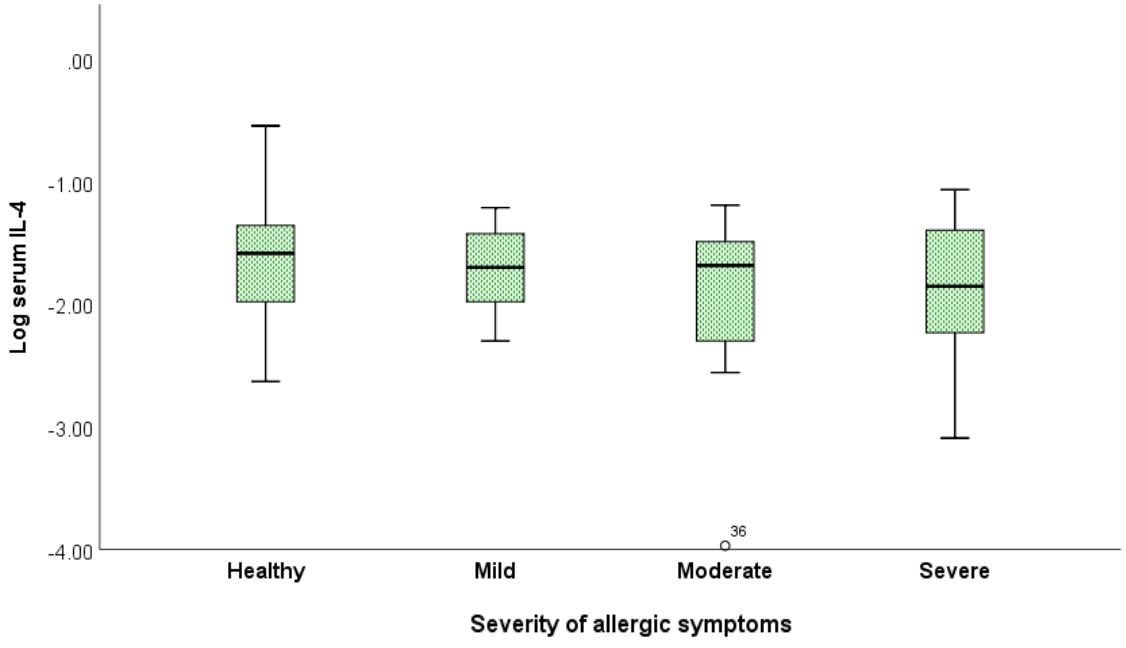
Figure 4.12 Serum levels of (a) CPA3, (b) IL-4, (c) IL-6 and (d) IL-8 in a range of allergic conditions.

The levels in patients with different allergic conditions were compared to those in healthy individuals. Statistical analysis was determined by the Kruskal-Wallis test on log-transformed data.

(a)


Log serum CPA3
4.00
3.00
2.00
1.00
.00
-1.00
-2.00
57
Healthy
Mild
Moderate
Severe

(b)


Log serum IL-4
.00
-1.00
-2.00
-3.00
-4.00
36
Healthy
Mild
Moderate
Severe
Severity of allergic symptoms

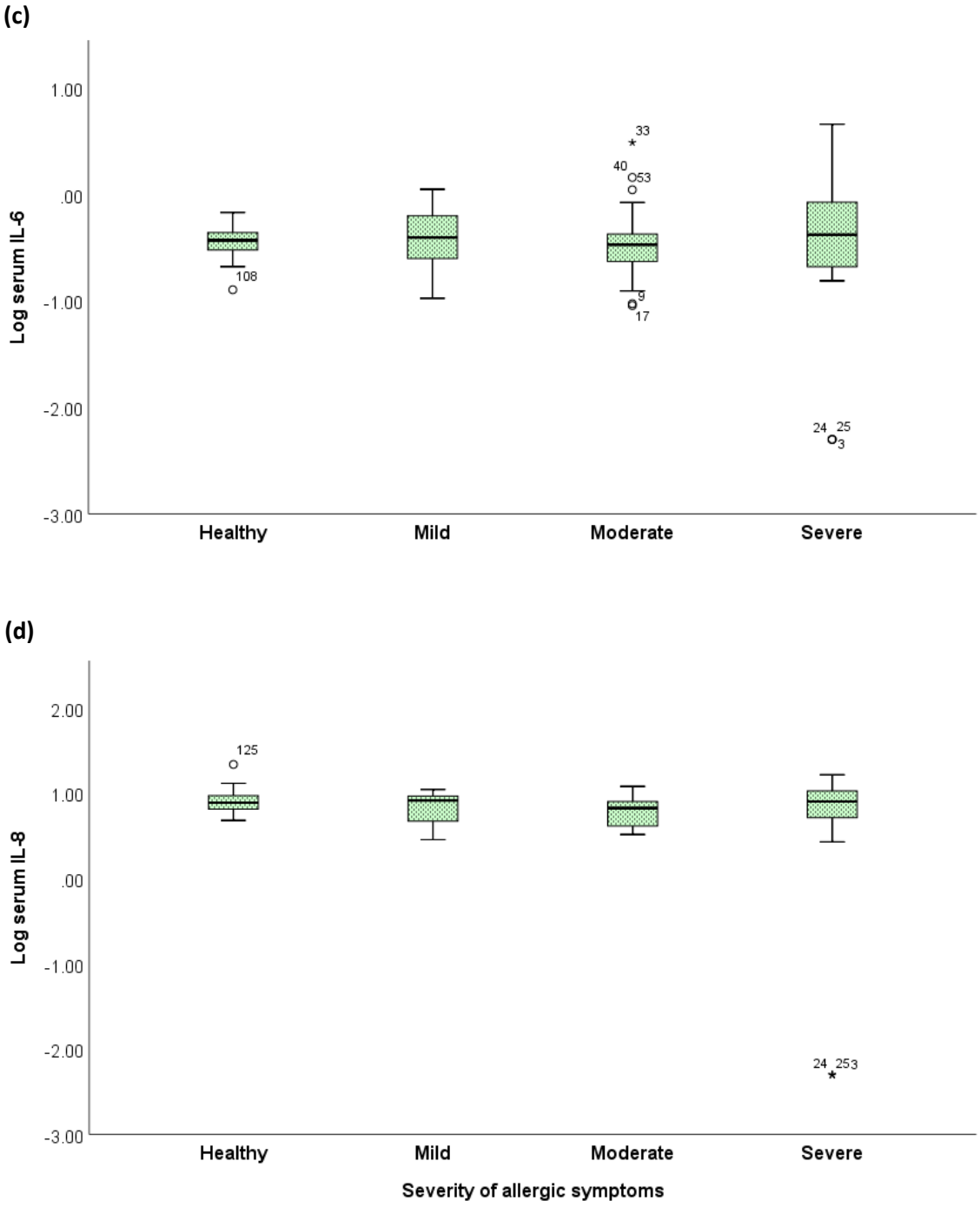


Figure 4.13 Serum levels of (a) CPA3, (b) IL-4, (c) IL-6 and (d) IL-8 in patients with a range of severity of allergic symptoms and in heathy subjects.

Statistical analysis was determined by the Kruskal-Wallis test on log-transformed data.

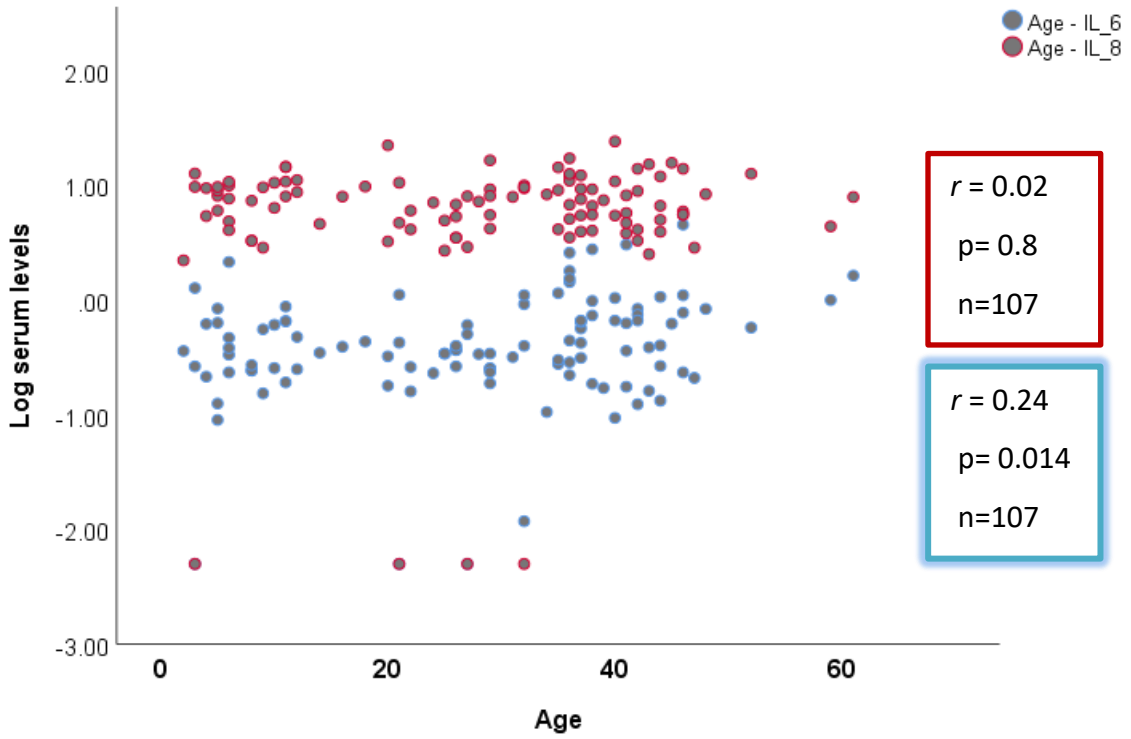
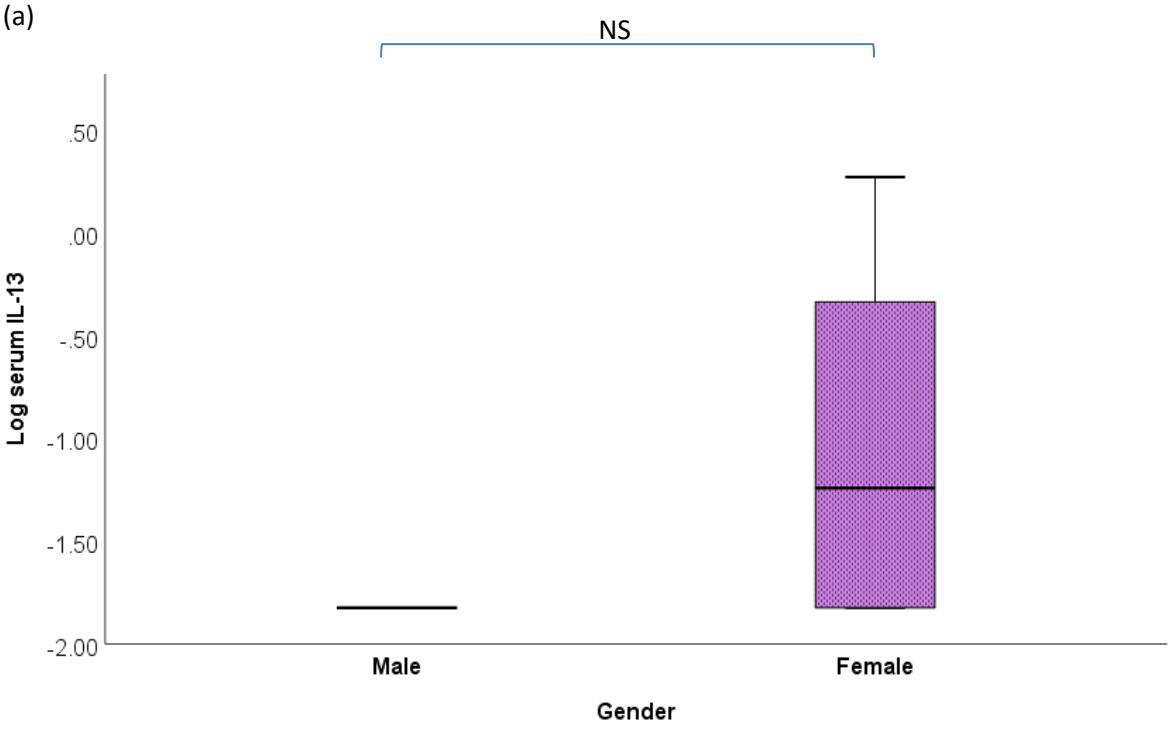


Figure 4.14 The association between serum levels of IL-6 and IL-8 with age of participants within the validation cohort.

Values for Spearman coefficient of rank correlation (*r*), *p*, and number of patients (n) are shown for analysis of log-transformed data.

(a)



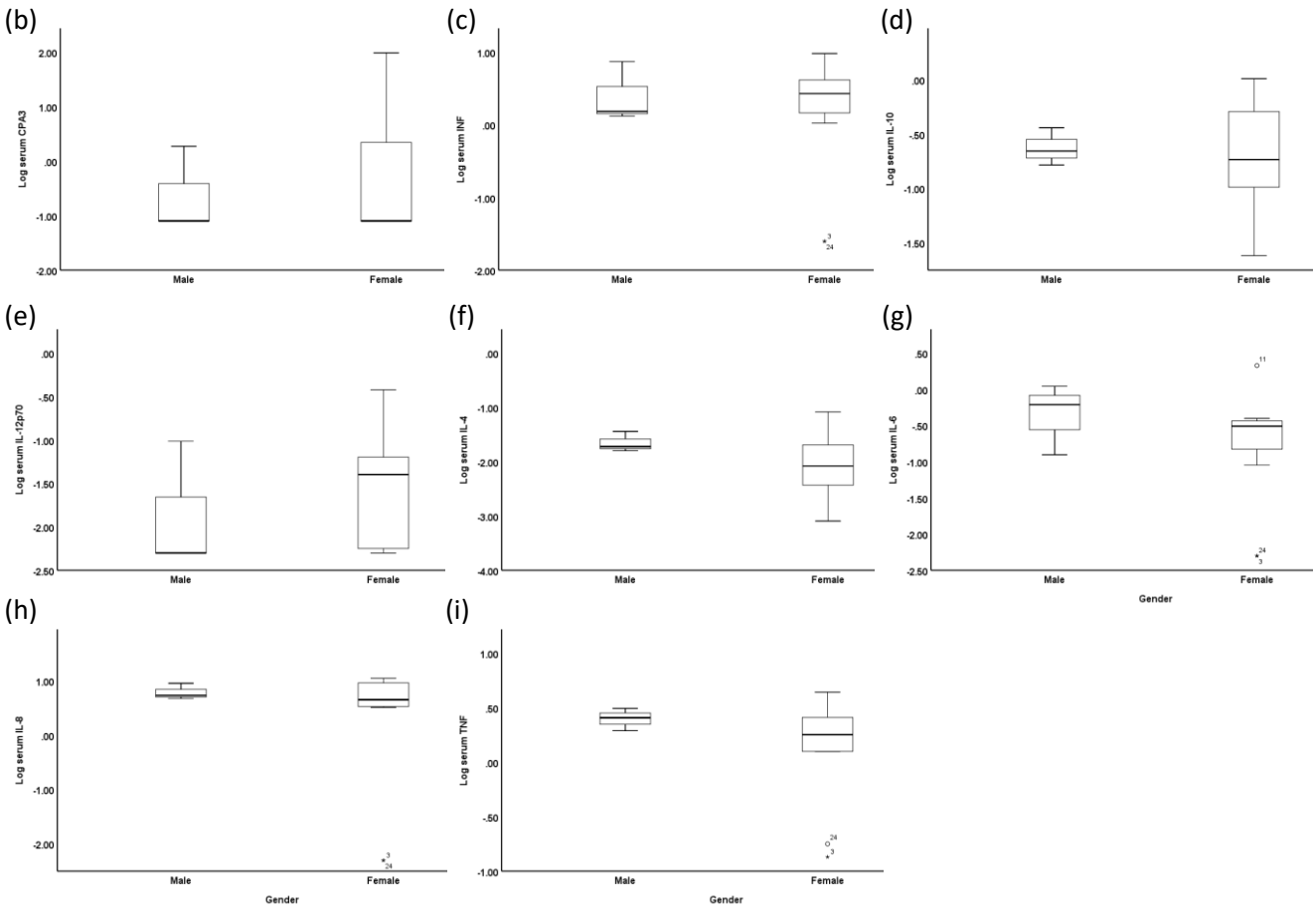


Figure 4.15 Gender-associated effects on the levels of mast cell proteases and pro-inflammatory cytokines in the validation cohort.

Levels for (a) IL-13, (b) CPA3, (c) INF, (d) IL-10, (e) IL-12p70, (f) IL-4, (g) IL-6, (h) IL-8, and (i) TNFα were analysed in patients (aged 35-55 years) who were diagnosed with anaphylaxis (n=15). Statistical analysis was determined by the Mann-Whitney U test on log-transformed data.

4.4.3 Topological data analysis to define clusters

When TDA was applied to the derivation cohort data sets, four multidimensional clinico-immunological clusters generated using 18 parameters (6 clinical features and 12 biomarkers) were identified. The demographics and clinical features of the four clusters are described in Table 4.4. These clusters were seen using baseline or post-challenge serum levels of measured biomarkers (Figure 4.16 a, b). The main features for each cluster are shown in Figure 4.16 c. The morphology of the clusters was mainly derived by the levels of CPA3 (Figure 4.17 a) and IL-13 (Figure 4.17 b), whereas other biomarkers were distributed throughout the clusters without a discrete pattern being apparent (illustrated for a representative biomarker in Figure 4.17 c). Excluding the levels of CPA3 and IL-13 from the analysis resulted in loss of the multidimensional structure of the data set. When subsequent TDA was applied on a geographically distinct validation cohort using 15, 5 clinical and 10 immunological, parameters, the four clusters were replicated (Table 4.5 and Figure 4.18).

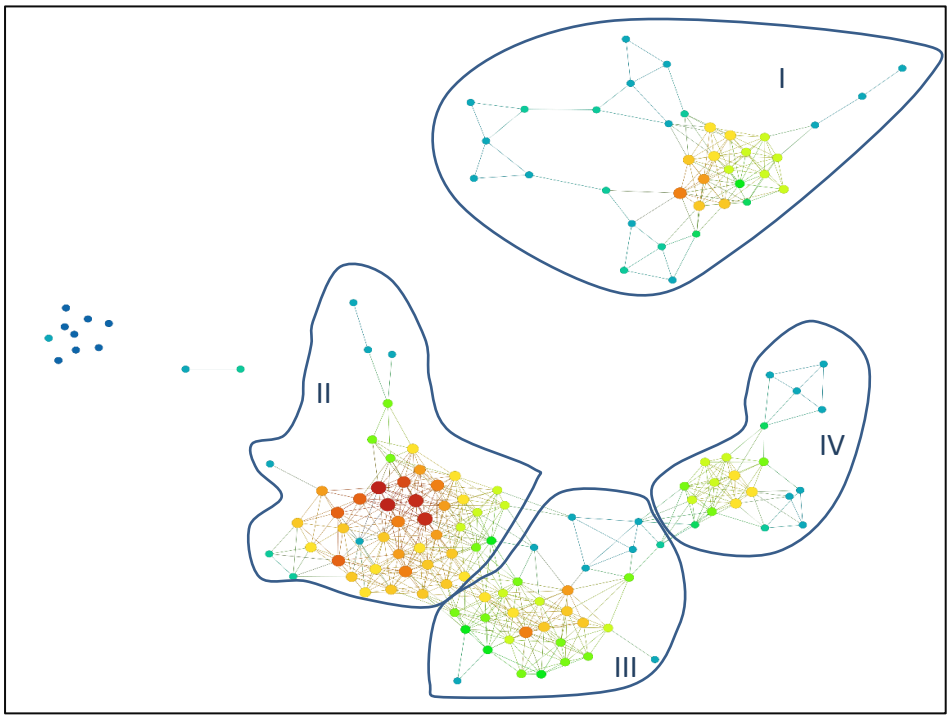
Of the four clusters, cluster I of the derivation cohort comprised participants with higher serum levels of CPA3 ($p< 0.0001$) and IL-13 ($p< 0.0001$), a majority of these were females. They were predominantly those undergoing drug challenges and they had had a more severe historical reaction compared to subjects in the other clusters. When characteristics of cluster II were analysed in relation to those of the other three groups, they were found to have higher serum levels of CPA3 ($p< 0.0001$), lower serum levels of IL-13 ($p< 0.0001$), a high proportion of males ($p= 0.0002$), there were the highest number of patients who tested for food allergic reactions and this cluster had the highest number of those who had had a severe historical reaction compared to the rest. Comparing the clinical and biochemical features for cluster III against the others showed lower levels of CPA3 ($p< 0.0001$) and IL-13 ($p< 0.0001$), a slightly younger age, a majority of females, a higher percentage of of patients undergoing food challenges than the other clusters and a lower percentage who had had a severe historical allergic reaction compared to clusters I and II. Cluster IV included subjects with lower serum levels of CPA3 ($p< 0.0001$), higher IL-13 ($p< 0.0001$), a majority of females ($p= 0.01$), a higher percentage of drug challenges and the least severity of historical allergic reactions ($p= 0.0008$) compared to the features of the other clusters. Most of the healthy control individuals were grouped in cluster IV ($p= 0.005$). Comparing the age and associated atopic illnesses across the four clusters showed little variation apart from Cluster III, which had slightly younger age median compared to the other groups.

For the clusters of the validation cohort, the biochemical characteristics seen in the derivation cohort clusters were replicated. The clinical data collected for the validation cohort were different from that for the derivation cohort and hence a complete replication could not be seen. However, clusters I and II showed a predominance for severe cases like anaphylaxis compared to the other clusters. Clusters II and III had higher percentages of cases of food allergy than the other two clusters. Comparing the percentage of patients with drug allergy across the clusters showed majority of cases were within cluster IV. In addition, cluster IV included most of healthy participants compared to the number found in the other three clusters. When the age of participants was compared across the clusters, cluster II had younger age group ($p < 0.01$) than the other clusters. Comparing the distribution of gender throughout the clusters showed an overall predominance of females over males. When ethnicity was analysed in relation to each cluster, no discrete distribution was found with middle eastern ethnic background being the predominant race across the clusters.

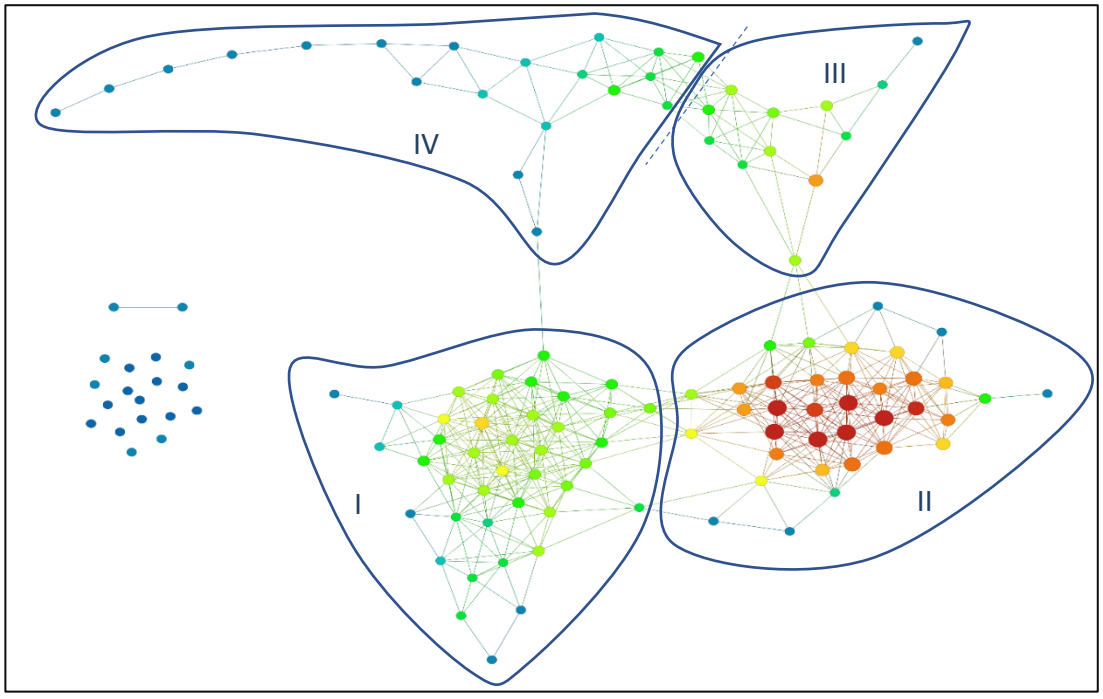
Table 4.4 Demographics and clinical characteristics of the clusters in the derivation cohort

Parameter	Clusters			
	I	II	III	IV
Number of participants	51	79	38	28
Number of healthy controls	2	5	6	9
Age (years) Median (min-max)	42 (8-78)	43 (7-86)	35 (8-78)	39 (11-67)
Gender (male/female) No. (%)	14 (26)/39 (74)	45 (54)/39 (46)	15 (35)/28 (65)	8 (22)/29 (78)
Allergen tested (drug/food) No. (%)	44 (83)/ 9 (17)	54 (68)/25 (32)	25 (66)/13 (34)	23 (82)/5 (18)
Challenge outcome (positive/negative) No. (%)	15 (30)/36 (70)	26 (33)/53 (67)	18 (49)/19 (51)	8 (30)/19 (70)
Severity of historical reactions Mild no. (%)	23 (46)	32 (42)	18 (50)	19 (68)
Moderate no. (%)	9 (18)	16 (21)	7 (19)	3 (11)
Severe no. (%)	18 (36)	28 (37)	11 (31)	6 (21)
Atopic illnesses (yes/no) no. (%)	28 (72)/11 (28)	46 (71)/19 (29)	18 (55)/15 (45)	16 (73)/6 (27)

(a)



(b)



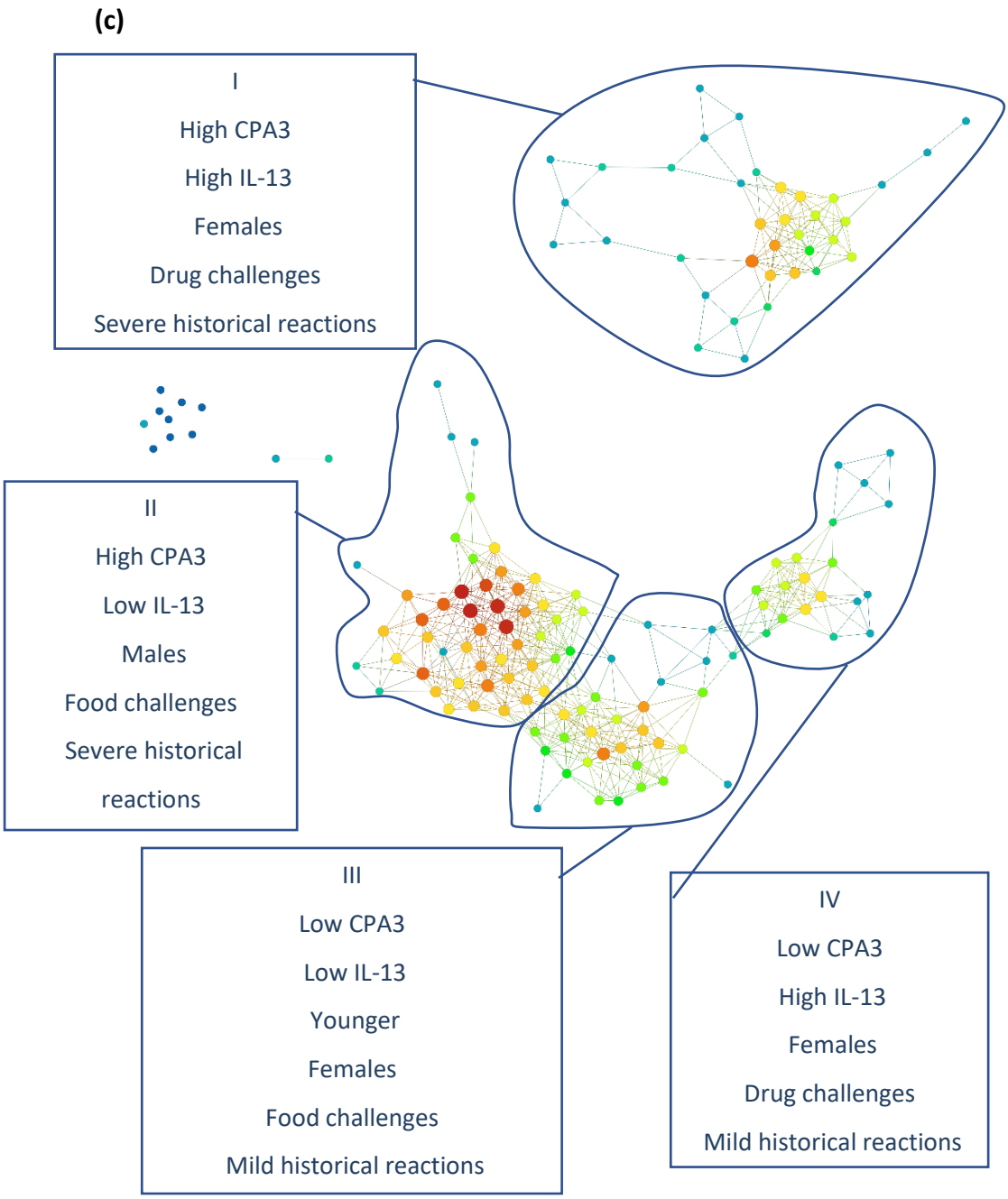
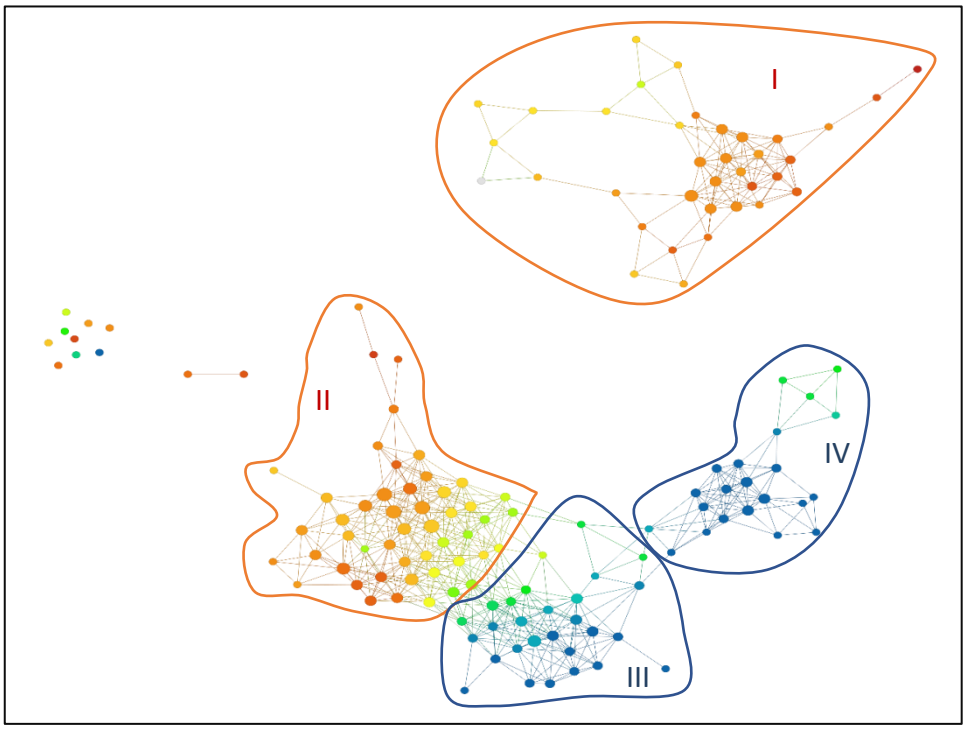


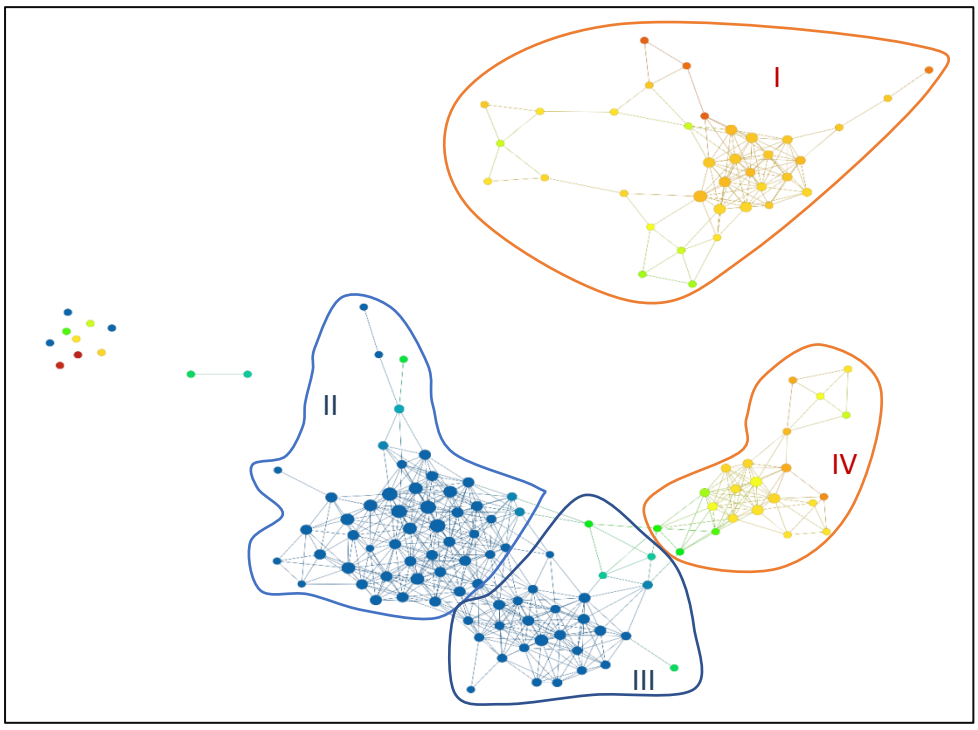
Figure 4.16 Multidimensional clinico-immunological clusters of patients with drug or food allergic reactions in the derivation data set (Southampton).

Two separate networks were built using (a) baseline and (b) post-challenge serum levels of measured biomarkers. Within each network, four clusters (I-IV) were identified. (c) The clinical and biochemical characteristics for each cluster are shown. The nodes lying outside the outlined clusters represent outliers. The nodes represent the average number of patients with colour scale ranging from blue (low values) to red (high values).

(b)



(c)



(d)

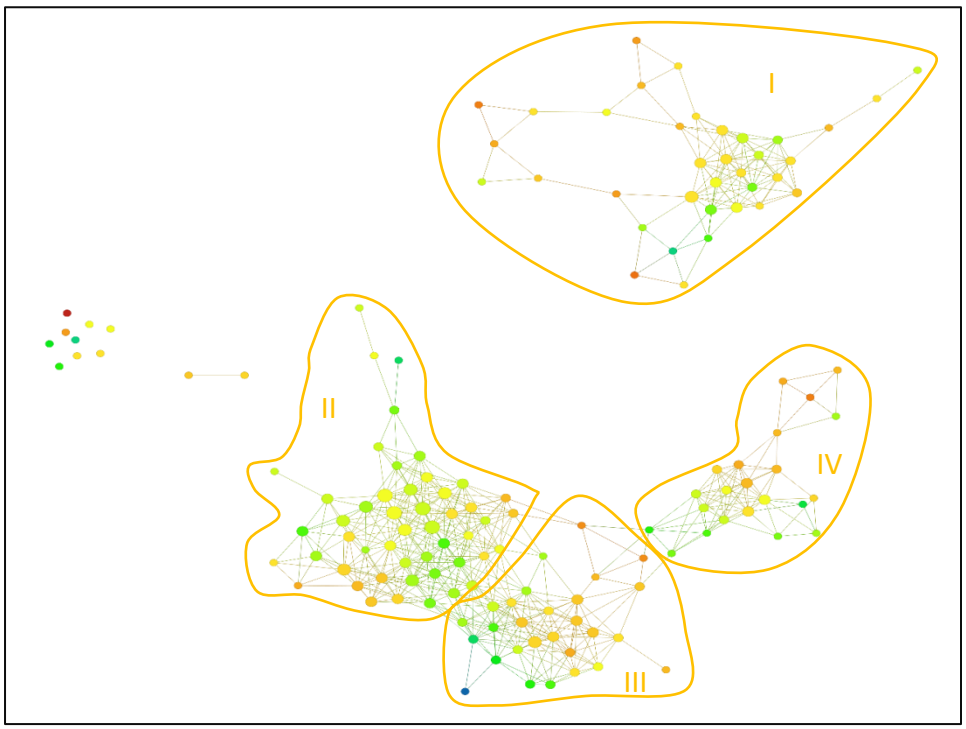


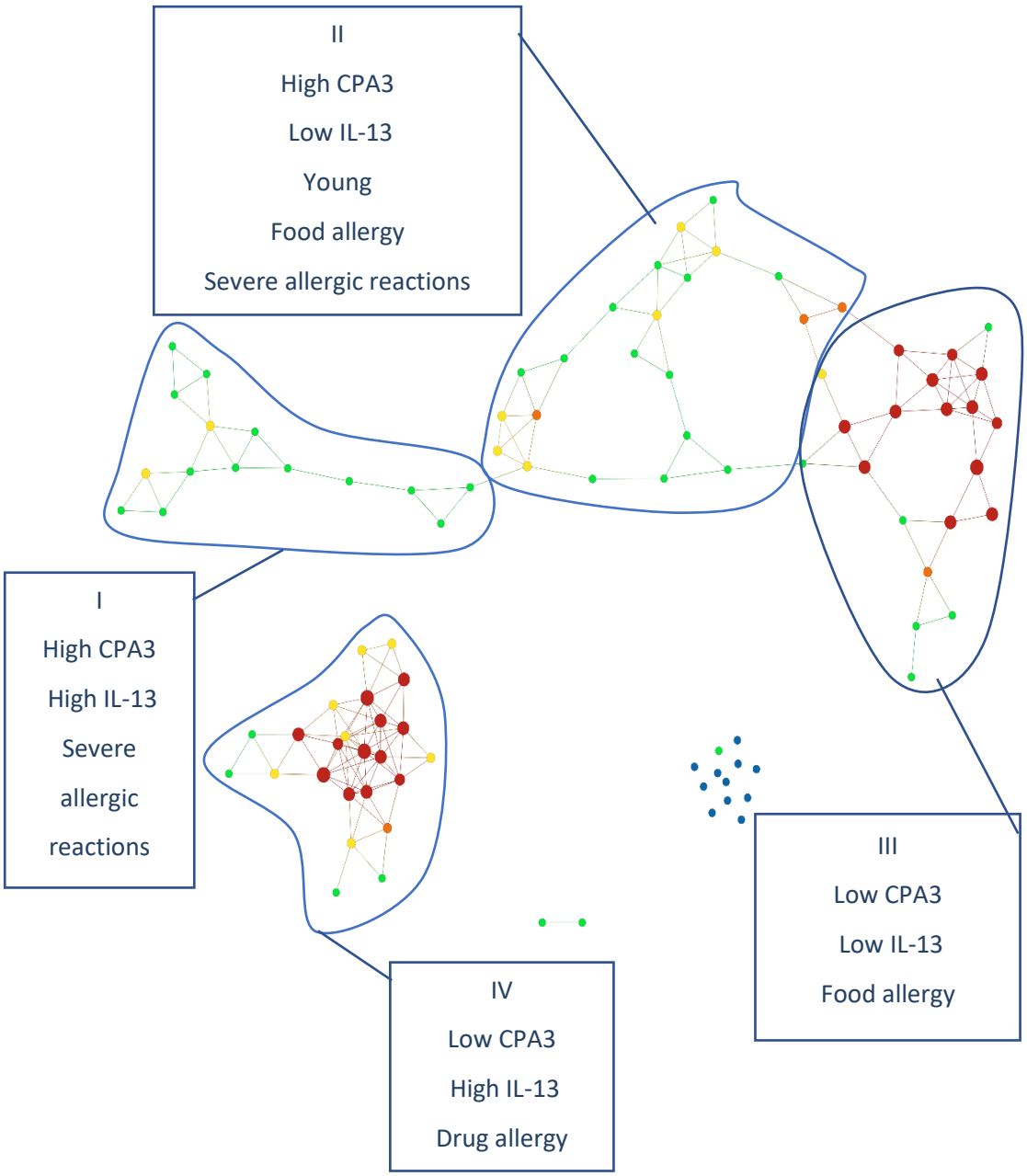
Figure 4.17 Multidimensional clusters of patients with drug or food allergic reactions in the derivation cohort (Southampton) coloured by serum levels of (a) CPA3, (b) IL-13 and (c) TNFα.

The distribution of the biomarkers in the four clusters is shown. The nodes lying outside the outlined clusters represent outliers. Colour scale ranges from blue (low values) to red (high values).

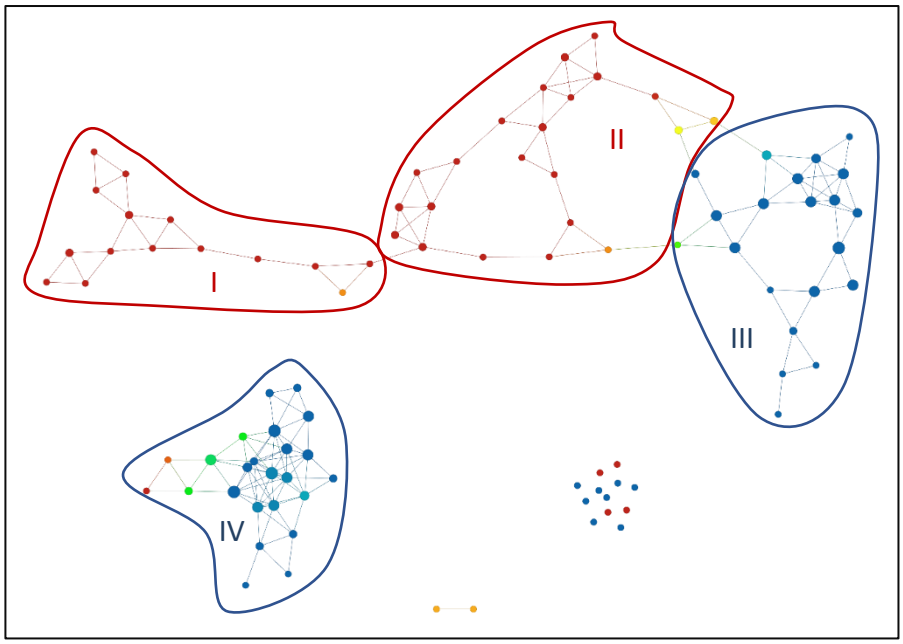
Table 4.5 Demographics and clinical characteristics of the clusters in the validation cohort

Parameter	Clusters			
	I	II	III	IV
Number of participants	16	23	36	32
Number of healthy subjects	2	4	3	11
Age (years) Median (min-max)	30 (3-46)	15 (4-46)	36 (5-61)	32 (2-52)
Gender (male/female) No. (%)	4 (24)/13 (76)	6 (23)/20 (77)	8 (23)/26 (77)	14 (36)/25 (64)
Ethnicity				
Caucasian no. (%)	5 (29)	7 (27)	4 (12)	7 (18)
Middle Eastern no. (%)	9 (53)	12 (46)	21 (62)	20 (51)
Asian no. (%)	3 (18)	4 (15)	7 (20)	7 (18)
African no. (%)	0 (0)	1 (4)	0 (0)	3 (8)
Hispanic no. (%)	0 (0)	2 (8)	2 (6)	2 (5)
Diagnosis				
Anaphylaxis no. (%)	5 (29)	6 (23)	7 (21)	3 (8)
Drug allergy no. (%)	1 (6)	1 (4)	4 (12)	6 (15)
Food allergy no. (%)	3 (18)	6 (23)	7 (21)	2 (5)
Atopic dermatitis no. (%)	4 (23)	2 (8)	7 (21)	6 (15)
Hay fever no. (%)	1 (6)	2 (8)	3 (8)	6 (15)
Asthma no. (%)	0 (0)	2 (8)	2 (6)	2 (5)
Insect bite no. (%)	1 (6)	3 (11)	1 (3)	3 (8)
Symptoms				
Skin (yes/no) no. (%)	13 (87)/2 (13)	21 (96)/1 (4)	29 (93)/2 (7)	26 (93)/2 (7)
Airway (yes/no) no. (%)	10 (67)/5 (33)	16 (72)/6 (27)	22 (71)/9 (29)	21 (75)/7 (25)
CVS (yes/no) no. (%)	6 (40)/9 (60)	5 (23)/17 (77)	13 (42)/18 (58)	12 (43)/16 (57)
GIT (yes/no) no. (%)	5 (33)/10 (67)	4 (18)/18 (82)	10 (32)/21 (68)	9 (32)/19 (68)
Other (yes/no) no. (%)	5 (33)/10 (67)	8 (36)/14 (64)	9 (29)/22 (71)	11 (39)/17 (61)

(a)


II
High CPA3
Low IL-13
Young
Food allergy
Severe allergic reactions
I
High CPA3
High IL-13
Severe
allergic
reactions
III
Low CPA3
Low IL-13
Food allergy
IV
Low CPA3
High IL-13
Drug allergy

(b)



(c)

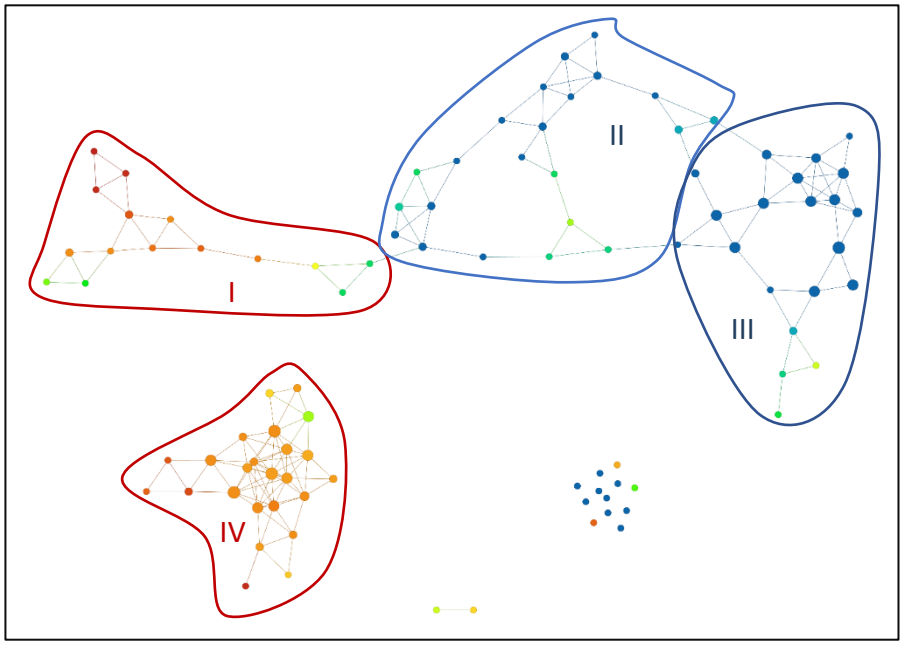


Figure 4.18 Replication of multidimensional clinico-immunological clusters of patients in the validation cohort (Qatar).

(a) The clinical and biochemical profile for each cluster, where nodes represent the number of patients, is shown. The distribution of the serum levels of (b) CPA3 and (c) IL-13, in which the nodes reflect the levels of biomarkers, is also shown. The nodes lying outside the outlined clusters represent outliers. Colour scale ranges from blue (low values) to red (high values).

Chapter 4: Multidimensional endotypes in patients with allergic reactions revealed by topological data analysis of clinical and immunological parameters

Chapter 4: Multidimensional endotypes in patients with allergic reactions revealed by topological data analysis of clinical and immunological parameters

Chapter 5: Factors modulating release and expression of mast cell mediators

5.1 Introduction

Measurement of mast cell mediators in body fluids has widely been considered as diagnostic tool for allergic reactions and particularly in the life-threatening condition of anaphylaxis (188). However, there have been cases where an increase in these markers could not be detected despite clinical manifestations of an allergic reaction (208). On the hand, being localized at the host-environment interface, mast cells are well placed to recognise allergens as well as pathogens, including bacteria. Several reports have indicated that mast cells have the ability to identify, engulf and destroy many bacterial species including *E. coli*, *Klebsiella pneumonia*, *Enterobacter cloacae* (225, 226), *Pseudomonas aeruginosa* (227), and *Mycobacteria tuberculosis* (228). Furthermore, there have been reports suggesting that bacterial exposure decreases IgE-mediated responses. These studies were performed using non-pathogenic bacteria like *E.coli* (150), *Lactobacillus rhamnosus* and *Bifidobacterium animalis* (151) in effort to emphasize probiotic administration and its role in allergic inflammation. However, the impact of pathogenic bacteria on mast cell allergic responses is yet to be elaborated.

Because of the costly and time-consuming nature of mast cell purification procedures, the study of human mast cell biology has relied on three major cell lines, HMC-1 (Human Mast Cell leukaemia-1) (229), LAD2 (Laboratory of Allergic Diseases 2) (230), and LUVA (Laboratory of University of Virginia) (231). HMC-1 cells have come to be considered as immature mast cells and their responses to IgE-dependent signals have not been observed consistently due to the immaturity of FcεRI receptor (229). They also express very low levels of most mast cell markers including tryptase and chymase (232). There are limited publications regarding LUVA cells and this might be a result of their variable response to stimuli. LAD2 cells can be considered to be relatively mature mast cells as they retain functioning FcεRI receptors and are able to respond to immunological stimuli (230). They also express many mast cell mediators including tryptase and chymase (230, 232).

In this chapter we have employed LAD2 cells to study the impact of *Staphylococcus aureus* infection on release and expression of mast cell mediators and highlighted possible underlying mechanisms. We also tested sensitisation of LAD2 cells with serum from allergic patients with the aim of developing a new means for investigating allergic sensitivity.

5.2 Methods

5.2.1 Testing FcεRI receptor in LAD2 mast cells

Sensitisation of LAD2 cells: LAD2 cells were cultured and harvested as described in Section 2.4.2. The cells were counted and adjusted to 1 x 10^6 cells per treatment and cultured in a 6-well plate using 5 ml/well StemPro-34 medium which had 50% less stem cell factor (50 ng/ml) than non-sensitised cells to prevent stem cell factor-driven mast cell activation (233-235). Human myeloma IgE (Merck Millipore, Hertfordshire, UK) was added to the cells at 1.5 μg concentration and incubated overnight at 37 °C and at 5% CO_2 atmosphere incubator. Non-sensitised cells (without addition of IgE) were cultured under the same conditions.

Harvesting LAD2 cells: The cells were harvested and centrifuged at 270 g for 10 minutes. After discarding the supernatant, the cells were resuspended with 10 ml Tyrode's buffer [0.8% (w/v) sodium chloride, 0.02% (w/v) potassium chloride, 0.007% (w/v) sodium dihydrogen phosphate dihydrate, 0.01% (w/v) magnesium chloride hexahydrate, 0.04% (w/v) calcium chloride dihydrate, and 0.24% (w/v) HEPES] and centrifuged again. The cells were resuspended in Tyrode's buffer and placed on a roller for 5 minutes.

Stimulation of LAD2 cells with anti-IgE: The cell suspension was added to a sterile V-bottom 96-well plate (180 µl/well). Serial dilutions of goat anti-human IgE (Bio-Rad laboratories Ltd., Hertfordshire, UK) in Tyrode's buffer (0.1, 0.3, 1, 3, and 10 µg/ml) were prepared and were added to the cells (20 µl/well). The spontaneous release of mediators was determined by adding 20 µl/well Tyrode's buffer alone to the cells, whereas the total release was determined following treatment with 20 µl/well 1% Triton X-100. All cell suspensions were incubated for one hour at 37 °C.

Collection of LAD2 cell supernatants: The plate containing the cells was centrifuged at 900 g for 10 minutes and the supernatants were transferred into either tubes or a 96-well plate and stored at -20 °C till the time of the assay. The procedures are indicated in a schematic diagram (Figure 5.1).

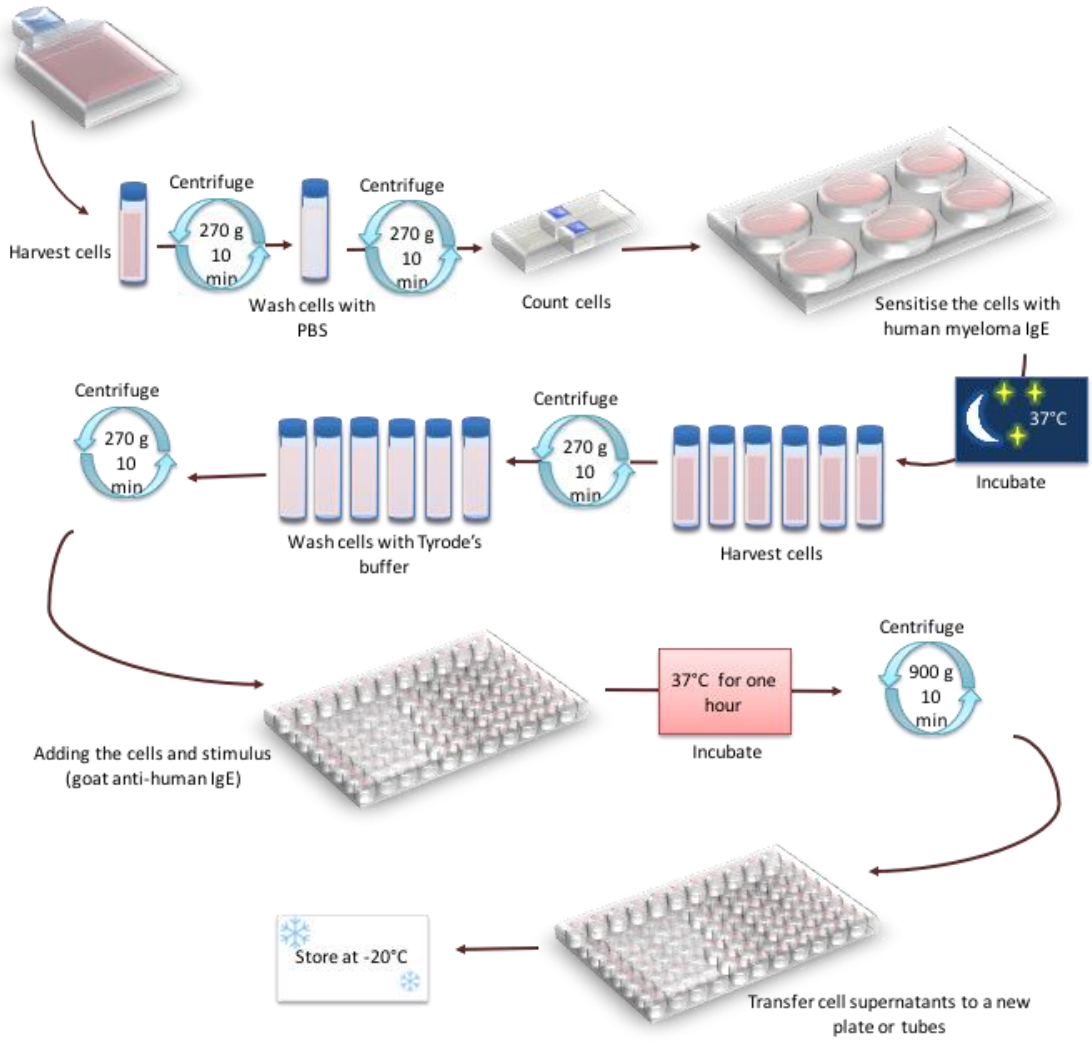


Figure 5.1 Sensitisation of LAD2 cells with human myeloma IgE antibody and their activation.

After being harvested, washed, and counted, the cells were incubated overnight at 37°C with human myeloma IgE. The cells were then harvested and washed with Tyrode's buffer before being challenged with goat anti-human IgE, which was incubated with the cells for one hour at 37°C. The cell supernatants were collected and stored at -20 °C till analysis.

5.2.1.1 Beta-hexosaminidase assay

Being one of the most abundant products released upon mast cell degranulation (57), β -hexosaminidase was used as an indicator of mast cell activation. Thirty µl/well cell supernatant was transferred into a flat bottom 96-well with 50 µl/well of 1.5 mg/ml p-nitrophenyl-n-acetyl-β-d-glucosaminide in 0.1 M sodium phosphate dibasic (pH 4.5). This was followed by an hour incubation at 37 °C, after which the reaction was stopped by adding 100 µl/well 0.2 M glycine (pH 10.7) and the reaction was detected on microplate reader at 410 nm wavelength. The absorbance was linear over the activities measured.

Net release of β-hexosaminidase in each sample was calculated by subtracting the absorbance of the spontaneous release from the absorbance of the sample, divided by the absorbance of the total release, and then calculating the percentage:

$$\text{Net release } (\%) = \frac{(\text{Abs sample} - \text{Abs spontaneous})}{\text{Abs total}} \text{x } 100$$

Where Abs refers to the absorbance.

5.2.2 Sensitisation of LAD2 mast cells with serum

Collection of serum samples: Serum samples were collected from subjects with a known history of allergy to house dust mite as well as from control subjects. The samples were processed (as described in Section 2.1.2) and stored at -80 °C freezer until analysis.

Sensitisation with serum: As with sensitisation with human myeloma IgE, LAD2 cells were harvested, washed, counted and cultured in 6-well plate using the same conditions described in Section 5.2.1, except for using 3 x 10^6 cells per well. The cell number was adjusted to at least 5 x 10^5 per condition for the subsequent experiments to allow better detection of β-hexosaminidase activity. For sensitisation with serum, there were six conditions including; total release, spontaneous release, three concentrations of allergen and one concentration of goat anti-human IgE (Figure 5.2). Serum was added to the cells in a range of concentrations (10%, 20%, and 40% serum) and incubated overnight at 37 °C. A set of cells were sensitised with 1.5 µg human myeloma IgE to act as a positive control. Cell preparation without sensitisation was acted as a negative control.

Harvesting and adding stimuli to LAD2 cells: The cells were harvested and washed with Tyrode's buffer and then resuspended with Tyrode's buffer as described in Section 5.2.1. A range of concentrations of house dust mite (*Dermatophagoides pteronyssinus*) allergen as well as 0.3

µg/ml goat anti-human IgE were used to stimulate the serum-sensitised cells. Human myeloma IgE-sensitised cells were stimulated with goat anti-human IgE. The cells were incubated with the stimuli for one hour at 37 °C. Determination of the spontaneous and total release of mediators was performed as explained in Section 5.2.1. As with sensitisation with human myeloma IgE, the cell supernatants were collected after centrifugation and stored at -20°C till the assay for β-hexosaminidase was performed.

5.2.3 Effect of bacterial infection on LAD2 mast cell sensitisation

Four sets of LAD2 cells were used to test the effect of infection with *Staphylococcus aureus* on the response of mast cells to IgE stimulation. These sets were (1) non-sensitised and non-infected, (2) non-sensitised and infected, (3) sensitised and non-infected and (4) sensitised infected. At least 4 x 10^6 cells per treatment were used. Sensitisation with human myeloma IgE was performed following the same protocol mentioned in Section 5.2.1, except for using antibiotic-free medium to culture the cells.

S. aureus cultures were grown by Mr. Tim Biggs (PhD student) who undertook infection of the cells (see Appendix E). The bacteria were incubated with the cells for two or four hours at 37°C and at 5% CO_2 atmosphere incubator. The cells were then harvested and washed with Tyrode's buffer as mentioned in Section 5.2.1, except for collection of cell culture supernatants after centrifugation. Each set of cells were stimulated with a range of goat anti-human IgE concentrations as well as calcium ionophore A23187 at two different concentrations for one-hour incubation at 37°C. The spontaneous and total release were determined separately for each group of cells. At the end of the incubation, the 96-well plate was centrifuged at 900 g for 10 minutes and supernatants were collected and stored at -20°C.

An assay for β-hexosaminidase release was performed for cell culture supernatants and for supernatants collected after stimulation following the same steps explained in Section 5.2.1.1. Quantification of protein analytes including IFNγ, IL-1β, IL-5, IL-6, MMP-7, TNFα and IL-17A using Luminex (R&D Systems, Minneapolis, MN, USA) in cell culture supernatants and post-stimulation supernatants was performed by Mr. Tim Biggs according to the manufacturer's protocol (see Appendix E).

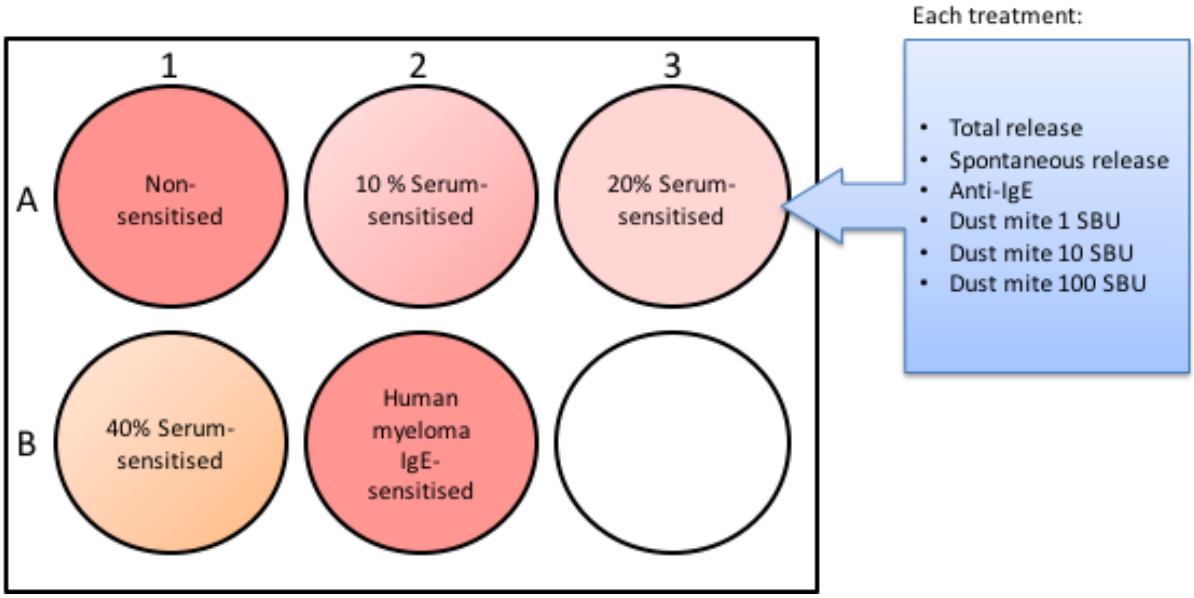


Figure 5.2 Sensitisation of LAD2 cells with serum.

LAD2 cells were cultured in a 6-well plate and sensitised with either different percentages of serum or human myeloma IgE. Cells without sensitisation were included as a negative control. For each condition, the cell number was adjusted to 5 x 10^5 cells and therefore the total number per well was 3 x 10^5 cells.

5.2.3.1 Gene expression of TNFα, IL-8 and IL-1β

Cell preparations including four treatment groups (non-sensitised non-infected, non-sensitised infected, sensitised non-infected and sensitised infected) were used to study the gene expression of TNFα, IL-8 and IL-1β. The experiment was performed as explained above, except for increasing the incubation time with the stimuli (goat anti-human IgE and calcium ionophore A23187) to two hours. After stimulation, the supernatants were collected and stored at -20°C as well as cell pellets. To preserve RNA, each cell pellet was treated with 150 µl RNA stabilisation solution (RNALater, Life Technology, Paisley, UK) and then stored at 4°C overnight and moved to -80°C. RNA extraction, reverse transcription, PCR and gene expression analysis were performed by Mr. Tim Biggs (See Appendix E).

5.2.3.2 Testing cell viability by lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) is an enzyme found in many cells including mast cells. When the plasma membrane breaks down, LDH is released and can be quantified in culture supernatants. The measurement is based on oxidation of lactate to pyruvate and reduction of NAD^+ to NADH through LDH-catalysed conversion. Then, diaphorase catalyses NADH-dependent reduction of tetrazolium salt (INT) to a red-coloured formazan product that can be detected at 490 nm (Figure 5.3). An increase in the number of dead cells results in increased LDH activity, which is directly proportional to the amount of formazan generated within a defined time period.

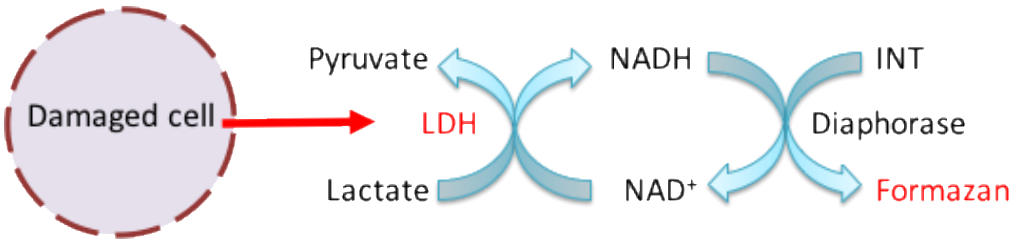


Figure 5.3 Principle of LDH assay.

LDH released from a damaged cell catalyses oxidation-reduction reaction of lactate to pyruvate and NAD^+ to NADH. The enzyme diaphorase uses NADH to reduce INT to formazan and generate a colour reaction that can be measured.

The release of LDH was determined using a Pierce LDH assay kit (Life Technology) according to the manufacturer's instructions. One hundred µl of cell culture supernatants collected after bacterial infection were added to a 96-well culture plate. Ten µl of lysis buffer (10x) were added and incubated for 45 minutes at 37°C. The maximum release of LDH was determined by treating 4 x 10^4 cells with 10 µl lysis buffer, whereas determination of the spontaneous release was performed by adding 10 µl ultrapure water to the cells.

At the end of the incubation, 50 µl of each sample were transferred to a new flat-bottom 96-well plate, where 50 µl reaction mixture were added next and incubated for 30 minutes at room temperature. The reaction was then stopped by adding 50 µl HCl (1N) stopping solution and the absorbance was measured using microplate reader at 450 nm with 595 nm correction wavelength. The linear portion of the absorbance curve was always employed.

Net release of LDH in each sample was calculated by subtracting the absorbance of the spontaneous release from the absorbance of the sample, divided by the absorbance of the total release and then calculating the percentage:

$$\text{Net release } (\%) = \frac{(\text{Abs sample} - \text{Abs spontaneous})}{\text{Abs total}} \text{x } 100$$

Where Abs refers to the absorbance.

5.2.3.3 Phosphorylation status of main protein kinases following bacterial infection of sensitised mast cells

Protein kinases play a key role in regulating cell function. They activate the target protein by adding phosphate groups and therefore altering the function of many proteins which are involved in a wide range of underlying cellular processes. Phosphorylation of mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1-3), and different p38 isoforms (α, β, δ, γ) was determined using a commercially available kit (Human Phospho-MAPK Array Kit, R&D Systems) according to the manufacturer's instructions.

Cells were prepared as discussed above in Section 5.2.3, except for using 5 x 10^6 cells for each treatment and using T25 flasks (Fisher Scientific) for cell culture. The culture medium was adjusted accordingly to 10 ml per flask. After overnight sensitisation, the cells were infected with *S. aureus* for four hours.

At the end of incubation, the cells were harvested and washed with 10 ml PBS and then resuspended with 500 µl lysis buffer (Buffer 6). The cell lysates were mixed gently by placing on a rocking platform for 30 minutes at 4°C. Cell supernatants were collected following centrifugation at 14,000 g for 5 minutes and transferred to new tubes, which were stored at -80°C till assay time.

On the day of the assay, the membranes supplied in the kit were transferred into a 4-well multidish containing 2 ml blocking buffer (Array buffer 5) to block non-specific binding sites. These were incubated for one hour at room temperature on a rocking platform. During this time, the samples were prepared by adding 400 µl of each cell supernatant to a separate tube and adjusting the volume up to 1.5 ml with diluent (Array buffer 1). Twenty µl of detection antibody cocktail were then added to each tube and incubated for an hour at room temperature. After blocking the membranes, the blocking buffer was removed and sample-antibody mixtures were added into the membranes which were incubated overnight at 4°C on rocking platform.

The membranes were washed three times with 1X wash buffer for 10 minutes. This was followed by incubation with Streptavidin-HRP diluted in blocking buffer to 1:2000 dilution for 30 minutes at room temperature on a rocking platform. After three washing steps, the membranes were incubated with Chemi Reagent Mix for one minute and then covered with a transparent film. Imaging was performed using image analysis unit (ChemiDoc Imaging system, Bio-Rad) with serial exposure up to 10 minutes.

Images were analysed using Image Lab version 6.0 build 26 (Bio-Rad laboratories) by applying volume tools to measure the intensity (pixels) for each sample. The volumes were adjusted by subtraction of background intensity.

5.2.3.4 Statistical analysis

The statistical significance of differences in response between the four treatment groups of LAD2 cells and their association with different concentrations of stimuli or with different genes was assessed by two-way ANOVA followed by Bonferroni's multiple comparisons test. A p value of less than 0.05 was considered significant.

5.3 Results

5.3.1 Sensitisation of LAD2 mast cells

Human myeloma IgE-sensitised LAD2 cells stimulated with a range of concentrations of goat anti-human IgE showed a stepwise increase in the net release of β-hexosaminidase with up to 6% at 1 µg/ml anti-IgE (Figure 5.3).

Using serum from house dust mite-allergic patients to sensitise LAD2 cells resulted in a dose-response increase in the release of β-hexosaminidase when the cells were stimulated with a range of concentrations of house dust mite. Higher concentrations of serum were associated with a higher percentage of β-hexosaminidase release. Non-sensitised cells did not show an increase in β-hexosaminidase activity above the spontaneous release (Figure 5.5a).

A graded increase in the response was also seen when serum-sensitised cells were challenged with 0.3 µg/ml goat anti-human IgE. A higher percentage of β-hexosaminidase release was measured from human myeloma IgE-sensitised cells compared to that released from serum-sensitised cells. The release of β-hexosaminidase from non-sensitised cells was equivalent to that of the spontaneous release (Figure 5.4b).

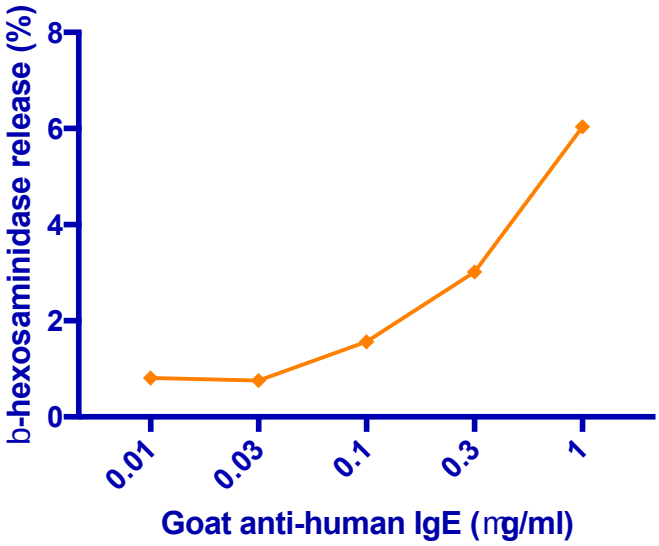


Figure 5.4 Net release of β-hexosaminidase from human myeloma IgE-sensitised LAD2 cells stimulated with goat anti-human IgE.

The release of β-hexosaminidase is expressed as a percentage of the total stores in the cells after subtracting the spontaneous release (6%). Data represent the mean for duplicate determinations.

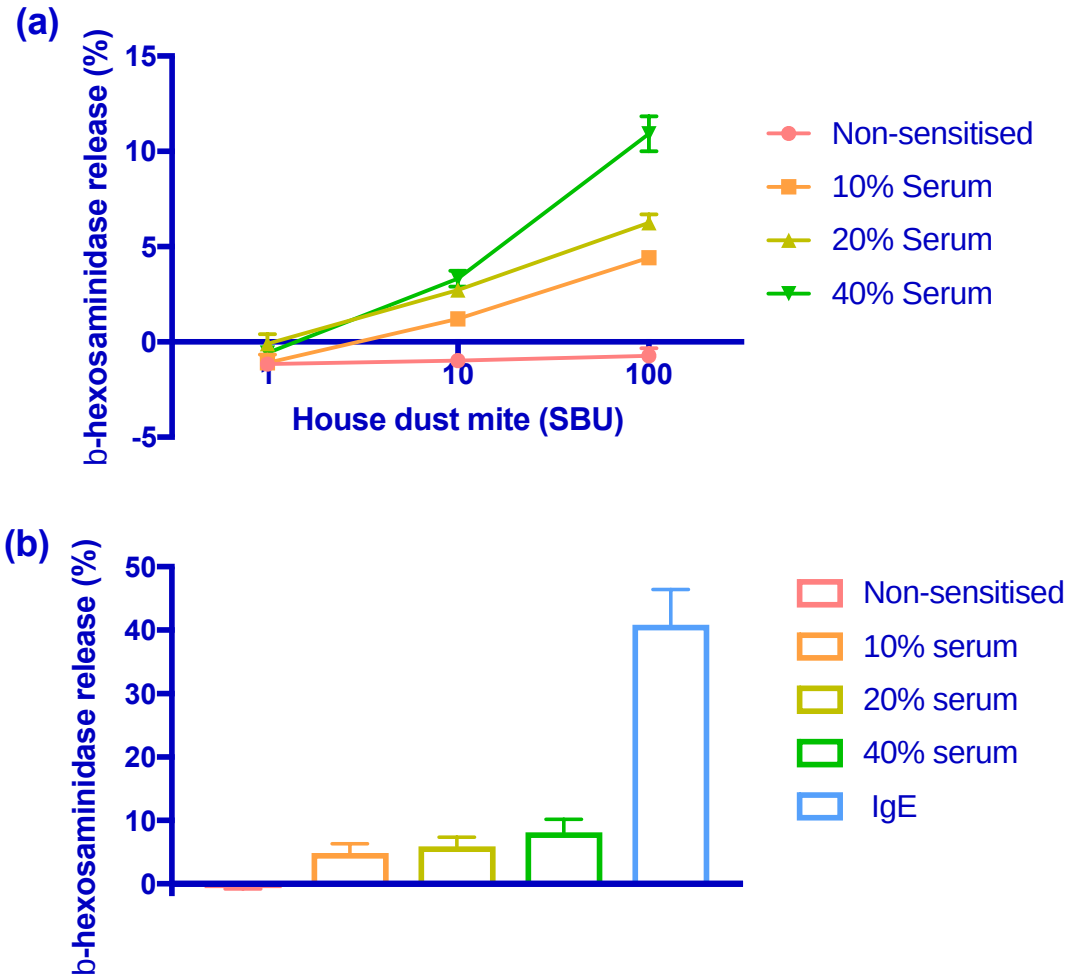


Figure 5.5 Net release of β-hexosaminidase from serum-sensitised LAD2 cells stimulated with (a) house dust mite or (b) 0.3 µg/ml goat anti-human IgE.

Mean ± SEM of spontaneous release was 12% ± 1, 16% ± 0.4, 12% ± 0.8, 11% ± 0.8, 15% ± 0.1 for non-sensitised, 10 % serum-, 20% serum-, 40% serum-, and IgE-sensitised cells, respectively. Data represent the mean ± SEM for three independent experiments.

5.3.2 The effect of bacterial infection on LAD2 mast cell sensitisation

5.3.2.1 The release of β -hexosaminidase

Stimulation of sensitised LAD2 cells infected with *S. aureus* for two hours with a range of concentrations of goat anti-human IgE was associated with release of β-hexosaminidase consistently less than that with the non-infected cells [F (1, 5) = 5.194, *p*= 0.07]. A significant difference in the release of β-hexosaminidase between the infected cells versus non-infected ones was seen at concentrations of 0.1 µg/ml (Mean difference= 2.07, 95% CI= 0.86 - 3.27, *p*= 0.0008) and 1 µg/ml of goat anti-human IgE (Mean difference= 1.67, 95% CI= 0.46 – 2.86, *p*= 0.005; Figure 5.6a]. Extending the period of infection to four hours resulted in a significant decrease in the release of β-hexosaminidase from sensitised cells compared to that from non-infected sensitised LAD2 cells [F (1, 5) = 9.17, *p*= 0.02]. Multiple comparisons between the sensitised LAD2 cells exposed to *S. aureus* infection and those non-infected cells showed significant differences in β-hexosaminidase release at the four different concentrations of goat anti-human IgE (Mean difference of 5.68, 95% CI= 1.68 - 9.68, *p*= 0.004; 6.05, 95% CI= 2.05 – 10.05, *p*= 0.002; 5.51, 95% CI= 1.51 – 9.5, *p*= 0.005; 7.77, 95% CI= 3.76 – 11.7, *p*= 0.0002 for 0.1, 0.3, 1, and 3 µg/ml; Figure 5.6b].

The comparison of β-hexosaminidase release from non-sensitised cells infected with *S. aureus* (for two or four hours) to that from non-infected cells showed no significant difference in percentages of release when the cells challenged with goat anti-human IgE antibody [F (1, 5) = 0.0003, *p*= 0.98 for two-hour infection and F (1, 5) = 1.68, *p*= 0.25 for four-hour infection; Figure 5.6a and b]. However, multiple comparisons between infected and non-infected LAD2 cells showed significant increase in the release of β-hexosaminidase in response to *S. aureus* infection at 0.1 µg/ml (2.4, 95% CI= 0.66 – 4.2, *p*= 0.005), 0.3 µg/ml (2.49, 95% CI= 1.17 – 4.7, *p*= 0.001), and 1 µg/ml (2.11, 95% CI= 0.33 – 3.8, *p*= 0.016) concentration of goat anti-human IgE (Figure 5.6b).

Calcium ionophore A23187 induced release was lower in LAD2 cells exposed to *S. aureus* than in non-infected cells at both concentrations of ionophore investigated. This effect was independent of sensitisation. As seen with stimulation with goat anti-human IgE antibody, apparent inhibition of β-hexosaminidase release was more pronounced after four hours of infection [F (3, 15) = 2.34, *p*= 0.11; Figure 5.7b) compared to that with two hours of infection [F (3, 15) = 0.37, *p*= 0.77; Figure 5.7a]. The apparent degree of inhibition in cells infected for four hours and stimulated with 0.03 µM calcium ionophore A23187 was greater than that with 0.3 µM [Mean difference of 47.62, 95% CI= 1.53 – 93.71, *p*= 0.04 for non-sensitized cells and 37.14, 95% CI= 6.55 – 67.76, *p*= 0.022 for sensitized cells].

There was little β-hexosaminidase release detected in cell culture supernatants from infected and non-infected cells collected before stimulation with goat anti-human IgE or calcium ionophore A23187. Comparisons of the release of β-hexosaminidase from infected cells to that from non-infected cells showed no significant differences [F (3, 9) = 0.9014, *p*= 0.47]. However, exposure of LAD2 cells to *S. aureus* for four hours was associated with more release of β-hexosaminidase compared to that from cells infected for two hours [F (1, 3) = 23.23, *p*= 0.017; Figure 5.8].

5.3.2.2 The release of pro-inflammatory cytokines

Most levels of pro-inflammatory cytokines released from LAD2 cells were below detection limit of the assay used (see Table 6, Appendix E), apart from TNFα and IFNγ. Before stimulation, levels of TNFα and IFNγ released from infected LAD2 cells were not different from those detected from non-infected cells [F (3, 6) = 2.09, *p*= 0.2; Figure 5.9a].

Using goat anti-human IgE to challenge human myeloma IgE-sensitised LAD2 cells infected with *S. aureus* for four hours was not associated with levels of TNFα and IFNγ different from those of non-infected cells. The TNFα and IFNγ release from non-sensitised cells after *S. aureus* infection were comparable to those released from non-infected cells [F (3, 6) = 1.72, *p*= 0.26; Figure 5.9b]. A similar trend was seen when the cells were stimulated with calcium ionophore A23187 [F (3, 6) = 2.04, *p*= 0.2; Figure 5.9c].

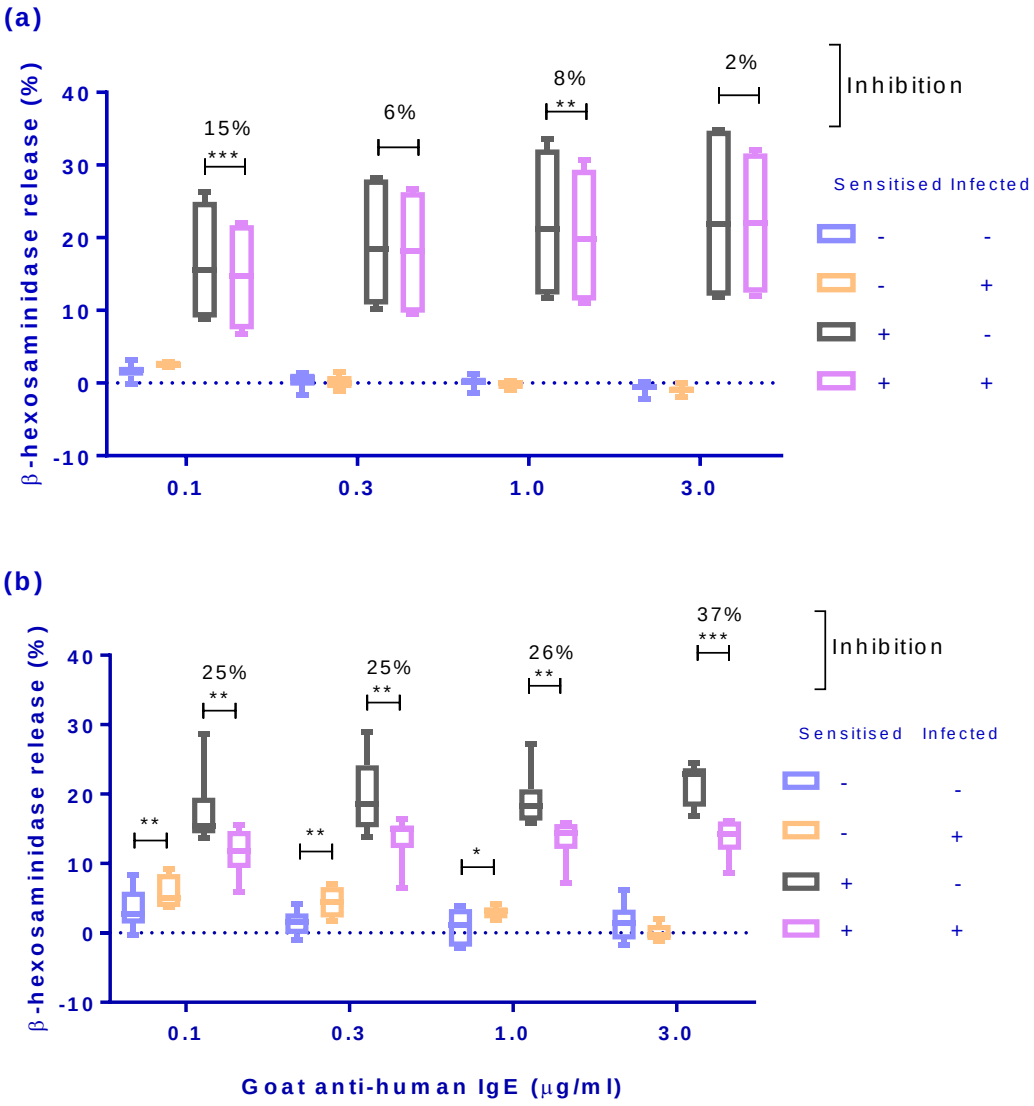


Figure 5.6 Net release of β-hexosaminidase from sensitised LAD2 cells with and without infection for (a) two and (b) four hours.

The degree of response of infected cells to stimulation with goat anti-human IgE was lower than in those without *S. aureus*. Apparent inhibition is indicated as a percentage. With a two-hour infection period, mean ± SEM of the spontaneous release for non-sensitised non-infected, non-sensitised infected, sensitised non-infected, and sensitised-infected cell groups was 5% ± 0.4, 9% ± 1.3, 8% ± 1.5, 7% ± 1, respectively. With a four-hour infection period, the corresponding values for the spontaneous release were 15% ± 1.3, 18% ± 3.3, 11% ± 0.6, and 13% ± 1.2. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test (* $p<0.05$, ** $p\leq0.01$, *** $p<0.001$).

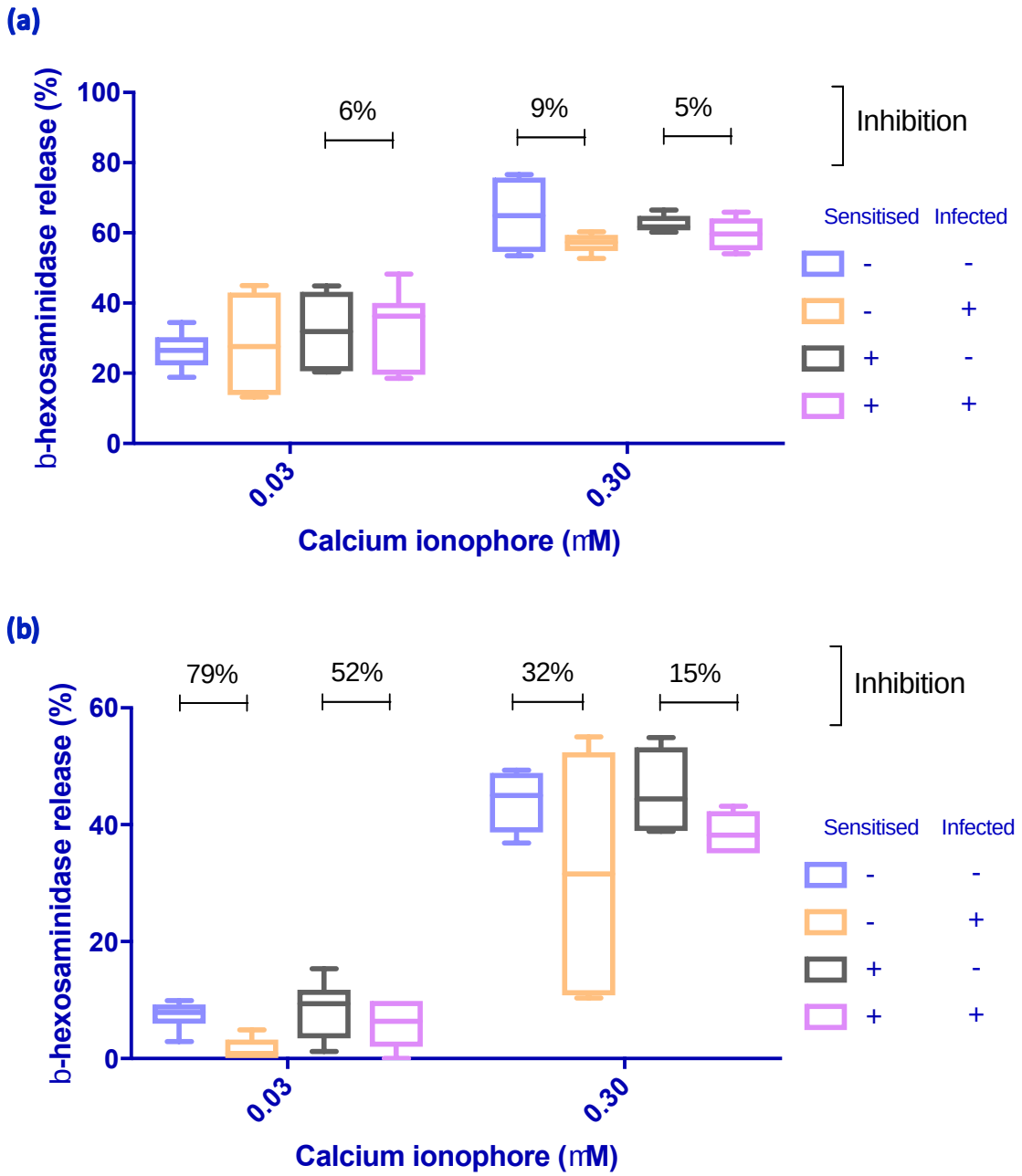


Figure 5.7 Net release of β-hexosaminidase from LAD2 cells with and without infection with *S. aureus* for (a) two and (b) four hours after stimulation with calcium ionophore A23187.

The degree of response of infected cells to stimulation with calcium ionophore A23187 was lower than in those without *S. aureus*. Inhibition is indicated as a percentage. Mean ± SEM of the spontaneous release after two hours of infection for non-sensitised non-infected, non-sensitised infected, sensitised non-infected, and sensitised-infected cell groups was 5% ± 0.4, 9% ± 1.3, 8% ± 1.5, 7% ± 1 respectively. At four-hour infection, the corresponding values for the spontaneous release were 15% ± 1.3, 18% ± 3.3, 11% ± 0.6, and 13% ± 1.2. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test.

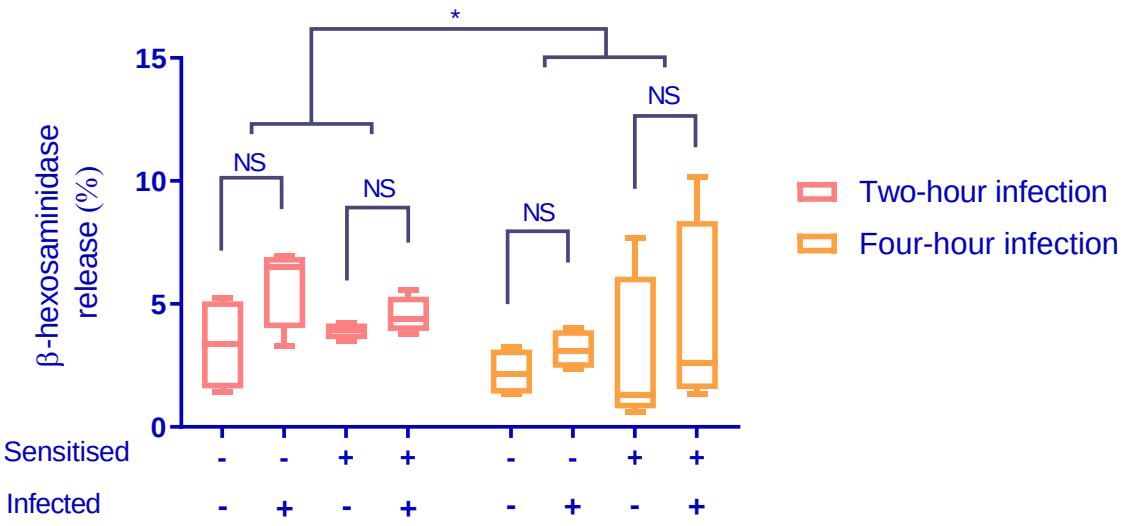


Figure 5.8 Net release of β-hexosaminidase in LAD2 culture supernatants collected before cell stimulation.

Cell culture supernatants were collected after two or four hours of infection with *S. aureus.* Mean ± SEM of the spontaneous release was 6 ± 2 at two-hour infection and 13 ± 0.7 at four-hour infection. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test (* $p<0.05$).

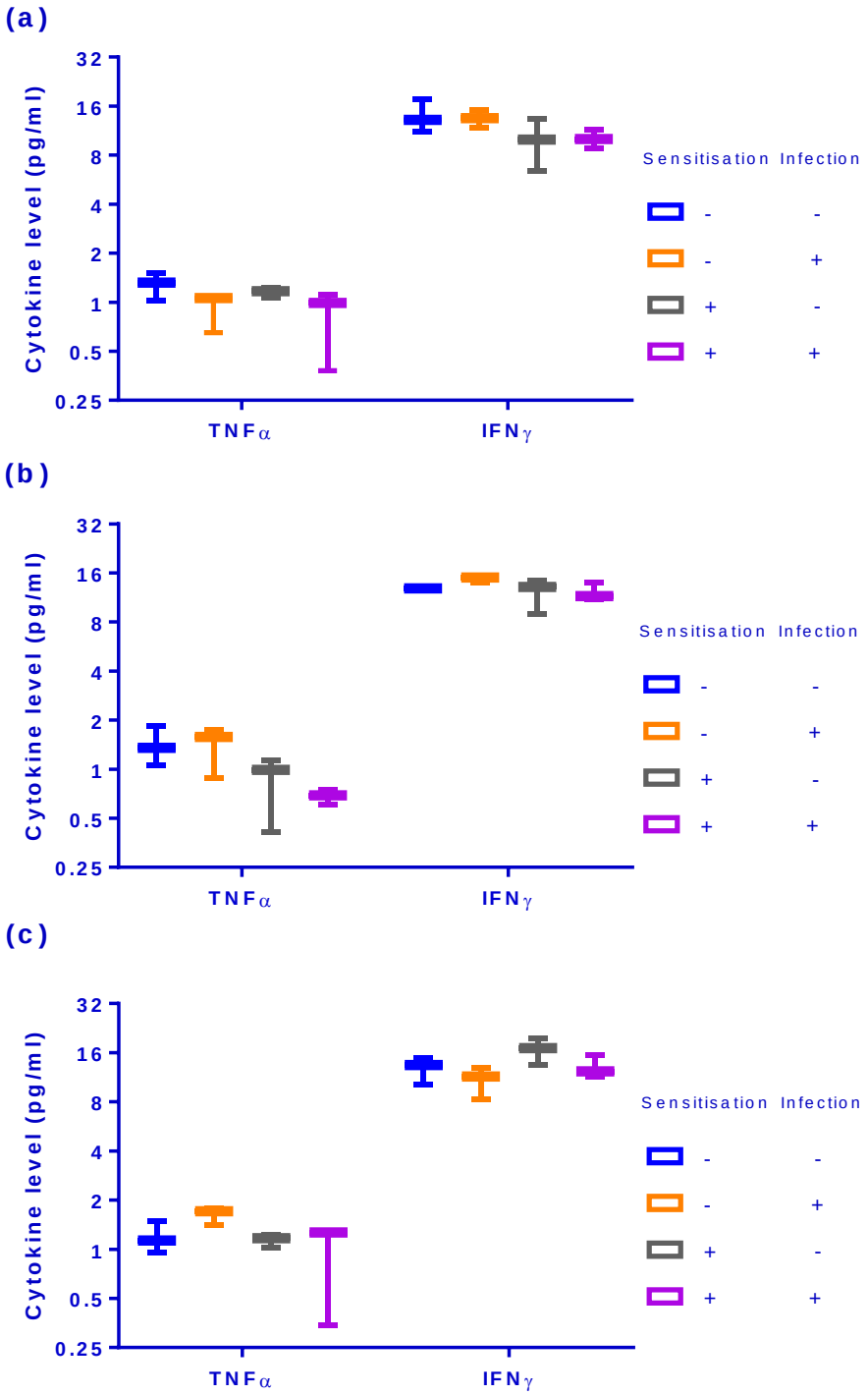


Figure 5.9 TNFα and IFNγ release from sensitised LAD2 cells with and without infection.

Measurements were performed in cell culture supernatants (a) before stimulation and in cell supernatants after stimulation with (b) 3 µg/ml goat anti-human IgE or (c) 0.03 µM calcium ionophore A23187 for one hour. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test.

5.3.2.3 Gene expression of TNFα, IL-8, and IL-1β

For sensitised LAD2 cells, stimulation following four-hour infection with *S. aureus* with goat anti-human IgE resulted in downregulation of TNFα, IL-8, and IL-1β gene expression [F (1, 2) = 1.88, *p*= 0.3]. Exposure of non-sensitised cells to *S. aureus* was associated with upregulation of gene expression of the cytokines of interest [F (1, 2) = 0.67, *p*= 0.49]. It is noteworthy that gene expression for TNFα, IL-8, and IL-1β in sensitised LAD2 cells infected with *S. aureus* was 45% (*p*= 0.002), 20%, and 36% (*p*= 0.011), respectively less than that in non-infected cells. On the other hand, gene expression of these cytokines was increased by 2%, 44%, and 16%, respectively over that seen in infected LAD2 cells without prior sensitisation compared to the non-infected counterpart (Figure 5.10a).

Gene expression for TNFα, IL-8, and IL-1β in LAD2 cells pre-infected with *S. aureus* and stimulated with calcium ionophore did not show consistent differences from those of non-infected cells [F (3, 6) = 1.011, *p*= 0.45; Figure 5.10 b].

5.3.2.4 LDH assay for cell viability

At the two-hour infection period, LDH activity detected in LAD2 cell supernatants exposed to *S. aureus* was comparable to that detected in supernatants of non-infected cells and was consistently low (less than 5%). A similar degree of LDH release was seen in supernatants from sensitised LAD2 cells (without infection) and was not different from the activity measured in supernatants of non-sensitised cells.

When the infection time was extended to four hours, LDH activity detected in LAD2 cell supernatants after infection with *S. aureus* was significantly greater than that in supernatants from non-infected cells (Mean difference of 11.35, 95% CI = 9.87-12.82, *p*<0.0001 for sensitized cells and 9.84, 95% CI= 8.37-11.32, *p*<0.0001 for non-sensitized cells). The release of LDH at four-hour infection was also significantly higher than the activity detected at two hours of infection [F (1, 7) = 97.4, *p*<0.0001]. However, the overall percentage of LDH release was persistently low. As seen with the two-hour infection period, supernatants collected from sensitised LAD2 cells had a consistently low LDH activity, which was not different from that detected in non-sensitised cell supernatants and was comparable to the rate seen with a two-hour duration of infection (Figure 5.11).

5.3.2.5 Protein kinase phosphorylation

Phosphorylation of 11 protein kinases in sensitised LAD2 cells infected with *S. aureus* for four hours was consistently lower than that seen in non-infected sensitised cells [$F(1, 10) = 15.5$, $p = 0.002$]. This effect was marked on the expression of ERK2 and reached statistical significance for the expression of Akt2 (Mean difference= 75220, 95%CI= 9828- 14×10^4, $p = 0.015$) and GSK-3α/β (Mean difference= 10.4×10^4, 95%CI= 3.8×10^4 – 16.9×10^4, $p = 0.0003$) in infected cells when compared to that in non-infected cells. A similar trend was seen in the phosphorylation patterns of protein kinases in pre-sensitised cells incubated with *S. aureus* compared to their non-sensitised counterparts [$F(1, 10) = 11.74$, $p = 0.0065$]. Sensitisation of LAD2 cells in the presence of *S. aureus* infection was associated with significantly lower expression of GSK-3α/β (Mean difference= 12.2×10^4, 95%CI= 5.7×10^4- 18.7×10^4, $p < 0.0001$) and GSK-3β (Mean difference= 8.9×10^4, 95%CI= 2.4×10^4- 15.5×10^4, $p = 0.002$) than that seen without sensitisation. There was little change in the phosphorylation pattern of protein kinases in infected cells without prior sensitisation in relation to that in non-infected cells. Similarly, the phosphorylation status in sensitised cells in the absence of *S. aureus* infection did not differ from that of non-sensitised cells.

Analysis was performed on the 11 protein kinases that were detected with non-treated LAD2 cells, as the signals for the remaining 15 protein kinases were not detected in these cells.

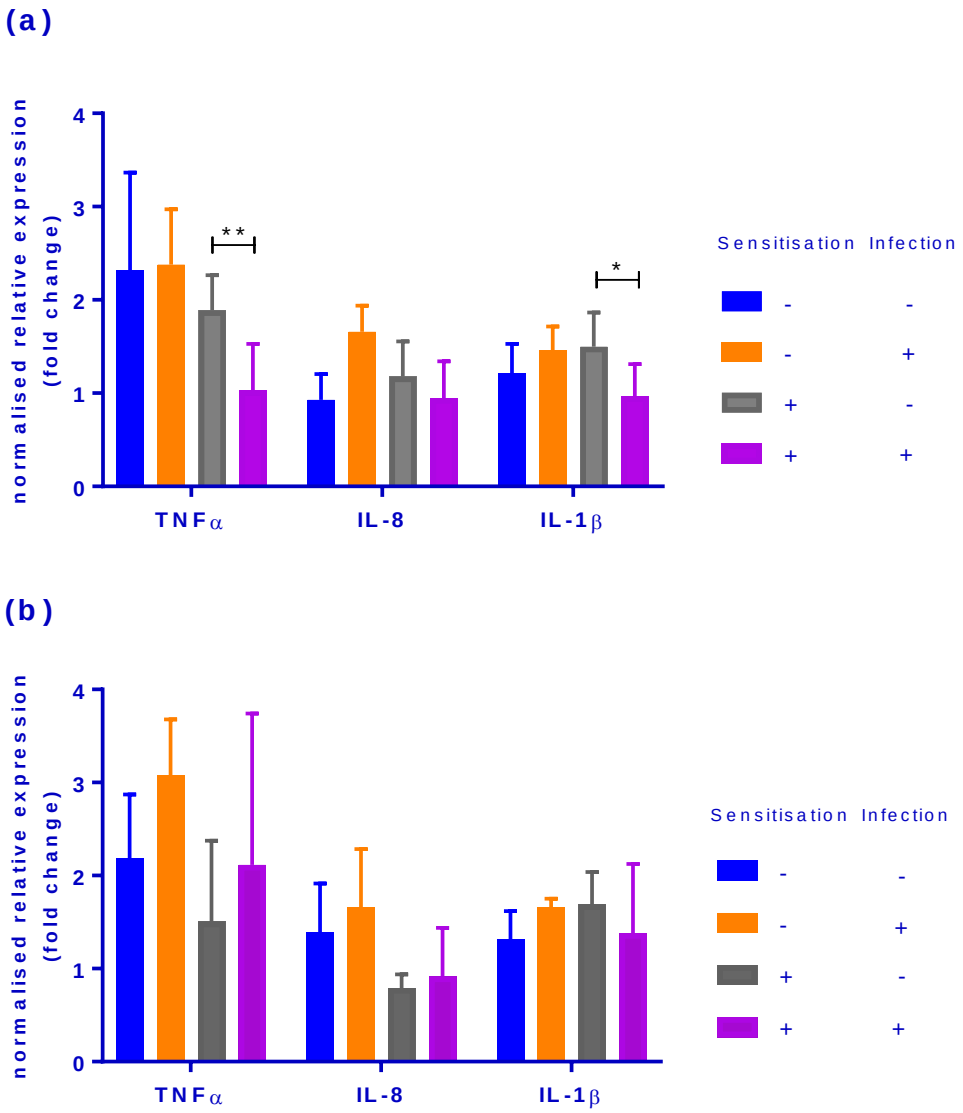


Figure 5.10 Effect of infection on gene expression for TNFα, IL-8, and IL-1β in sensitised LAD2 cells.

Total RNA was extracted from cells following stimulation with (a) 3 µg/ml goat anti-human IgE or (b) 0.03 µM calcium ionophore A23187. Beta-actin served as a housekeeping gene in TNFα, IL-8, and IL-1β gene expression. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test (* $p<0.05$, ** $p<0.01$).

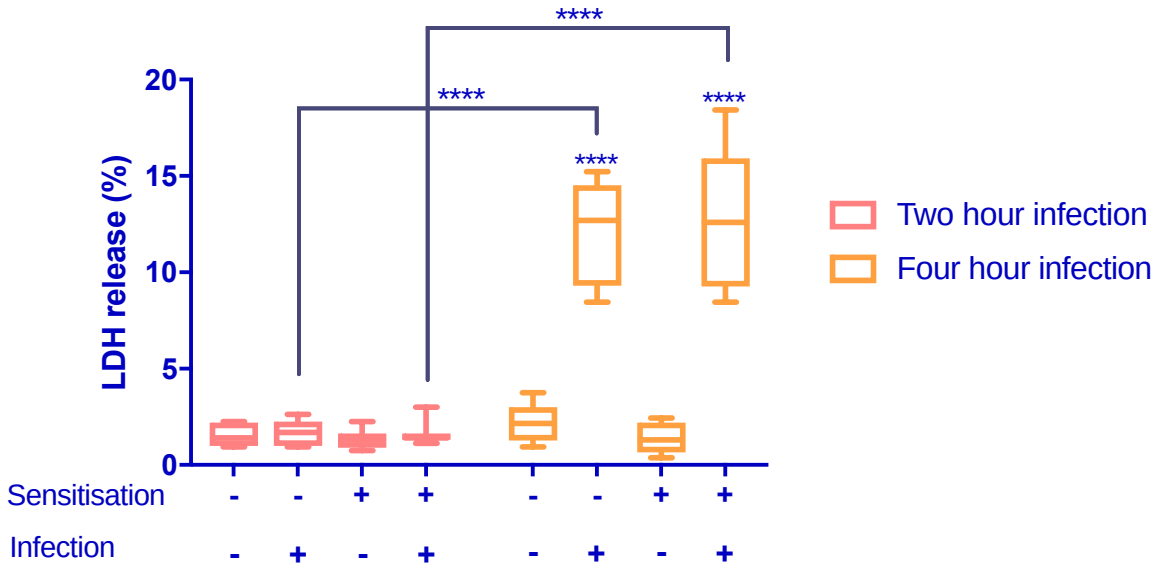


Figure 5.11 The release of LDH into cell culture supernatants collected after two or four hours of infection.

Mean ± SEM of the spontaneous release was 9 ± 0.2. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test (**** $p < 0.0001$).

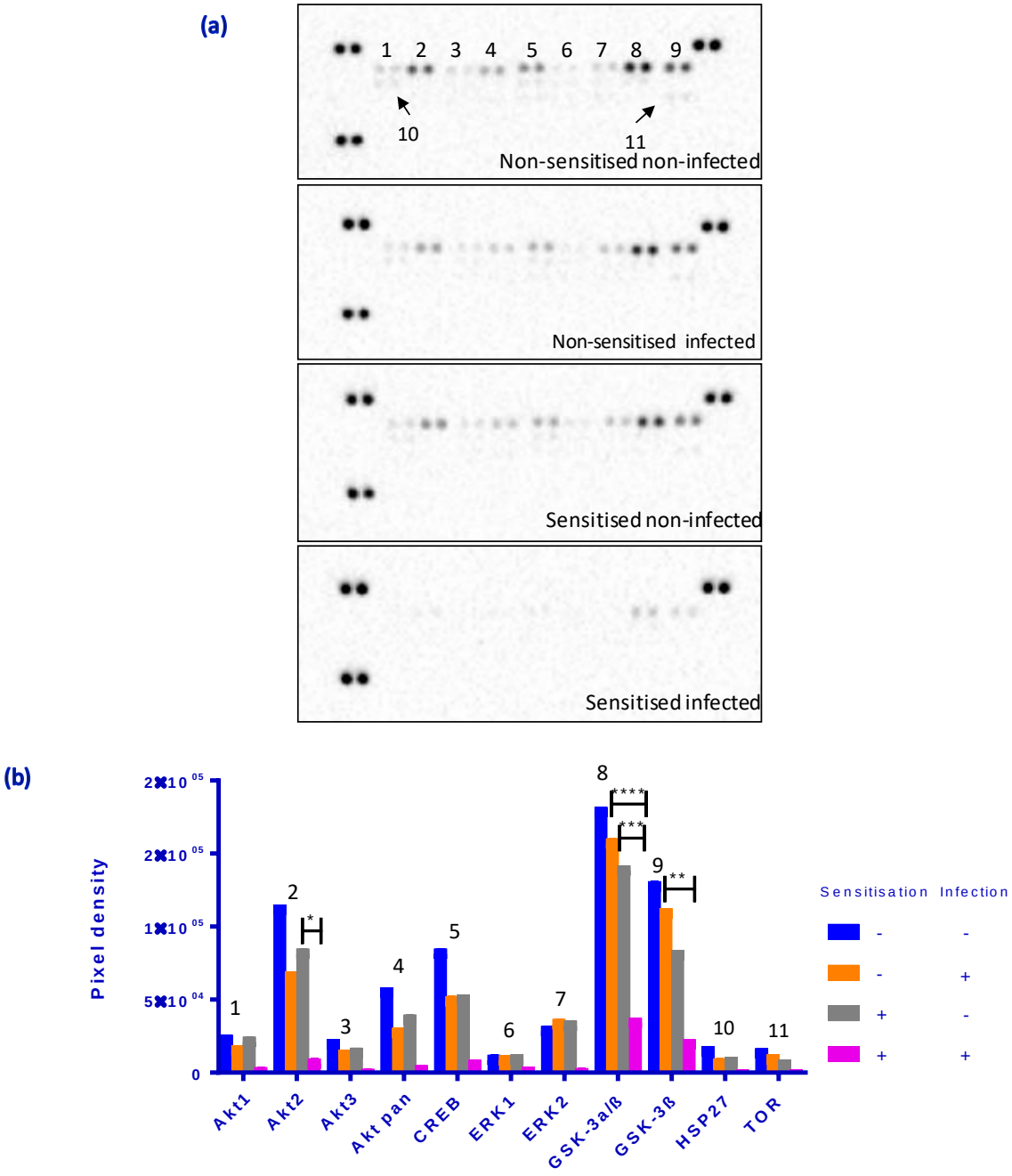


Figure 5.12 Effect of infection on protein kinase phosphorylation in sensitised LAD2 cells.

(a) Images for membranes showing phosphorylation of 11 main protein kinases in response to cell sensitisation and infection. (b) Corresponding analysis of differences in pixel density within the four groups of cells. Data shown were from a 76.2 second exposure. Images were analysed using Image Lab software. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test. (* $p = 0.02$, ** $p = 0.002$, *** $p = 0.0003$, **** $p < 0.0001$).

Chapter 6: The subcellular localization of mast cell proteases

6.1 Introduction

Mast cells play a fundamental role in the mechanism of immune-mediated conditions. Once activated, mast cells release a wide spectrum of chemical mediators which can orchestrate immune responses against environmental insults. Tryptase, CPA3 and chymase are well-established mast cell proteases that are secreted upon degranulation. However, their subcellular distribution is still unclear. DPPI has been little investigated as a secreted protease. Roles for DPPI have been proposed in the activation mast cell proteases from their inactive form (134, 135) and in controlling CPA3 levels (128). However, it is unclear to what extent the principal actions of DPPI are intracellular or if this protease may also have extracellular actions. It has been suggested that DPPI can cleave extracellular matrix proteins in uninflamed dog airways (133), but the studies supporting this were limited and relied on use of a preparation of partially purified DPPI. Precise location of DPPI within mast cells and its relationship with other mast cell proteases remain poorly defined.

In the present chapter, we have used LAD2 cells to investigate for the first time the subcellular location of DPPI and to determine the degree to which it may be co-localized with other mast cell proteases.

6.2 Methods

6.2.1 Immunofluorescence staining with antibodies specific for tryptase, chymase, CPA3 and DPPI

We have applied immunocytochemistry to LAD2 cells using specific antibodies for tryptase, chymase, CPA3 and DPPI that were developed in our laboratory. LAD2 cells are non-adherent cells; hence we have modified and optimised a protocol (236) to allow them to attach to coverslips. Details of LAD2 cell culture procedure are described in Section 2.5.2. An aqueous solution of 0.5% (w/v) Alcian blue was used to coat 8-well chamber slides (Ibidi, Martinsried, Germany) and was applied for at least 15 minutes at room temperature then the slides were washed four times with diH_2O. The cells were harvested and centrifuged at 400 g for 5 minutes.

After removal of the medium, the cells were resuspended with PBS and centrifuged again using the same conditions. This washing step was repeated twice. The cells were then counted and adjusted to 1 x 10^6 cells/ml PBS before applying them on Alcian blue pre-coated slides. Another set of cells were counted and immediately added to Alcian blue pre-coated slides. Both sets were centrifuged at 20 g for 5 minutes to allow gentle deposition of the cells onto the slide surface. An opaque monolayer of cells was apparent to the naked eye when the slide was viewed from the side. Details of LAD2 cell processing for immunocytochemistry and imaging are described in Sections 2.6 and 2.7 and the procedures are summarised in Figure 5.1.

Optimisation of the working dilution for each antibody was performed initially to get the best signal from LAD2 cells (Table 5.1). Testing of different antibodies for each protease was performed as an additional step for validating their specificity. The secondary antibodies included anti-mouse Alexa Fluor 555 (1/1000 dilution) and anti-rabbit Alexa Fluor 647 (1/1000 dilution). Initially, LAD2 cells were stained for a single marker, then staining was performed for three markers with a combination of antibodies specific for tryptase, chymase and CPA3, or DPPI, chymase and CPA3, or tryptase, DPPI and CPA3. Interactions of subcellular protein markers were analysed using co-localization plugin (Coloc 2) of Fiji (216) to implement and perform pixel intensity correlation over space methods of Pearson's coeffiecient (237), Manders (238) and Costes tests (239).

6.2.2 Immunofluorescence staining with avidin-sulforhodamine

Avidin-sulforhodamine 101 (Avidin-Texas red conjugate) is a highly cationic glycoprotein that can specifically bind to mast cell granules (240). Therefore, LAD2 cells were labelled with avidin-sulforhodamine to identify mast cell granules and also with DPPI combined with two other mast cell proteases to specifically locate DPPI within the cell. Initially the concentration of avidin-sulforhodamine was optimised by staining LAD2 cells with 5, 10 and 20 µg/ml. Then, staining was performed using a combination of DPPI, chymase and CPA3 antibodies with avidin-sulforhodamine at the optimised concentration. Coloc2 plugin in Fiji was used to analyse co-localization between markers.

6.2.3 Fractionation by differential pelleting

Fractionation using density gradient centrifugation was employed on LAD2 cells to determine the subcellular localization of DPPI and its relationship with other mast cell proteases. A protocol for fractionation of HeLa cells (241) was adopted and optimised to be applied on LAD2 cells (Figure 5.2). The cells were harvested as mentioned in Section 2.4.2, adjusted to 10 x 10^6 cells and incubated with 5 ml of ice-cold hypotonic lysis buffer (25 mM Tris hydrochloride, pH 7.5, 50 mM sucrose, 0.5 mM magnesium chloride, 0.2 mM EGTA) for 5 minutes. This was followed by centrifugation at 270 g for 10 minutes to pellet the crude cell mixture, which was resuspended with 4 ml pre-chilled hypotonic lysis buffer. The cell mixture was transferred into a Dounce homogeniser (Sartorius, Goettingen, Germany) and homogenised with 30 strokes. Then, sucrose concentration was adjusted to 250 mM with ice-cold hypertonic sucrose buffer (2.5 M sucrose, 25 mM Tris pH 7.5, 0.5 mM magnesium chloride, 0.2 mM EGTA) to generate a density gradient and help separation of subcellular organelles during high speed centrifugation.

Crude cell homogenate was centrifuged over a range of centrifugation force (listed in Figure 5.2) using an ultracentrifuge (Beckman Coulter, Indiana, Unites states) with TLA-110 rotor (Beckman Coulter). After each centrifugation, the supernatant was transferred into a new ultracentrifuge tube, and the pellets were dissolved in SDS buffer (2.5% SDS, 50 mM Tris pH 8.1) and heated for 5 minutes at 72 °C. The supernatant collected after the last centrifugation run (78,400 g for 30 minutes) was considered to be the cytosolic fraction. The protein concentration for each fraction was determined using the BCA assay as described in Section 2.3. The presence of immunoreactivity of tryptase, chymase, CPA3 and DPPI was determined in the seven fractions by dot blot following the main steps described in Section 2.4.

Table 6.1 Dilutions for the primary antibodies used in immunocytochemistry.

Mast cell protease	Antibody	Dilution
Tryptase	JJ3	1/2000
	AA5	1/500
CPA3	CA5-AF* 488	1/300
	CA2	1/2000
Chymase	CC1	1/500
DPPI	Rabbit polyclonal anti-DDPI	1/500

* AF = Alexa Fluor

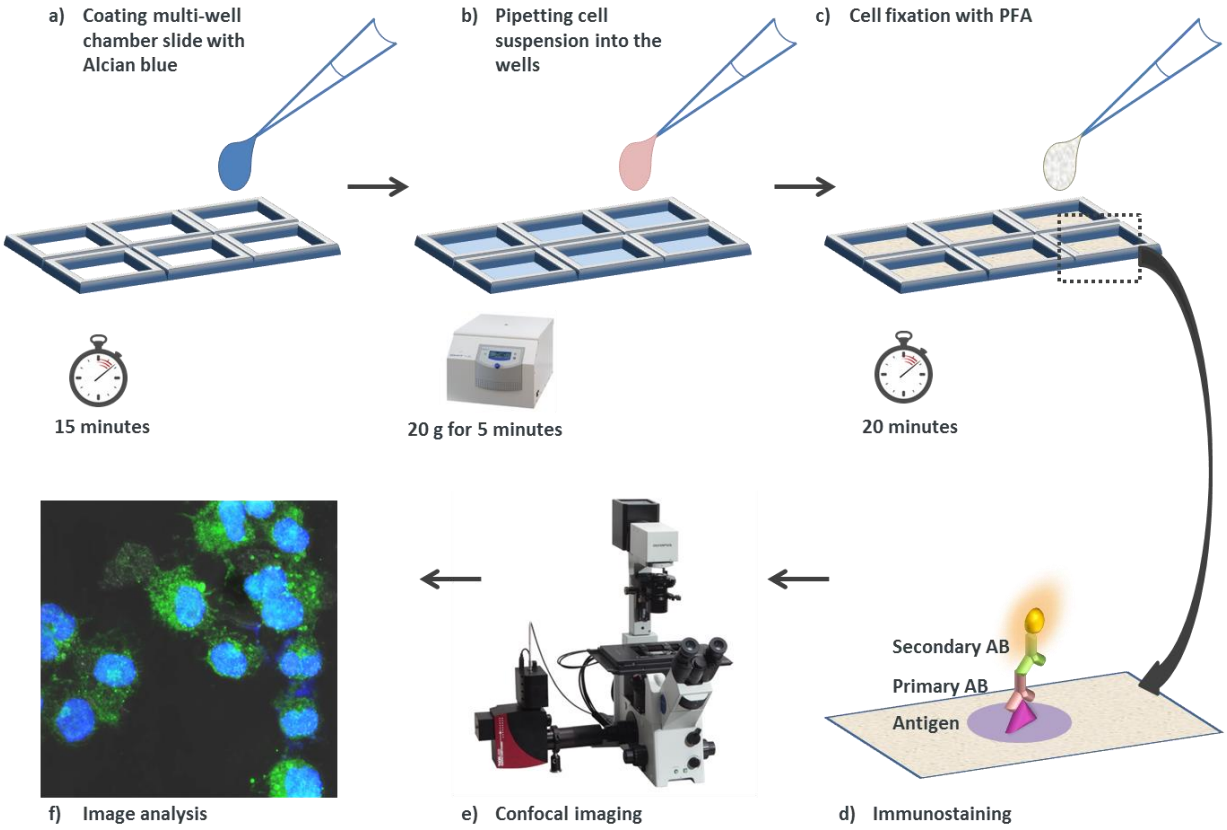


Figure 6.1 Schematic diagram of steps in immunocytochemical analysis of LAD2 cells.

(a) Alcian blue solution was applied to multi-well chamber slides and incubated for at least 15 minutes before (b) LAD2 cell suspension was applied and centrifuged at low speed to deposit the cells into the surface of the slide. (c) The cells were fixed with 2% paraformaldehyde (PFA) for 20 minutes and further processing including permeabilisation and blocking was performed after which (d) the cells were immunostained with the primary and secondary antibodies. (e) A confocal microscope was used for imaging and (f) images were analysed using LAS X Core software and Fiji.

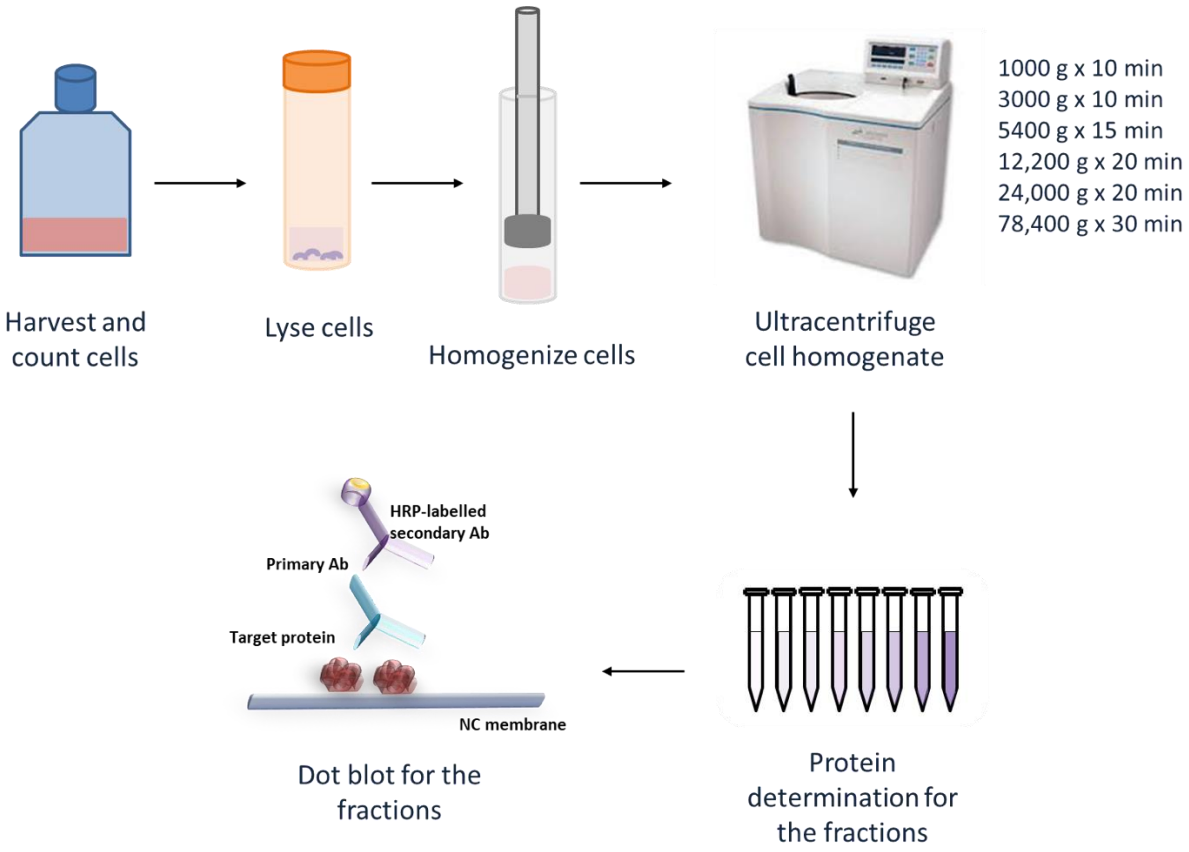


Figure 6.2 Schematic illustration of LAD2 cell fractionation.

The cells were harvested and adjusted to 10 x 10^6 cells, which were lysed using hypotonic lysis buffer. The crude cell mixture was homogenised mechanically and, after restoring sucrose concentration, centrifuged at selected forces and times to separate subcellular organelles. Protein content for the resulted fractions was determined and immunoreactivity of each fraction to antibodies specific for mast cell proteases was detected by dot blot. NC= nitrocellulose, Ab= antibody.

6.3 Results

6.3.1 Immunofluorescence single staining of LAD2 cells

When LAD2 cells were added onto Alcian blue pre-coated slides without prior centrifugation or washing (modified protocol) and immunostained with tryptase, their structure appeared to be intact with intracellular tryptase staining could be seen (Figure 5.3a). In contrast, most LAD2 cells which were centrifuged and washed before their adherence to the slides (original protocol) appeared to be contracted or degranulated and showed minimum intracellular tryptase staining (Figure 5.3b).

Using tryptase specific antibodies in immunostaining of LAD2 cells showed a predominant cytoplasmic distribution within the cells and the presence of tryptase in all the cells (Figure 5.4a). Surprisingly, there was nuclear staining apparent in some cells. Taking images of those cells at different focal planes (Z stack) confirmed the presence of nuclear tryptase staining (Figure 5.5) and allowed exclusion of granular material being present on top of the nucleus during imaging at a defined focal plane.

When chymase immunostaining was applied, majority of LAD2 cells showed a cytoplasmic distribution of the marker but some cells were lacking chymase (Figure 5.4b). CPA3 immunostaining of LAD2 cells mirrored that of chymase with some cells were having less CPA3 than the others (Figure 5.4c). When DPPI specific antibody was used in immunostaining of LAD2 cells, the fluorescent signal was observed in the cytoplasm of many cells. Interestingly, DPPI was not only found in the cytoplasm but also seen in the nuclei of some cells (Figure 5.4d). Z stack images of LAD2 cells labelled with DPPI antibodies showed persistent staining through the nuclear planes (Figure 5.6).

6.3.2 Immunofluorescence multi-staining of LAD2 cells

When a combination of antibodies specific for tryptase, chymase and CPA3 was applied, LAD2 cells showed co-localization of the three markers but it was more apparent between chymase and CPA3 (Figure 5.7a). Some cells were predominantly stained for tryptase compared to the staining with the other two markers. When interactions of pixel intensities for tryptase, chymase and CPA3 were analysed, a strong correlation between tryptase and chymase (Manders' tM1 = 0.8 ± 0.17, Manders' tM2 = 0.7 ± 0.23), a moderate correlation between tryptase and CPA3 (Manders'

tM1 = 0.8 ± 0.14, Manders' tM2 = 0.7 ± 0.1) and a very strong correlation between chymase and CPA3 (Manders' tM1 = 0.8 ± 0.2, Manders' tM2 = 0.8 ± 0.2) were seen (Figure 5.7b).

Immunofluorescence staining of LAD2 cells with DPPI, chymase and CPA3 antibodies was associated with a partial co-localization of the three immunolabels and variable staining intensity for each of them (Figure 5.8a). Analysis of the co-localization of pixel intensities for DPPI, chymase and CPA3 showed moderate correlations between DPPI compared with each chymase (Manders' tM1 = 0.6 ± 0.2, Manders' tM2 = 0.4 ± 0.34) and CPA3 (Manders' tM1 = 0.6 ± 0.3, Manders' tM2 = 0.6 ± 0.3). As seen with the previous staining preparation, chymase correlated strongly with CPA3 (Manders' tM1 = 0.95 ± 0.1, Manders' tM2 = 0.6 ± 0.2; Figure 5.8b).

A partial co-localization between tryptase and DPPI was also seen when the cells were labelled with specific antibodies (Figure 5.9a). When positional relation of pixel intensities for tryptase and DPPI was analysed, a moderate correlation (Manders' tM1 = 0.9 ± 0.1, Manders' tM2 = 0.7 ± 0.1) was seen (Figure 5.9b).

6.3.3 Immunofluorescence staining of LAD2 cells with avidin-sulforhodamine

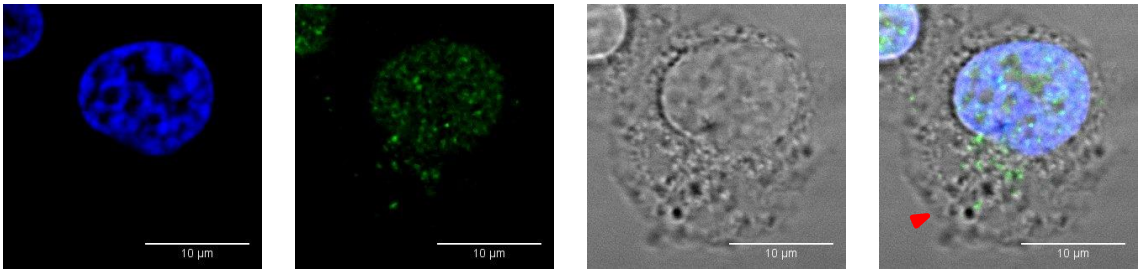
When three different concentrations of avidin-sulforhodamine were used to immunostain LAD2 cells, a concentration of 10 μg/ml was sufficient to label the granules with minimal background staining (Figure 5.10b). Using a low concentration of avidin-sulforhodamine was associated with poor staining of LAD2 granules (Figure 5.10a), whereas immunostaining with a high concentration resulted in background and non-specific staining (Figure 5.10c).

Immunofluorescence staining of LAD2 cells with avidin-sulforhodamine combined with specific antibodies for DPPI, chymase, and CPA3 indicated the presence of co-localization between avidin-sulforhodamine and DPPI. This co-localization was comparable to that observed between avidin-sulforhodamine with chymase or with CPA3 (Figure 5.11a). Analysis of interactions of pixel intensities for avidin-sulforhodamine with the three markers showed strong correlation of avidin-sulforhodamine with DPPI (Manders' tM1 = 0.6 ± 0.1, Manders' tM2 = 0.4 ± 0.1), CPA3 (Manders' tM1 = 0.6 ± 0.2, Manders' tM2 = 0.6 ± 0.2) and chymase (Manders' tM1 = 0.5 ± 0.3, Manders' tM2 = 0.5 ± 0.2; Figure 5.11b)

6.3.4 Fractionation of LAD2 cellular content by differential pelleting

When LAD2 cells were centrifuged over increased gradient of centrifugational force, seven fractions were collected. These fractions included nuclear (pellet collected at 1000 g centrifugation), organellar (pellets collected between 3000 and 24,000 g centrifugation) and cytosolic (supernatant collected at 78,400 g centrifugation) fractions based on the classification described for fractionation of HeLa cells (241). When the immunoreactivity to tryptase, chymase, CPA3 and DPPI antibodies was analysed by dot blot, reactions of nuclear fraction with the four antibodies and cytosolic fraction with with tryptase and DPPI antibodies were observed. In addition, weak reactions were seen with organellar fractions to DPPI antibody and the signal was too poor to be observed against tryptase, chymase or CPA3 antibodies (Figure 5.12).

(a) Modified protocol



(b) Original protocol

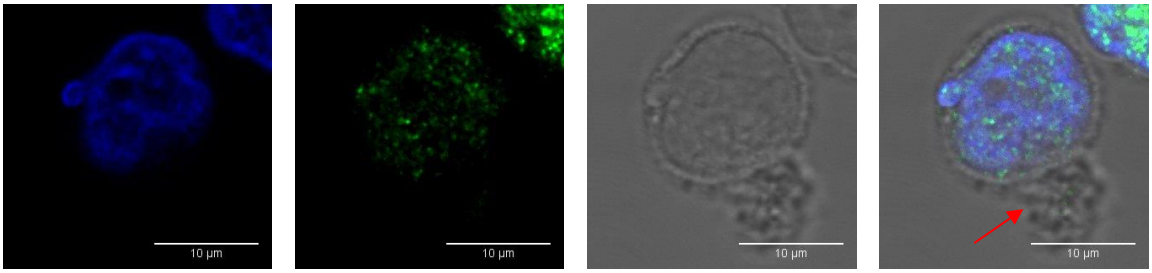


Figure 6.3 Optimising the technique for fixing LAD2 cells on slides for confocal imaging.

(a) Preserved structure of LAD2 cell (arrowhead indicates intact cellular boundaries) with intracellular tryptase staining (green). (b) Apparent degranulation (indicated by the arrow) and lacking cytoplasmic tryptase staining. DAPI (blue) was used as nuclear counterstain. Images are representative of three independent experiments. Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens. Scale bars = 10 µm

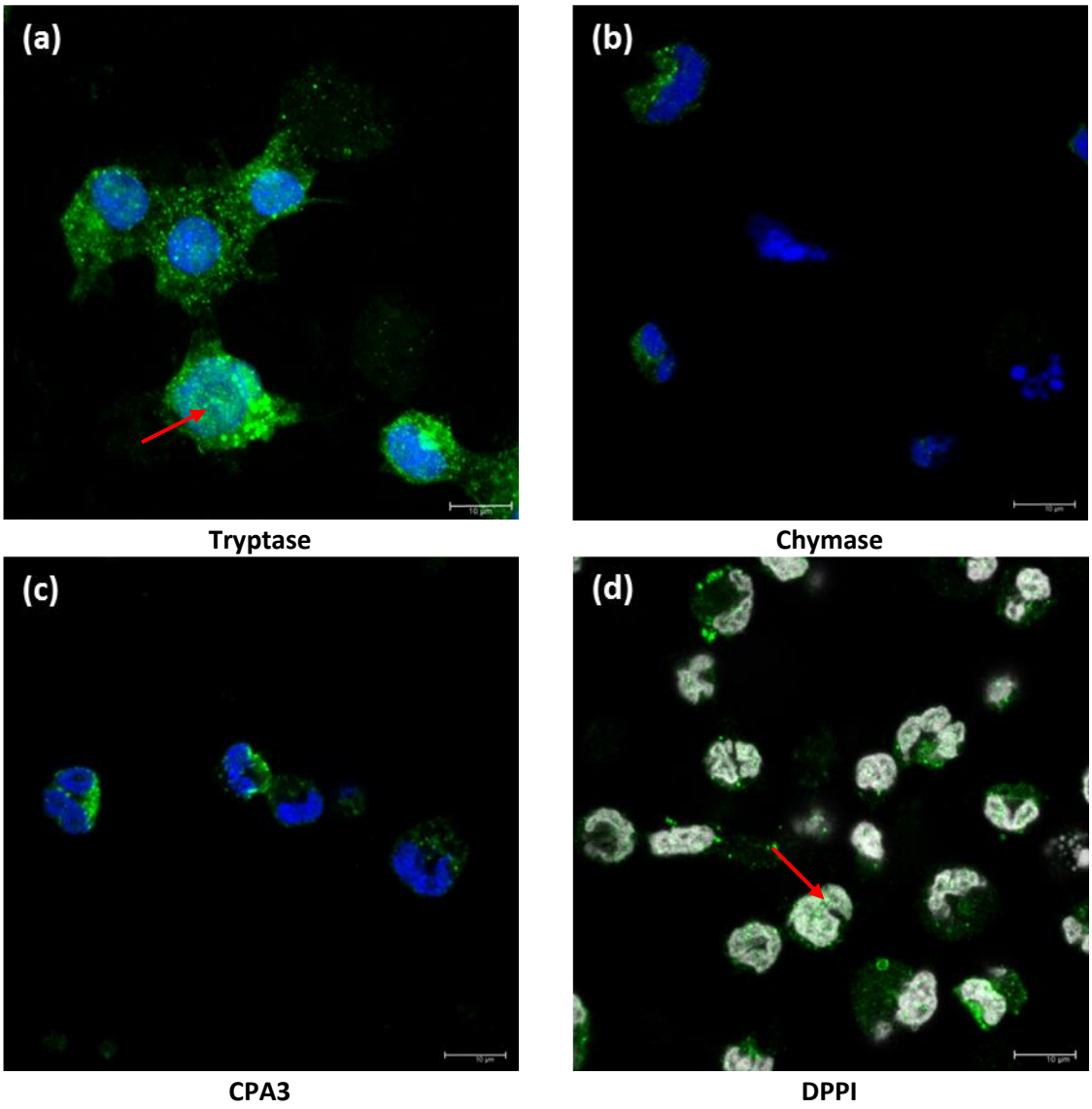


Figure 6.4 Immunofluorescence single staining of LAD2 with (a) tryptase (green), (b) chymase (green), (c) CPA3 (green) and (d) DPPI (green) antibodies.

In addition to the cytoplasmic staining, some nuclear staining was apparent with tryptase and DPPI (indicated by the arrows). DAPI (blue and white) was used as a nuclear counterstain. Images are representative of three independent experiments. Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens. Scale bars = 10 µm.

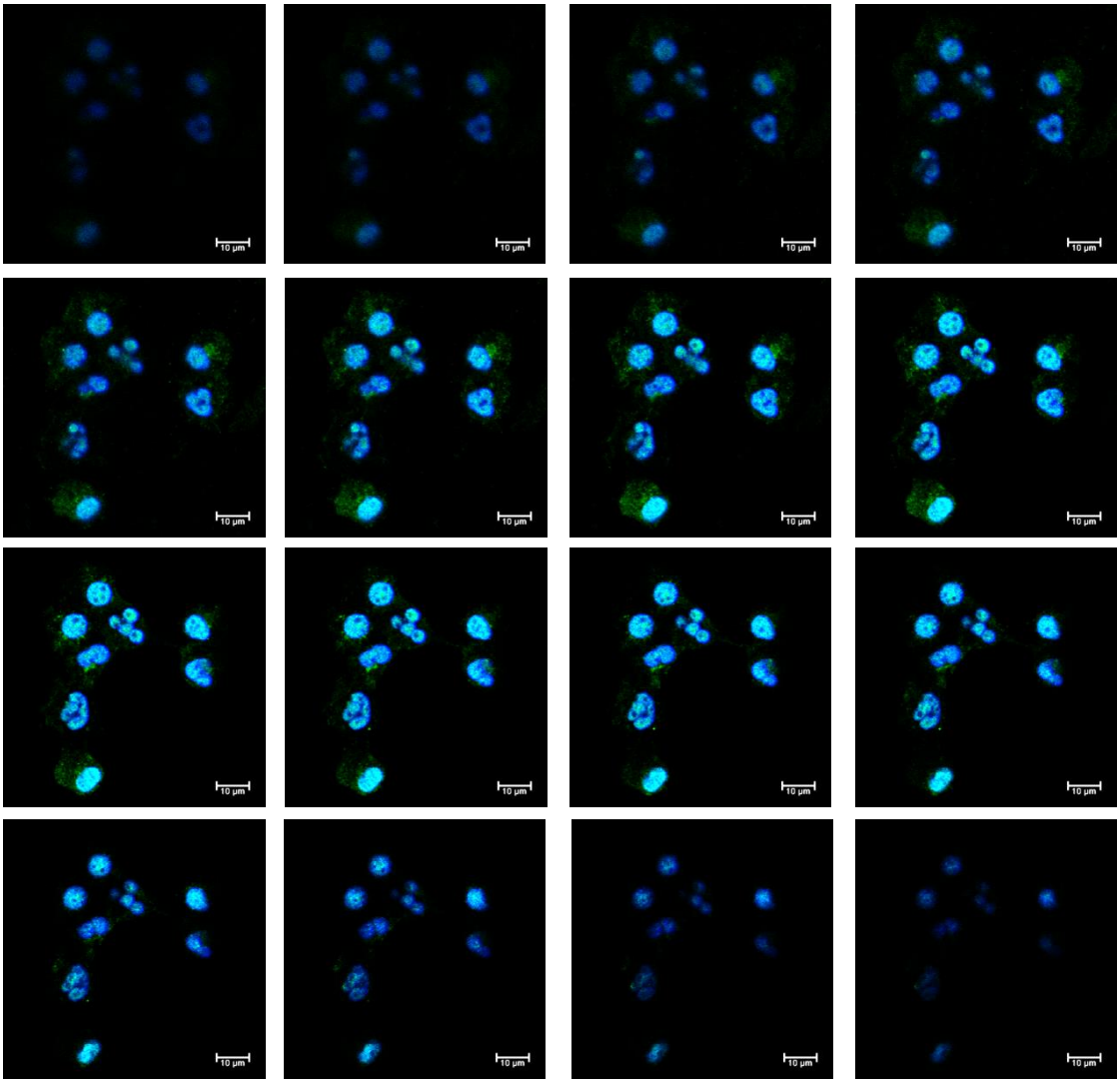


Figure 6.5 Z stack of LAD2 cells immunostained with tryptase (green) antibodies. Images were taken at 0.5 μm intervals. DAPI (blue) was used as a nuclear counterstain. Images are representative of three independent experiments. Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens. Scale bars = 10 μm.

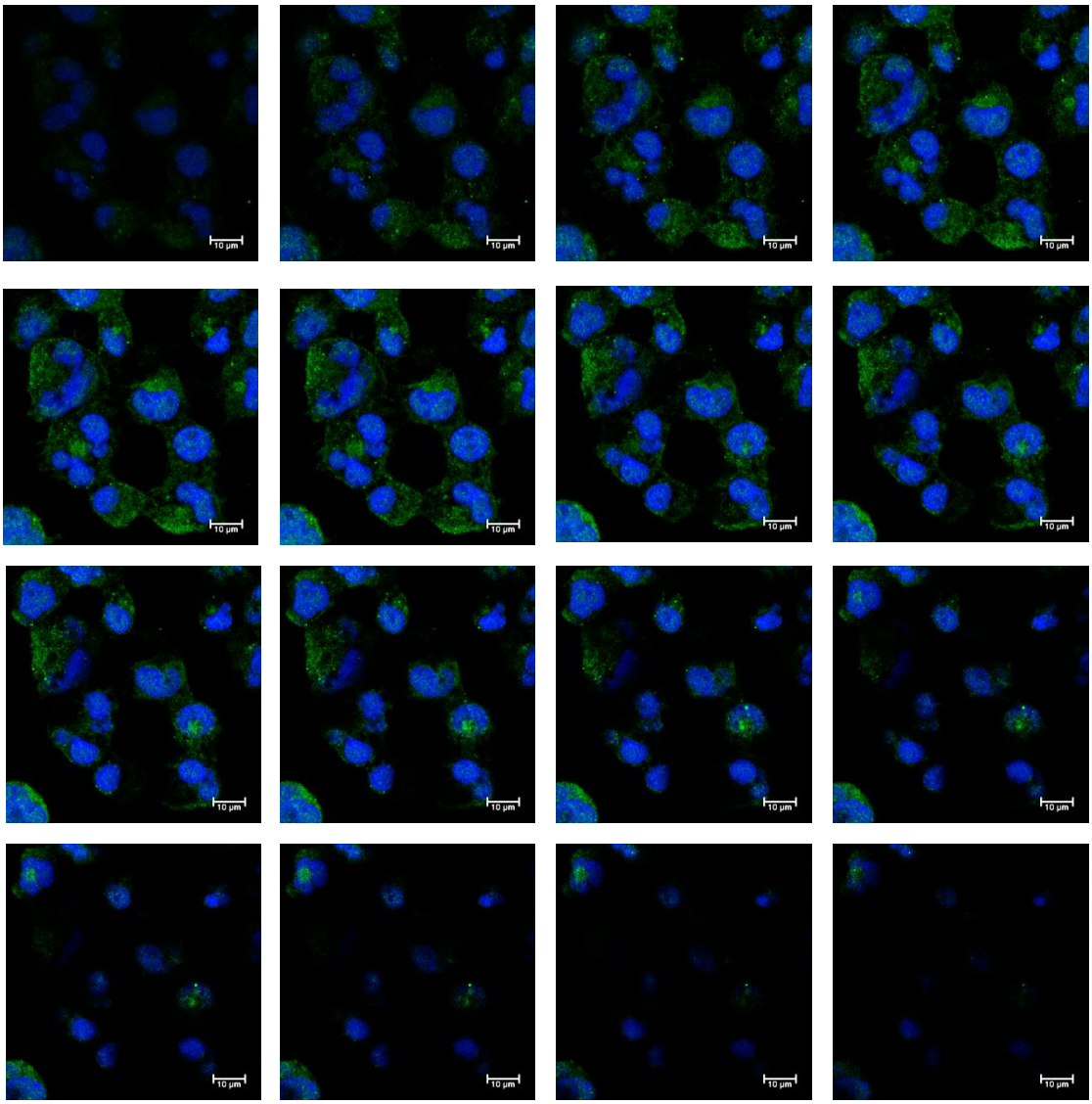
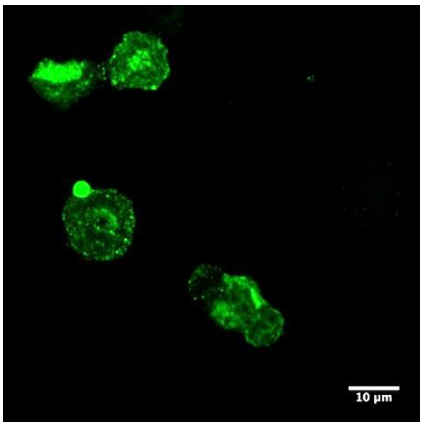
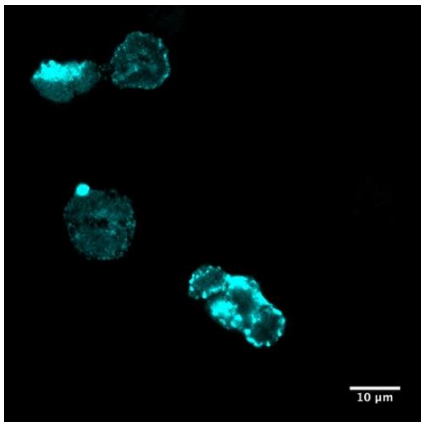


Figure 6.6 Z stack of LAD2 cells immunostained with DPPI (green) antibodies. Images were taken at 0.5 µm intervals. DAPI (blue) was used as a nuclear counterstain. Images are representative of three independent experiments. Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens. Scale bars = 10 µm.

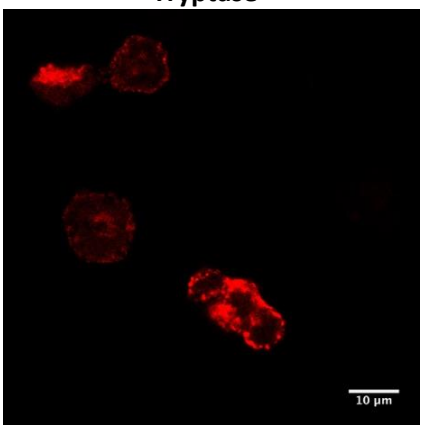
(a)



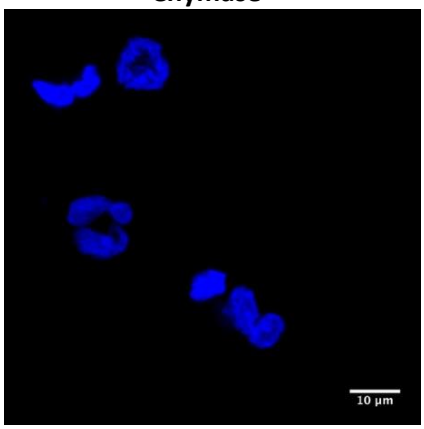
Tryptase



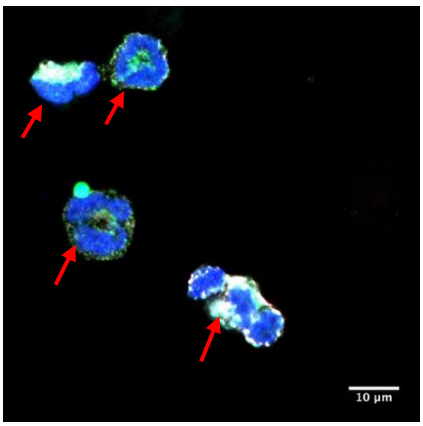
Chymase



CPA3



DAPI



Merge

(b)

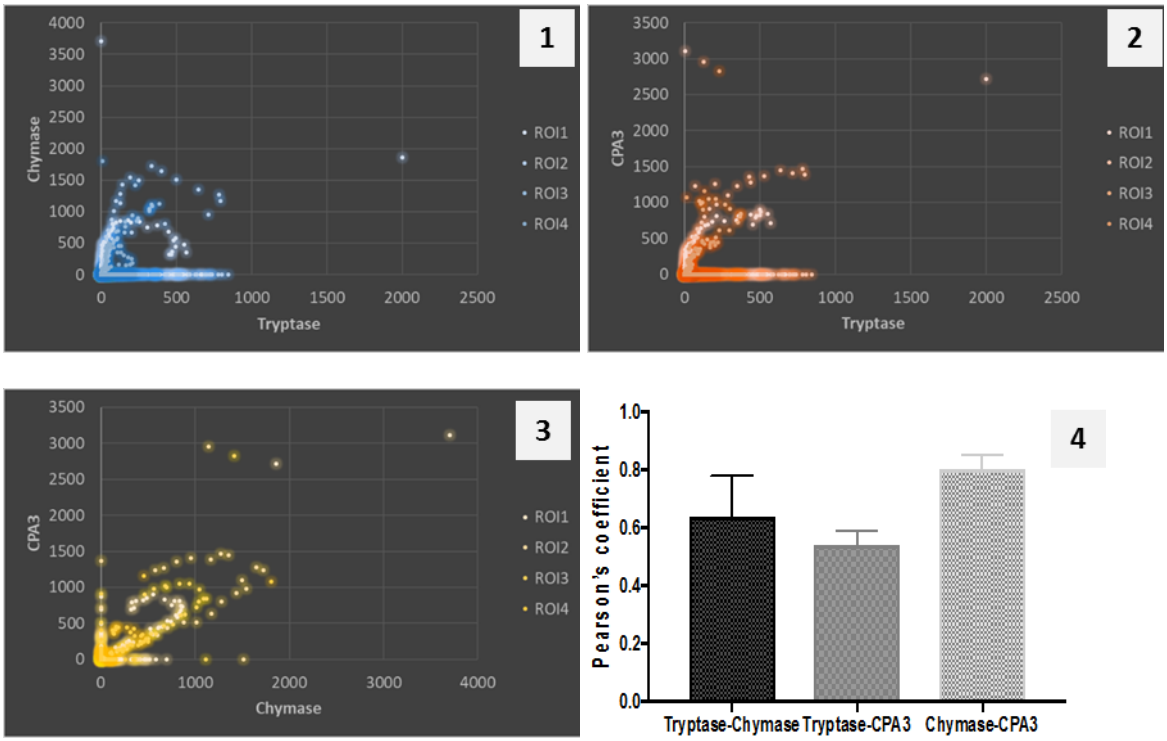
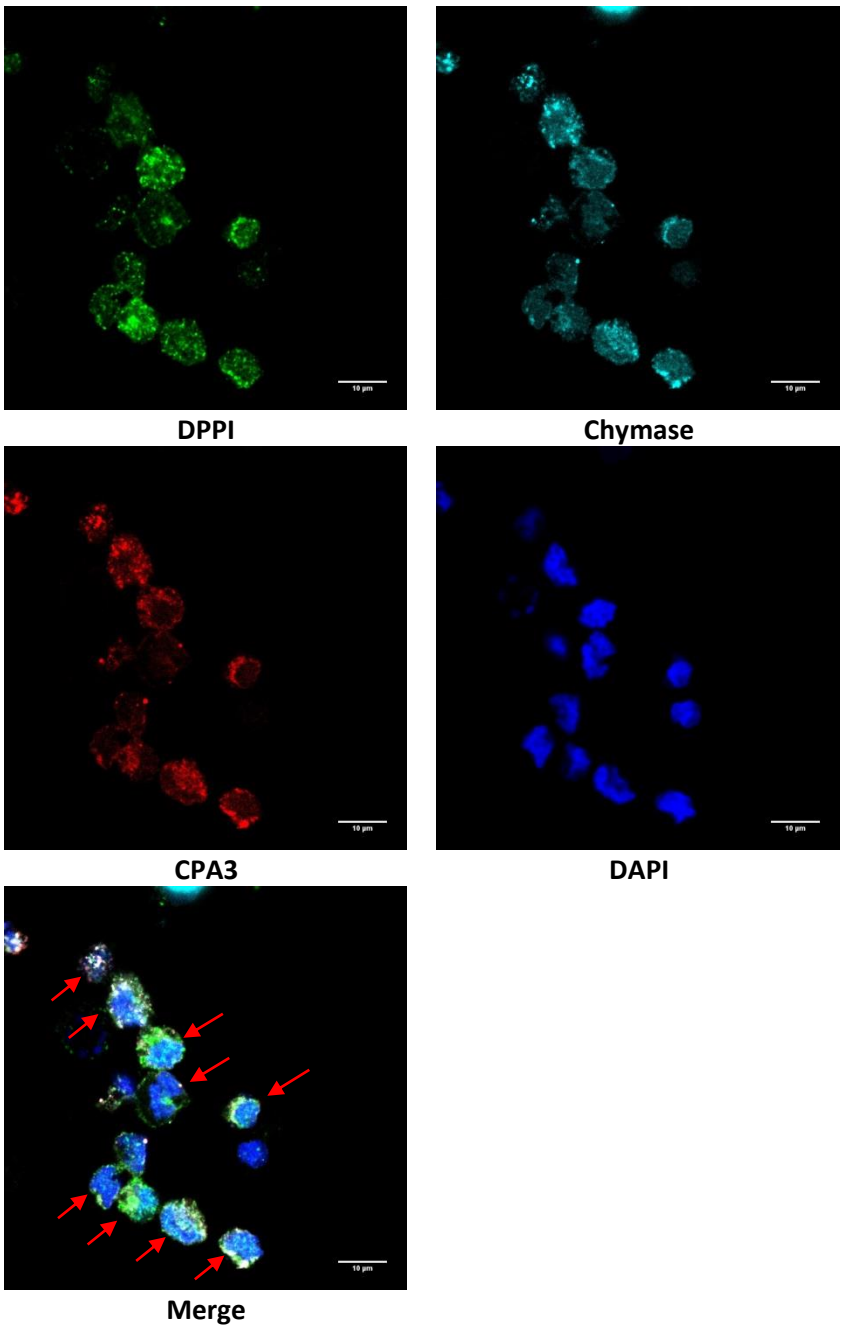


Figure 6.7 Triple immunofluorescence staining of LAD2 cells with tryptase, chymase and CPA3 antibodies.

(a) Confocal images of LAD2 cells immunostained with tryptase (green), chymase (cyan) and CPA3 (red) antibodies. DAPI (blue) was used as a nuclear counterstain. Arrows indicate regions of interest (ROI). (b) Co-localization between the markers in the indicated ROIs is represented in scatter plots of pixel intensities for (1) tryptase and chymase, (2) tryptase and CPA3, and (3) chymase and CPA3 and in (4) a bar chart of Pearson's correlation coefficient. Data is representative of three independent experiments. Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens. Scale bars = 10 µm.

(a)



(b)

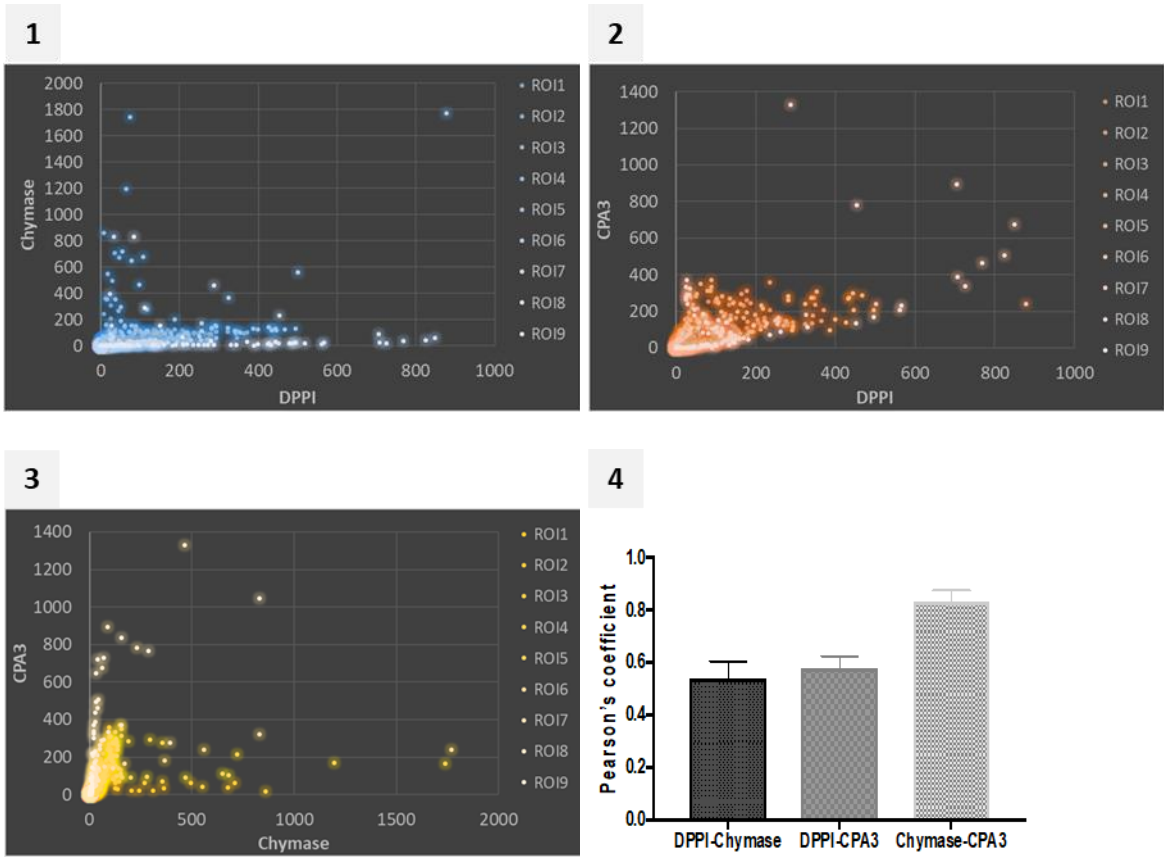
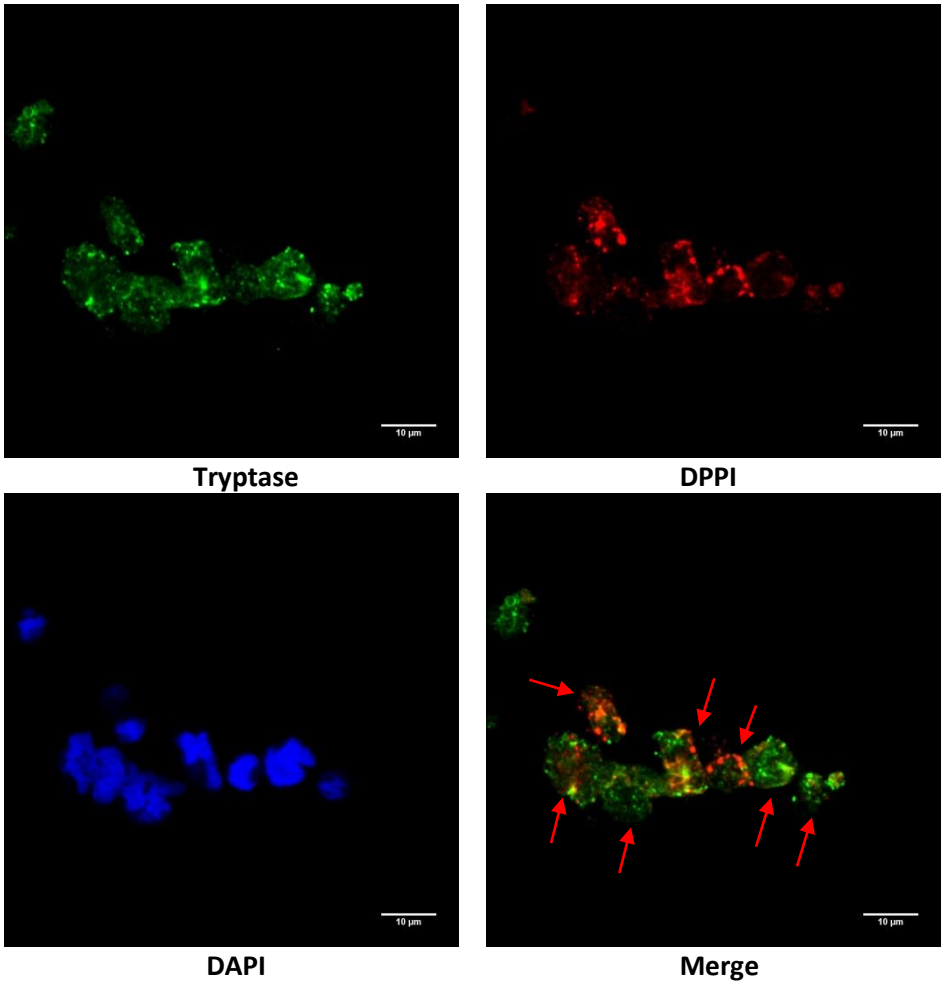


Figure 6.8 Triple immunofluorescence staining of LAD2 cells with DPPI, chymase and CPA3 antibodies.

(a) Confocal images of LAD2 cells immunostained with DPPI (green), chymase (cyan) and CPA3 (red) antibodies. DAPI (blue) was used as a nuclear counterstain. Arrows indicate regions of interest (ROI). (b) Co-localization between the markers in the indicated ROIs is represented in scatter plots of pixel intensities for (1) DPPI and chymase, (2) DPPI and CPA3, and (3) chymase and CPA3 and in (4) a bar chart of Pearson's correlation coefficient. Data is representative of three independent experiments. Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens. Scale bars = 10 µm.

(a)



(b)

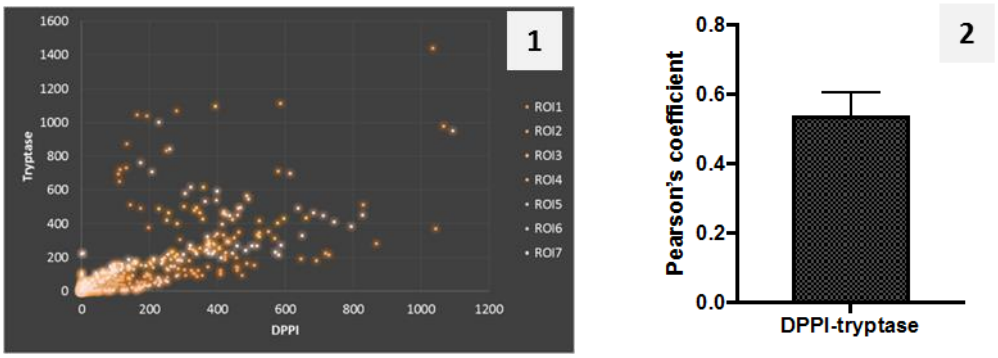


Figure 6.9 Immunofluorescence staining of LAD2 cells with tryptase and DPPI antibodies.

(a) Confocal images of LAD2 cells immunostained with tryptase (green) and DPPI (red) antibodies. DAPI (blue) was used as a nuclear counterstain. Arrows indicate regions of interest (ROI). (b) Analysis of co-localization between the markers in the indicated ROIs represented in (1) a scatter plot of pixel intensities for tryptase and DPPI and (2) a bar chart of Pearson's correlation coefficient. Data is representative of three independent experiments. Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens. Scale bars = 10 µm.

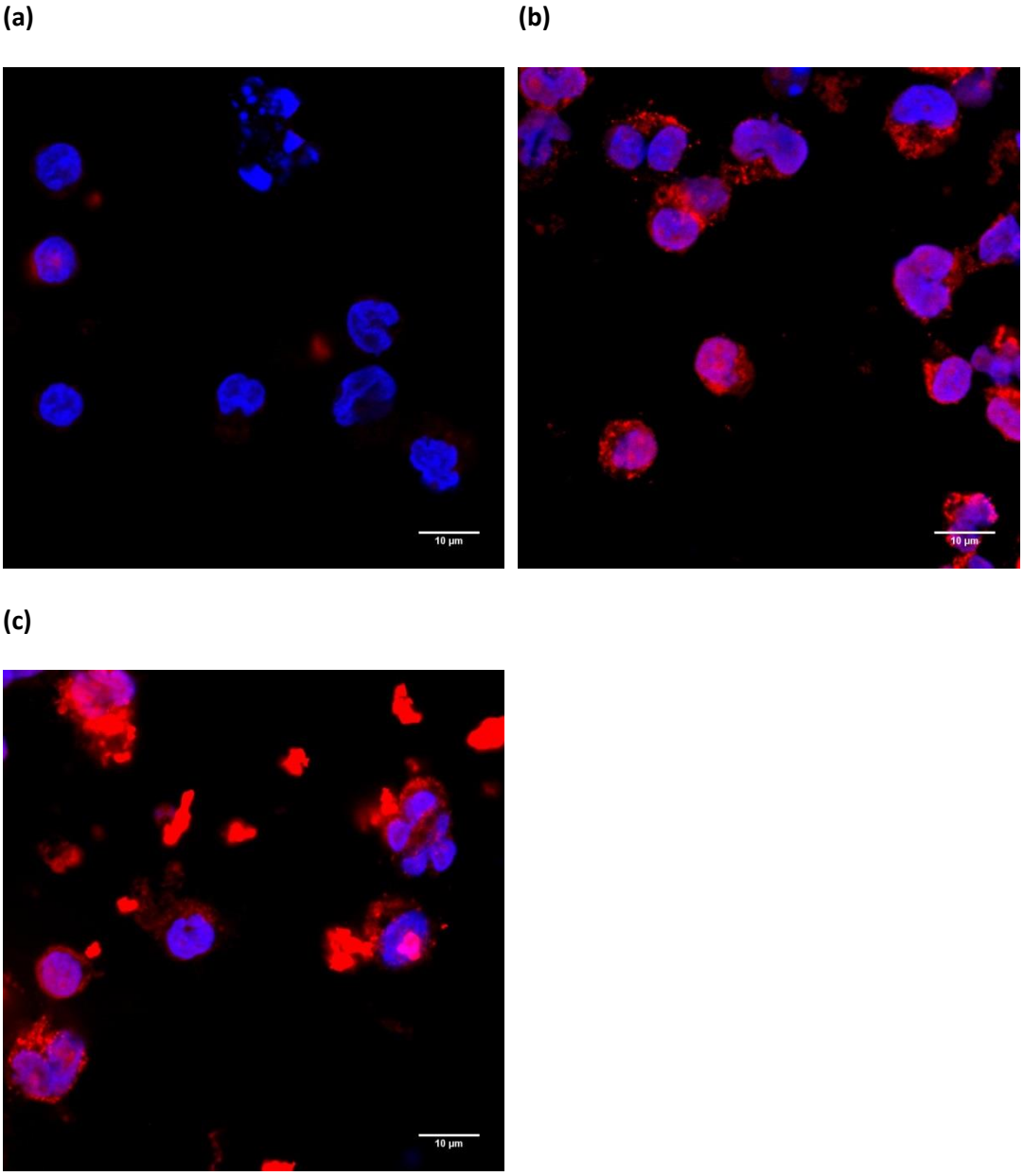
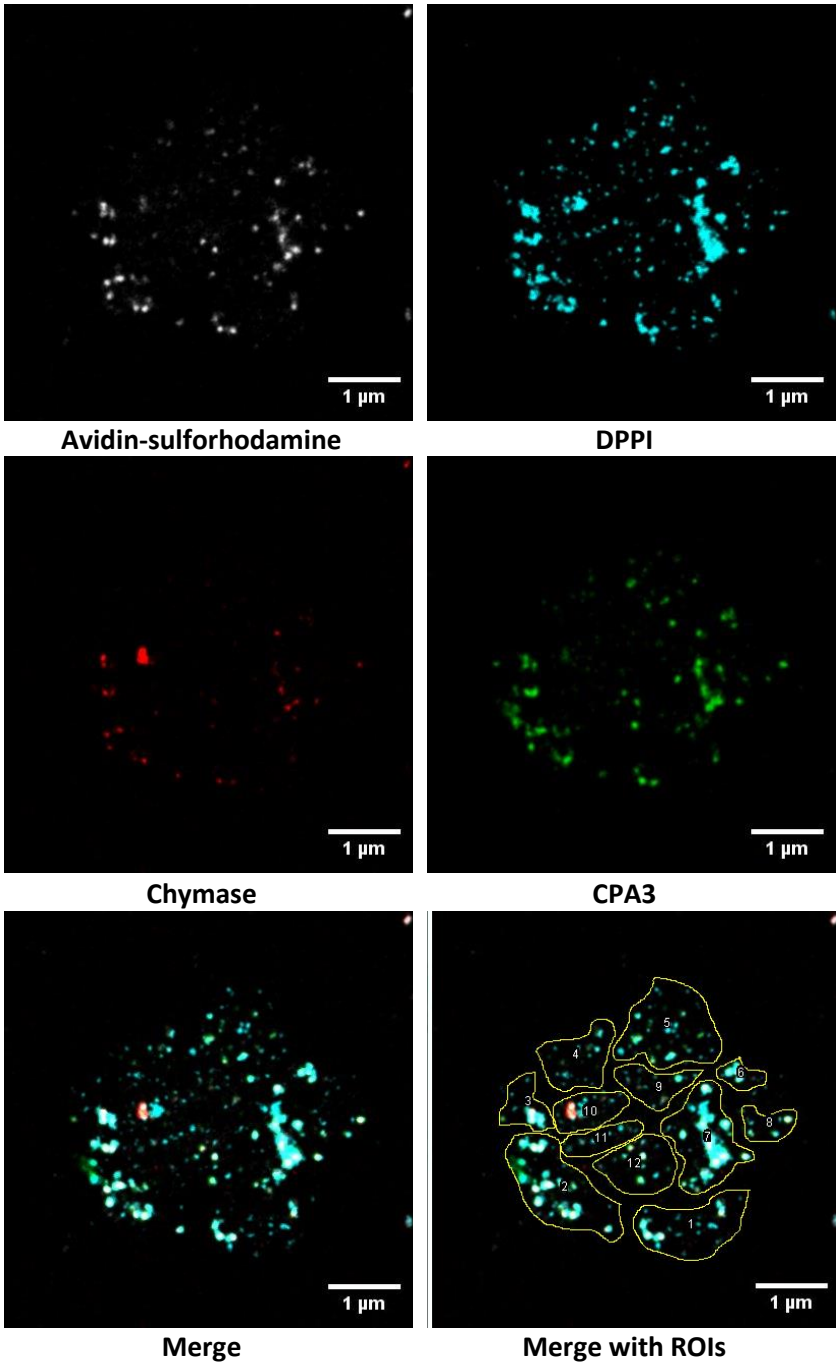


Figure 6.10 Optimising the concentration of avidin-sulforhodamine immunostaining for LAD2 cells.

Staining with (a) 5 µg/ml was not sufficient to label LAD2 cell granules, whereas staining with (c) 20 µg/ml resulted in high background with non-specific staining. LAD2 cells stained with (b) 10 µg/ml avidin-sulforhodamine showed the optimum staining for the granules. Images are representative of three independent experiments. Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens. Scale bars = 10 µm.

(a)


1 µm
Avidin-sulforhodamine
DPPI
Chymase
CPA3
Merge
Merge with ROIs

(b)

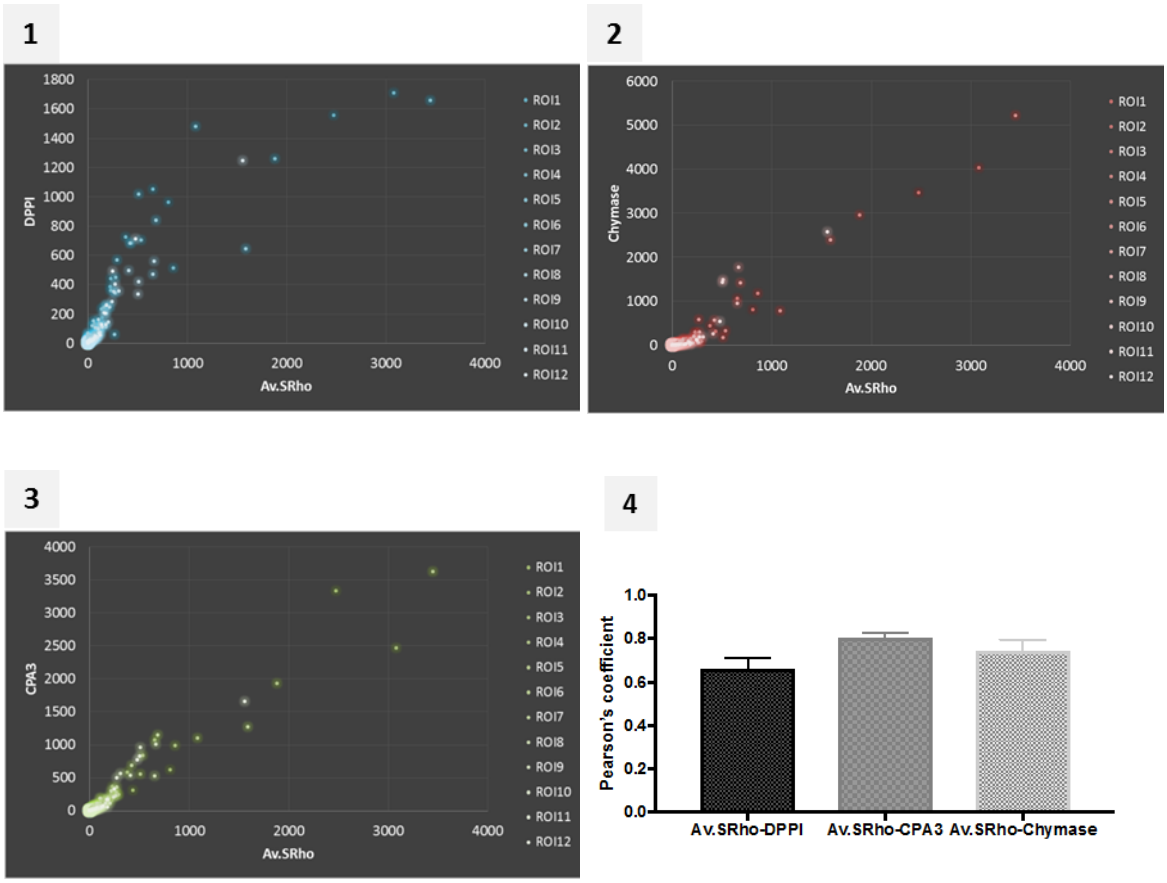


Figure 6.11 Multi-immunofluorescence staining of LAD2 cells with avidin-sulforhodamine, DPPI, chymase, and CPA3.

(a) Confocal images of LAD2 cells immunostained with avidin sulforhodamine (Av.SRho) (white), DPPI (cyan), chymase (red) and CPA3 (green) antibodies. Arrows indicate regions of interest (ROI). (b) Co-localization between the markers in the indicated ROIs is represented in scatter plots of pixel intensities for (1) Av.SRho and DPPI, (2) Av.SRho and chymase, and (3) Av.SRho and CPA3 and in (4) a bar chart of Pearson's correlation coefficient. Images are representative of three independent experiments. Images were captured using stimulated emission depletion (STED) microscopy technique. Scale bars = 1 µm.

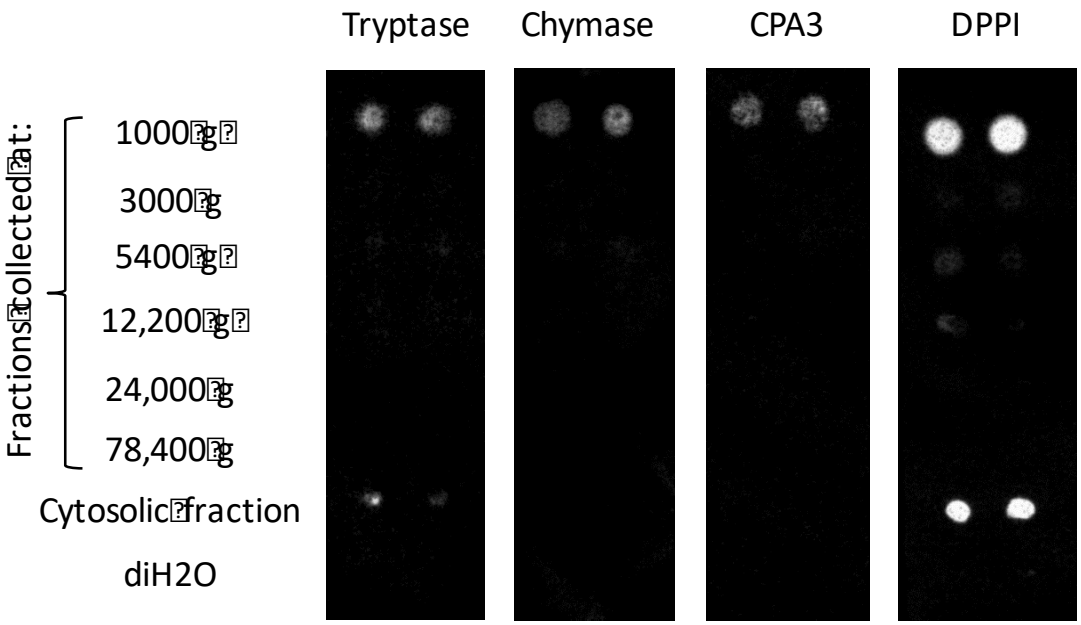


Figure 6.12 Dot blot analysis of subcellular fractions of LAD2 cells.

Fractions collected after ultracetrifugation at wide range of centrifugal forces were analysed for their reactivity to specific antibodies of mast cell proteases (tryptase, chymase, CPA3 and DPPI). Ultrapure water was used to exclude non-specific binding of the antibodies.

Chapter 7: Discussion

Following development and optimisation of assays for detecting mast cell mediators, we have been able to apply them to define patterns of disease in patients with drug and food allergy and assess their diagnostic utility. The finding that baseline levels of CPA3 may be closely associated with the severity of historical allergic reactions to both injected and ingested allergens, suggests that measurement of serum levels of this protease may be of value in predicting the severity of future allergic reactions. We have also identified and replicated four novel endotypes of allergic reactions through the analysis of the clinical features of the patients who have allergic reactions in combination with the measurements of mast cell proteases and pro-inflammatory cytokines by the application of TDA. Our studies on LAD2 mast cell line have shown an inhibitory effect of *S. aureus* on the release and expression of mast cell mediators. Subcellular investigations of LAD2 cells have indicated the presence of DPPI in association with other mast cell proteases, suggesting that DPPI can be released during mast cell degranulation. Here we discuss the context and implications of our findings.

7.1 Development and optimisation of assays for mast cell proteases

As assays for the mast cell proteases studied presented particular challenges, assay components were prepared in the laboratory rather than being available in a standardised form from commercial suppliers. The CPA3 sandwich ELISA was successfully optimised and validated for measurement of levels in patient serum samples. Similarly, a sensitive assay for tryptase was developed using reagents prepared in-house and applied to measure tryptase levels in serum. On the other hand, a sandwich ELISA for chymase and for DPPI did not prove possible within the time available.

Since our assays were dependent on reagents that have had to be developed in our laboratory, there has been a need for careful standardisation and optimisation. The performance characteristics for each reagent were investigated in accordance with the guidelines of the Food and Drug Administration (242). A sandwich ELISA for tryptase using polyclonal and monoclonal antibodies was employed, but for assay consistency we wanted to develop a monoclonal antibody-based assay. All monoclonal antibodies tested bound to tryptase in an indirect ELISA. However, when two of the monoclonal antibodies (AA8 and AA9) were employed as capture antibodies in a sandwich ELISA with monoclonal antibody AA5 as detecting antibody, there was no detection of tryptase. This could be due to AA8 and AA9 binding to similar epitopes as AA5.

Therefore, we decided to focus on the development of new polyclonal antibodies that could be combined with our monoclonal AA5 antibody in a sandwich ELISA.

Following immunisation of three rabbits and screening of the antisera collected, the specificity of antibody binding to tryptase was confirmed by binding to recombinant tryptase in an indirect ELISA. Being able to include a recombinant protease as a standard in the assay would improve consistency of the supply. It would also avoid biohazards and ethical issues related to the use of human tissues in the assay. Tests for tryptase have not to date been developed with recombinant tryptase as a standard, but our assay has shown the potential for development of a sandwich ELISA with the recombinant protein. The assay applied to clinical samples in the present study employed native tryptase as the standard. With production of sufficient quantities of recombinant tryptase we anticipate that the assay could be adjusted accordingly.

The sandwich ELISA for tryptase as optimised to measure levels in serum samples had a lower limit of detection of 0.38 ng/ml (compared to a 1 ng/ml lower limit of detection for the only diagnostic assay that is commercially available; ImmunoCAP Tryptase assay, Phadia). This sensitivity should facilitate detection of small increases in tryptase levels. When a range of serum samples were serially diluted and spiked with known amounts of tryptase, a 1/5 dilution was found to be the optimal dilution for most serum samples. Undiluted serum or serum samples which had been little diluted were avoided because of the limited availability of some serum samples and to overcome the interference caused by other proteins present in the serum. However, lower dilutions had to be employed in the assay for a few serum samples with undetectable levels at 1/5 dilutions.

In the current study we investigated the best conditions for the sandwich ELISA for CPA3 and found that a 1/5 serum dilution was optimal for measurement of levels in most serum samples. The assay was sensitive enough to detect levels as low as 0.23 ng/ml. This assay together with that of tryptase were employed in generating the data presented in chapter 4. As with tryptase, interference in the assay was less evident when diluting serum samples to 1/5 as indicated by the recovery of a known concentration of purified CPA3 in the spiked sample compared to unspiked control samples.

Despite extensive attempts, we have not so far been able to develop sandwich ELISAs for either chymase or DPPI. Though the monoclonal antibodies were stored at -20ºC, several cycles of freeze-thawing might have affected the stability of the antibodies. Antibodies of the IgM class such as monoclonal antibodies CC4, DD1 and DD3 may be particularly susceptible to loss of antigen-binding capacity after repeated freezing and thawing. Purification of the antibodies involved filtration and dialysis steps which might have also affected the integrity of the antibodies

rendering them unable to bind to the proteins of interest. Antibodies tend to stick to the membranes of dialysis tubing which can reduce the final quantity of the antibodies. However, using antibody supernatants rather than purified antibodies did not improve reactivity to the proteases. This might be attributed to changes in the stability of the hybridoma cells including mutations in the genes for the antibody (243).

In seeking to develop an assay for chymase, we investigated the binding of antibodies to various chymase preparations but only one of the monoclonal antibodies tested (CC1) reacted. A commercially available polyclonal antibody specific for chymase failed to cooperate with CC1 antibody in sandwich ELISA. An explanation may be that CC1 and the polyclonal anti-chymase antibodies could have changed the structure of the chymase molecule leading to loss of the epitope and failure of the sandwich ELISA. However, we think that these antibodies may work best as detection antibodies and we need to develop or find alternatives to act as capture antibodies.

In seeking to develop the assay for DPPI, the monoclonal antibodies were not able to recognise recombinant DPPI. As the recombinant protein has less a compact structure and consequently fewer binding sites compared to the three-dimensional structure of a native protein (244, 245), further studies are required to investigate the binding of these antibodies with native DPPI.

At this point, assay for measurement of levels of tryptase in serum has been successfully developed and optimised. In parallel, CPA3 assay has also been best optimised for determination of serum levels. Therefore, the next stage comprised of application of these assays on clinical samples to determine the diagnostic and/or predictive value of tryptase and CPA3 measurements.

7.2 Multidimensional endotypes in patients with allergic reactions revealed by topological data analysis of clinical and immunological parameters

We have found that patients with more severe historical reactions have high serum levels of CPA3 compared to healthy individuals. We have determined a role of baseline CPA3 levels in predicting the severity of allergic reactions to drugs or food. We have also found that patients who had one or more concomitant atopic illnesses have increased levels of CPA3. Furthermore, we have found that the severity of allergic reactions experienced is strongly associated with the presence of concomitant atopic illnesses. Therefore, measurement of CPA3 serum levels and assessment of the atopic status in patients with allergic reactions can give an indication on the risk of

anaphylaxis in the future and may provide insights into new approaches for diagnosis and management.

We have identified four novel clinico-immunological endotypes in two distinct populations of allergic patients by applying TDA on large size data sets. The structure of multidimensional endotypes or clusters was mainly derived from the serum levels of CPA3 and IL-13: Cluster I high CPA3 and IL-13 serum levels, cluster II high CPA3 and low IL-13 serum levels, cluster III low CPA3 and IL-13 serum levels, and cluster IV low CPA3 and high IL-13 serum levels. This analytical approach showed that clusters I and II included the majority of patients with moderate or severe historical reactions to drugs or food, whereas subjects with milder allergic reactions were distributed within cluster III and IV. Using TDA as exploration tool to investigate large data set has been reported in many studies. Hinks and colleagues have reported six novel endotypes of asthma by analysing 103 clinical, physiological and inflammatory parameters using TDA (235). Three new phenotypes of hip osteoarthritis have been reported through multidimensional analysis of biochemical and biomechanical biomarkers of 102 subjects (221). Useful insights have been provided for diagnosis and treatment of ovarian (223) and oral cancers (222) through the application of topological analysis. TDA has been also employed to analyse anatomical magnetic resonance imaging (MRI) data of 42 young boys with fragile X syndrome (224). The study has reported two distinct subgroups of fragile X syndromes that can be used to predict outcomes and design targeted therapies. The present study demonstrates the associations between specific biomarkers and clinical characteristics of patients with allergic reactions to drugs or food and applies topological analytic technique to identify multidimensional endotypes of allergic reactions.

Analysis of the clinical and biochemical parameters in two large geographically distinct cohorts using a statistically unbiased approach is a major strength to the study by including an external validation of the derivation cohort rather than using single data set. The use of a validation cohort has been reported in several studies to help ensure that the findings were due to true biological variation and not due to experimental artefact. In a US-based retrospective study of patients admitted to the medical service over six years with the aim of developing a prediction score for *Clostridium difficile* infection, the test was validated the test by dividing the data set into two and the analysis run separately (246). Validation of a prediction model for 30-day mortality in adult patients admitted with lower gastrointestinal bleeding to an academic medical centre in Chicago over seven years has been reported by randomly splitting the cohort into derivation and validation cohorts (247). This internal validation can be subjected to selective reporting and publication bias, which may provide insufficient and weak replication. In contrast, external validation involves a separate analysis of two different data sets. For example, six novel endotypes of asthma have been reported in a cross-sectional observational study, in which

volunteers with mild to severe asthma were assessed clinically and their blood, sputum and bronchoalveolar lavage samples were collected at two geographically distinct centres in the UK (220). The approach in the current study was to investigate the clinical and immunological characteristics in two quite different cohorts of patients with allergic reactions. Though additional data on blood counts and total IgE would enable more detailed description of the clusters, the sensitivity of TDA to detect a structure in the data sets, and the external validation provides confidence in the features described.

Our study found that the percentage of females among those undertaking drug challenge, a group comprised mostly of adults, were higher than that of males. On the other hand, males dominated the food challenges, in which the median age for the participants was 14 years old. This dimorphic sex distribution has been reported in a systematic review that included almost 600 publications of food-related allergic reactions, in which 65% of adults were females whereas 64% were males among children (248). It has been also reported that females self-reported drug allergies at a significantly higher rate than that of males (22). The gender-mediated differences in the incidence of allergic reactions could be due to sociocultural, hormonal or immunological determinants that influence the host risk.

The finding that the serum levels of mast cell proteases and of most of pro-inflammatory cytokines showed little change following a positive drug or food challenge reflects the mild nature of the allergic reaction elicited. The challenges were designed to cause only local skin reactions which were probably not sufficiently severe to stimulate mast cell release of mediators and other pro-inflammatory cytokines to reach detectable levels in serum. Challenges that involved oral administration of the drug or food tested were associated with mild systemic symptoms like a tingling sensation or palpitations. This is consistent with the findings of a study on the effect of insect sting challenge on the levels of mast cell mediators in which a rise in serum levels of tryptase and histamine was found only in cases with severe reactions and not in mild reactions (249). However, the finding of significant changes in the serum levels of IL-6 following both positive and negative challenges indicates the presence of other causative factors other than the allergic reactions. The stress associated with the challenges can be a main factor leading to the increase in levels of IL-6 observed. There have been several studies on the effect of stress on reactivity of IL-6 (250, 251). Eighteen studies published between 1993 and 2006 with different numbers of healthy individuals examined for their circulatory inflammatory responses following induced laboratory stress have shown that serum levels of IL-6 increased post-task and the increase was higher if the samples were taken after a longer period following the induced stress (251). Though the exact mechanism underlying this increase during acute stress is not well-

defined, the documentation that IL-6 is secreted from immune and non-immunological cells like endothelial and epithelial cells (252) might explain the additional levels in the circulation.

The current study shows that baseline serum levels of CPA3 were higher in patients with moderate or severe historical reactions to drugs or food compared to the levels seen in healthy individuals. A cutoff value of ≥ 6.5 ng/ml for CPA3 serum levels in patients with drug reactions had a sensitivity of 61% and a specificity of 60%, and a cutoff value ≥ 3 ng/ml in patients with food reactions had 68% and 70% sensitivity and specificity, respectively. The differences between the CPA3 cutoff value for the patients who underwent drug challenge from that for those who underwent food challenges and, as will be discussed later, the lack of association between CPA3 levels and age or gender, may reflect a genuine trigger-mediated variation. Though measurement of serum tryptase has been employed to predict the severity of allergic reactions among children triggered by food (6) or venom (7), the cutoff values for tryptase were not the same for age-similar studies and the sensitivity of the test in prediction the severity of an allergic reaction was either missed (6) or low (7). The present study had the advantage of including a wide age range and more than one trigger of allergic reaction. Therefore, measurement of serum levels of CPA3 might give an indication of the severity of the allergic reaction if the susceptible subject came in contact with the eliciting agent. The replication of a similar trend for CPA3 levels within the validation cohort when the severity of symptoms experienced was graded has strengthened this suggestion. The lack of statistical significance within the validation cohort might be attributed to the smaller sample size compared to that of the derivation cohort.

Levels of tryptase were very low for most of the participants and the relationship with the severity of historical reactions could not be assessed. This was in contrast with several studies in which tryptase, as a well-known biomarker for mast cell degranulation, has been postulated to be a predictive marker for the severity of certain allergic reactions (6, 7). This might be attributed to the sensitivity of the tryptase assay employed in the current study. Measurement of more than one marker could increase the sensitivity of a test to predict the severity of future allergic reactions. As CPA3 is released upon mast cell activation together with other mast cell products *in vitro* (253), levels might reflect those of other mediators *in vivo*. It has been reported that high serum levels of CPA3 were seen in patients with severe allergic reactions even when levels of tryptase had been low (208). Our study is the first to investigate the role of baseline serum levels of CPA3 in predicting susceptibility to severe allergic reactions.

The finding that patients with concomitant atopic conditions had higher levels of CPA3 compared to the levels seen in non-atopic control subjects is in accord with the previous studies indicating an increased degree of mast cell activation in allergic conditions and also a greater potential for

mast cell mediator release. Thus, for instance, it has been reported in an *in vitro* model that mast cells derived from patients with atopic dermatitis had an elevated total granular content of β-hexosaminidase, histamine and tryptase than those from healthy individuals (254). Moreover, higher levels of tryptase have been reported in both bronchoalveolar lavage (255) and serum (256) from asthmatic patients compared to those in non-atopic subjects. Serum levels of CPA3 in patients with one or more atopic conditions have not been investigated before and our findings suggest a role for CPA3, as one of the major mediators released from mast cells, in the mechanism underlying atopic inflammations.

Investigations of the various pro-inflammatory cytokines in patients with allergic reactions to drugs or food indicates that levels of IL-4, IL-6, and IL-8 were lower in patients with more severe historical reactions compared to those in healthy control subjects. There was a trend also for these biomarkers to be lower in patients with one or more atopic conditions regardless of the severity of previous reactions. These findings suggest the presence of coordinated regulation for the release of IL-4, IL-6 and IL-8 by mast cells. It has been reported that IL-4 was degraded by the chymase contained within skin mast cells. Incubation of IL-4 with a lysate of purified mast cells resulted in time- and concentration-dependent decreases in the bioavailability of IL-4, an effect that was prevented by the addition of inhibitors of chymase (257). It has been also reported that levels of IL-6 in supernatants of activated mast cells were markedly reduced after one hour and the reduction was more pronounced after four or 24 hours (258). On the other hand, levels of IL-6 in supernatants from activated mast cells of serglycin-deficit mice have been found to be less affected, suggesting the presence of serglycin proteoglycan regulatory mechanism that had an impact on the levels of IL-6. A parallel study has shown that incubation of exogenous cytokines including IL-6, IL-13 and GM-CSF with a releasate of activated skin-derived mast cells can result in complete loss of IL-6 and IL-13, whereas GM-CSF levels were unaffected (259). This supports the idea that mast cell proteases may be involved in the regulation of certain cytokines. To identify which serglycin-dependent serine proteases were responsible for the degradation, Schwartz and his colleagues incubated IL-6 with chymase, tryptase or cathespsin G (259). They showed that there was a significant degradation of IL-6 over the course of 24 hours induced by chymase and cathepsin G, but not by tryptase, and that the degradation was abrogated by chymotryptic inhibitors. The the potential of IL-8 to be cleaved by mast cell proteases has not been investigated, though a single study has shown that β-Tryptase involved in the regulation of IL-8 expression in airway smooth muscle cells (260).

As IL-4, IL-6, and IL-8 are key cytokines involved in the pathogenesis of allergic reactions and can promote both acute and chronic inflammation and structural changes (261, 262), mast cell proteases-mediated regulation of their bioavailability may represent a protective mechanism.

Though an assay for chymase could not be developed during this study, the high levels of CPA3 seen in patients with severe historical reactions or with atopic conditions compared to the levels in healthy subjects are likely to reflect the release of the other mast cell proteases.

In the present study, the median age for the control subjects (33 years old) was different from that of the participants with food challenges (14 years old) and it was critical to investigate the effect of age on the levels of measured markers. The finding that levels of IL-6 and IL-8 correlated with age of participants indicates the potential for an age-related effect. This is consistent with the finding of a study of the influences of aging on cytokine profiles in 73 healthy individuals with a wide age range, in which it has been reported that serum levels of IL-6 were positively correlated with age (263). Another study on plasma levels of cytokines among 138 Swedish participants (aged 86, 90 and 94 years) and 18 healthy Swedish volunteers (32-59 years old) has indicated significantly higher levels of IL-6 in the study group compared to the control subjects (264). Consistent with this, in a study of the effect of aging and menopause on the serum levels of IL-6 in 220 women (25-107 years old), it has been reported that median levels of IL-6 were increased ten-fold increase with age (265). It has been suggested that age could also affect cytokine levels in the exhaled breath condensates from healthy subjects. In a study of 30 healthy adults, levels of exhaled IL-8, TNFα and nitric oxide have been reported to correlate with age and significant differences in the levels of markers have been found when older age groups (over 60 and 30-60 years) were compared with those below 30 years old (266). The effect of aging on pro-inflammatory cytokine production has been also investigated *in vitro*. Fibroblasts from three age groups (under 28 days, 6 months – one year, and 7- 17 years) cultured from foreskin samples collected from boys undergoing elective circumcision has shown an age-related increase in the production of IL-6 and IL-8 following stimulation with platelet-derived growth factor. Furthermore, the gene expression for IL-6 and IL-8 was greater in fibroblasts derived from the older age groups (267). Taken together, this indicates the presence of changes in some components of immune response during aging process, which might be additional factor causing the low levels of IL-6 and IL-8 seen in the patients who were tested for food allergic reactions.

It is noteworthy that in the present study there was no association with age for serum levels of mast cell proteases and, or for the pro-inflammatory cytokines investigated apart from that with IL-6 and IL-8. Therefore, comparison of the levels of these biomarkers in patients who underwent drug or food challenge with those in the healthy control subjects can be considered valid. This gives strength to the finding of significantly increased levels of CPA3 and decreased levels of IL-4 in the patients with moderate or severe historical reactions compared to those in healthy subjects. On the other hand, there were lower levels of IL-8 in the patients who underwent drug challenge, despite their being older than those of the healthy control group. This suggests that

age differences were not a factor, and the differences in levels of IL-8 observed may possibly be due to a mast cell-mediated regulatory mechanism

The finding that levels of IL-13 were significantly higher in female participants than those in male participants within the derivation cohort and the replication of a similar trend in the validation cohort could explain reports of the higher incidence of allergic reactions among adult females, that was also reflected in our data. A role for IL-13 in inducing allergic responses has been suggested by the ability of this cytokine to stimulate the induction of IgE synthesis in cultures of purified human B cells (268). Targeted expression of IL-13 in the lungs of transgenic mice has been reported to cause airway inflammation and mucous hypersecretion (269). Administration of anti-IL-4 monoclonal antibody did not block the effect of IL-13 indicating the presence of an IL-4-independent mechanism for the induction of IgE synthesis (268). Intranasal administration of soluble IL-13 receptor fusion protein prior to challenge of sensitized mice with ovalbumin has been reported to significantly reduce the extent of eosinophil influx into bronchoalveolar lavage, and the number of goblet cells in histological sections of murine lungs (270). In agreement with our finding, it has been reported that IL-13 could influence the susceptibility of female mice to experimental autoimmune encephalomyelitis (271). However, the observed lack an association between IL-13 and age observed did not explain the high percentage of males among participants less than 18 years old. Therefore, further investigations are needed to define the role of gender in the pathogenesis of allergic reactions.

In the present study, it was found that the severity of historical allergic reactions to drugs or food was strongly related with the presence of one or more atopic conditions. In a UK-based retrospective study of 177,000 patients with a diagnosis of asthma (aged 10-79 years old) and 200,000 age- and sex-matched non-asthmatic individuals, it has been reported that the incidence of anaphylaxis was greater in those with asthma. In addition, the risk of anaphylaxis has been found to be increased in patients who had allergic rhinitis or atopic dermatitis (12). In a study of the incidence of fatal anaphylaxis in patients requiring hospital admission from 1992 to 2012, it has been reported that 75% of the fatal cases of food-induced anaphylaxis had asthma (10). As high CPA3 levels were seen in patients with more severe historical reactions and in those with atopic diseases, the measurement of CPA3 serum levels and a detailed history could provide important insights in the management of those who are more susceptible to severe allergic reactions.

Several studies have suggested that certain ethnicities may have a higher risk of anaphylaxis (15, 25, 26). The risk of anaphylaxis in a population with South Asian ethnicity living in the UK has been reported to be double that of the indigenous white population (25). In the United States, deaths

from food related anaphylaxis have been reported to be strongly associated with African American race (15). However, these findings derived from epidemiological studies that have not included an immunological perspective of anaphylaxis. In contrast, the current study compared the levels of a range of biomarkers among patients with allergic reactions and found limited effect of ethnicity on the levels of mast cell proteases and pro-inflammatory cytokines among the validation cohort, which included participants with diverse ethnicities. Such a comparison was not performed for the derivation cohort because all the participants were Caucasians.

There were few differences in the levels of mast cell proteases and pro-inflammatory cytokines measured in the patients with the different conditions in the validation cohort. This might be due to the small sample size for each condition, unlike the derivation cohort which included larger number of patients. The validation cohort included patients who had experienced anaphylaxis, drug/food allergic reactions, insect bite and atopic conditions rather than just patients who were undergoing drug or food challenge testing as in the derivation cohort. Details of associated atopic illnesses were lacking. The patient characteristics recorded for the validation cohort were not the same as for the derivation cohort, but using Brown's grading criteria to classify those with anaphylaxis, or drug/food allergic reactions it would be possible to compare data with that of the derivation cohort. Similar trends for levels of CPA3 and IL-4 in relation to severity of symptoms experienced to that of Southampton cohort were seen in the validation cohort. Though not statistically significant, these two mediators were present at higher levels in the patients than in the healthy subjects. The association between serum levels of IL-6 and age was also replicated in the validation cohort, but not that for IL-8, possibly on account of the small sample size, as mentioned earlier.

The present study has allowed identification of four novel clinico-immunological endotypes for allergy-related conditions through the application of TDA. The replication of the four clusters in a geographically distinct cohort, which was not identical to the derivation cohort, provides a strength to our findings. The distribution of CPA3 and IL-13 serum levels strongly influenced the shape of the data set and the composition of these clusters, whereas other biomarkers were normally distributed throughout the clusters. This highlights the importance of considering these two biomarkers when phenotyping individual patients.

Patients who had suffered more severe historical reactions were present in increased numbers in clusters I and II, and had significantly higher serum CPA3 levels. On the other hand, those who had suffered more mild historical reactions were in clusters III and IV, which were associated with significantly lower levels of CPA3. Levels of IL-13, gender, and type of the allergen challenge were the main components that distinguish cluster I from II and cluster III from IV. Given the

predominance of females within those undergoing drug challenges and males undergoing food challenges, the distribution of the challenges among the clusters might have been driven by the effect of gender rather than the type of the allergen per se. The strong association of high levels of IL-13 with female gender might be additional component influencing the shape of the clusters. The clinical features of the clusters in the validation cohort were not as clearcut as in the derivation cohort due to the diversity of conditions included, though a similar trend for the severity of the allergic reactions could be seen with clusters I and II. The reason why the majority of the healthy individuals mapped in cluster IV is not clear. Though it has been reported that serum levels of IL-13 measured in 44 asthmatic patients were not significantly different from those in 44 non-asthmatic healthy controls, the latter had higher levels than the patients (272). Therefore, factors affecting the levels of IL-13 including gender should be further investigated in the future.

The findings of our study underline the connection between CPA3 levels, atopy and the severity of allergic reactions, and highlight a possible new approach to devising management plans. The strong influence of gender on IL-13 levels may underlie the increased the susceptibility of adult females to drug or food allergic reactions, but not predict severity. Further assessment and investigations are required to define other factors affecting the release of mast cell mediators.

7.3 Factors modulating the release and expression of mast cell mediators

We have found that *S. aureus* infection of mast cells is associated with a reduced potential for degranulation in response to both IgE- and non-IgE-dependent mast cell activation. We have also found that bacterial infection affects the degree of gene expression for TNFα, IL-8, and IL-1β, major cytokines with established roles in anti-bacterial defence mechanisms. Furthermore, we have found that bacterial exposure can alter the extent to which protein kinases are phosphorylated downstream of FcεRI engagement, and in particular with those involved in regulation of proliferation, differentiation, survival, apoptosis and cytokine production. This has implications on the diagnosis and as well as on understanding the pathogenesis of allergic reactions as measurement of mast cell mediators may thus be affected in the presence of concurrent bacterial infection. The current study presents evidence for an inhibitory effect for *S. aureus* infection on mast cell responses.

The LAD2 mast cell line was employed to study the effect of bacterial infection on mast cells responses due to the difficulties of sample collection from allergic patients who have concurrent *S. aureus* infection. The use of LAD2 cells in *in vitro* models of mast cell degranulation, receptors, cell signalling and genetic studies has been widely accepted and validated (273). Therefore, we have considered using LAD2 cells for being flexible and highly productive cell line which could provide an insight on the factors that affect mast cell degranulation and expression of mast cell mediators.

Investigations on the integrity of FcεRI in LAD2 cells using β-hexosaminidase as a marker for mast cell activation have indicated that LAD2 cells express functional FcεRI. This was demonstrated by Kirshenbaum and his colleagues when LAD2 cells were first characterised. He reported an increase of 40% in the release of β-hexosaminidase when FcεRI expression was upregulated (230). It is of note to mention that attempts to measure tryptase and CPA3 in LAD2 lysates were not successful with the number of cells employed, levels were too low to be detected using tryptase and CPA3 assays. It has been reported that histamine measurements were comparable to those of β-hexosaminidase (230). Therefore, we relied on release of β-hexosaminidase to detect LAD2 cell degranulation.

Our findings indicate that LAD2 cells can be employed as a new mean to investigate allergic sensitivity. LAD2 cells could be sensitised with house dust mite specific IgE present in the serum of house dust mite sensitive subjects, and the percentage of β-hexosaminidase released was highest from cells sensitised with 40% serum when stimulated with house dust mite. This indicates the presence of more IgE available to sensitise the cells. These findings were in agreement with a study conducted by Dr. Laurie Lau on grass pollen-allergic subjects in which LAD2 cells were sensitised with serum and the release of histamine was determined in cell lysate after stimulation with grass pollen (data not published). Taken together, these data suggest that LAD2 cells can be used as a mean to confirm allergic sensitivity, especially in cases where results of skin testing were inconclusive.

Investigation of the effect of bacterial infection on allergen-mediated response of LAD2 mast cells revealed that mast cell exposure to *S. aureus* led to consistent decreases in the release of β-hexosaminidase from sensitised LAD2 cells when stimulated with anti-IgE antibody. A similar effect has been reported previously when LAD2 cells were co-cultured with non-pathogenic *E.coli* and stimulated via IgE-dependent pathway (172). Furthermore, it was found that murine-derived mast cells infected with non-pathogenic *E.coli* had less serotonin release than that from non-sensitised cells (147). On the other hand, exposure to *S. aureus* significantly induced β-hexosaminidase release from non-sensitised cells. This was consistent with the findings of other

studies in which it was reported that infection with *S. aureus* can activate mast cells (274, 275). Incubation of peritoneal cell-derived (274) or human cord blood-derived mast cells (275) with *S. aureus* induced the release of pro-inflammatory cytokines. It is noteworthy to mention that mast cells were co-cultured with *S. aureus* at a multiplicity of infection (MOI) ratio of 1:1 in the present study compared to ratios of 1:10 (275) and 1:25 (274) employed by other studies.

Bacterial infection appeared to inhibit the release of β-hexosaminidase from LAD2 mast cells after stimulation with calcium ionophore A23187 and the inhibitory effect was more evident at the lower concentrations of calcium ionophore A23187. This might be due to potent degranulation induced by calcium flux at 0.3 µM calcium ionophore A23187 that bacteria could not compete against. On the other hand, the apparent inhibitory effect of bacteria was most pronounced at a concentration of 0.03 µM calcium ionophore A23187. This was in agreement with the finding of a decrease in serotonin release from mouse peritoneal mast cells infected with non-pathogenic *E.coli* that had been reported when cells were stimulated with 0.01 µM calcium ionophore A23187 (147).

The findings that *S. aureus* co-incubation with LAD2 cells had an inhibitory effect on the activation of IgE-dependent and IgE-independent mast cell activation is at variance with the idea that mast cells are sentinel cells in the innate immune response (Sheppard 2012). Mast cells degranulate and release a broad spectrum of mediators in response to pathogens resulting in the initiation of the early phase of the inflammatory response. It has been mast cell degranulation and killing of the extracellular bacteria in *in vitro* and *in vivo* murine models (176, 177). It has been also shown that alive and dead *S. aureus* can stimulate the release of TNF-α and IL-8 from human cord blood derived mast cells. Nakamura and colleagues have reported that culture supernatant of *S. aureus* can stimulate the release of β-hexosaminidase from cultured murine mast cells (nakamura, 2013). Moreover, intradermal administration of δ-toxin derived from *S. aureus* has resulted in mast cell degranulation as indicated by the leakage of Evan's blue dye at the site of injection in wild type mice, but not in mast cell-deficient mice. Though the author has suggested that *S. aureus* could promote allergic skin diseases through activation of mast cells, that study did not compare the results obtained with that in *in vitro* or *in vivo* models of concomitant IgE-derived mast cell activation and bacterial incubation or administration. On the other hand, it has been suggested that peptidoglycan from *S. aureus*, lipopolysaccharide from *E. coli* and lipoarabinomannan from *Mycobacterium smegmatis* failed to stimulate mast cell degranulation and histamine release from mature rat mast cells, but could induce the generation and release of significant amounts of leukotrienes when compared to calcium ionophore A23187-induced generation (179). Therefore, mast cells could be involved in the clearance and killing of bacteria through recruitment of other immune cells to the site of infection. Mast cells roles in immune responses against pathogen

could also include phagocytosis. It has been reported that human cord blood mast cells can phagocytise and kill various bacteria including *S. aureus*, *Streptococcus faecium, Klebsiella pneumoniae, Citrobacter freundii*, and *Escherichia coli* (arock, 1998). However, other reports have suggested that the bacteria might utilize mast cells as a vehicle and a safe intracellular environment protecting them from other immune cells (176, 177). At this point, it is clear that there is a lot of controversy over the nature of the interactions between mast cells and bacteria. This could be attributed to the differences in experimental conditions, origin of mast cells, bacterial incubation (live, dead, toxins or bacterial wall), and the mediators analysed. It should be taken into account that the present study focused on the effect of bacterial incubation with cultured mast cells in the presence of IgE-dependent and IgE-independent stimulation.

The inhibitory effect of *S. aureus* with the two-hour infection period was less apparent. This is consistent with the findings reported from co-cultures of rodent mast cell line (RBL-2H3) with *Mycoplasma pneumonia* or *Streptococcus pneumoniae* (276, 277), in which four hours were needed to induce significant degranulation. Furthermore, it was reported that there was a higher percentage recovery of *S. aureus* after three hours co-incubation with human cord blood-derived mast cells than at a 30-minute, after which the cells were incubated with gentamicin to kill any extracellular bacteria (275). This suggests that bacterial internalization occurred after three hours.

There have been suggestions that *S. aureus* could induce apoptosis through the release of toxins (278). The finding that LDH and β-hexosaminidase release in the cell culture supernatants was low following infection and before addition of stimuli indicates that the altered mast cell responses were due to the effects of *S. aureus* on degranulation rather than because of cell death. LDH release from infected cells was significantly higher after four hours exposure to *S. aureus* than after two hours of infection. However, LDH release was in general quite low (< 15%) and could have been a consequence of *S. aureus* infection. Furthermore, β-hexosaminidase release from LAD2 cells after infection mirrored that of LDH, with no significant differences between the groups and with a percentage of less than 5%. Taken together, it seems likely that the responses of LAD2 cells were not simply a consequence of cell death.

The present study focused on the release of selected cytokines that are known for their effect on mast cells and their bacterial associated interactions. TNFα is a pro-inflammatory cytokine, and is one of pre-formed cytokines found within mast cell granules (279). IFNγ is a cytokine classically associated with T_H1-mediated inflammation. IFNγ has been found to enhance the antibacterial and pro-inflammatory responses of mast cells against *S. aureus* infection (280). IL-1β, a cytokine implicated in a number of inflammatory conditions and produced by monocytes and macrophages, is important in the defence against bacterial infections at mucosal surfaces (281).

Moreover, when its activity is inhibited in animal models there is increased susceptibility to bacterial infections, including those caused by *S. aureus* (282, 283). IL-8 (CXCL8) acts as a chemotaxis factor for phagocytic cells, namely neutrophils but also mast cells, drawing them to the site of infection (284, 285).

Analysis of the levels of cytokines released from LAD2 cells infected with *S. aureus* following stimulation indicated that levels of these mediators were very low. These findings were similar to those of a study on mast cell activation in which it was reported that stimulation of LAD2 cells with anti-IgE even for 24 hours resulted in low levels of TNFα, whereas other cytokines did reach levels that could be detected (286). On the other hand, it has been reported that activation of mast cells by neuropeptides can result in high levels of cytokines being secreted after 24 hours of stimulation (286-288). Therefore, the period of stimulation and the type of stimulus may account for the differences seen between our results and those of previous studies.

Our study is the first to investigate the influence of *S. aureus* infection on mast cell gene expression for TNFα, IL-8, and IL-1β during IgE-mediated responses. There was an apparent infection-induced downregulation of TNFα, IL-8, and IL-1β gene expression in sensitised LAD2 cells when stimulated with anti-IgE compared to non-infected ones. Our findings were in contrast to those of a study in which it was found that co-incubation of LAD2 cells with non-pathogenic *E. coli* led to upregulation of many genes, including TNFα and IL-8, in sensitised cells (172). Such variation in the findings could be attributed to bacterial pathogenicity. During IgE-mediated responses, non-pathogenic *E. coli* might not alter the innate immune response of mast cells to bacterial exposure at the level of gene expression, whereas *S. aureus* might have more a potent effect on mast cell responses, including both gene expression and mediator release, leading to inhibition of natural immune responses. This could represent a survival mechanism for *S. aureus*.

In the present study, there was a trend for increased gene expression of TNFα, IL-8, and IL-1β in LAD2 cells infected with *S. aureus* and without prior sensitisation. This is consistent with the findings of a study on the global effect of *S. aureus* on mast cell gene expression, in which it was found that 52 genes, including those for various cytokines and chemokines, were significantly upregulated after four hours of infection (274). This suggests that *S. aureus* promotes a strong pro-inflammatory response in mast cells in the absence of IgE-mediated activation.

Activation of LAD2 cells with calcium ionophore A23187 resulted in little changes in the gene expression for TNFα, IL-8, and IL-1β when infected with *S. aureus*. There have been few studies on the effect of calcium ionophore A23187 on gene expression, but one might suspect that the overwhelming effect of calcium flux could be too quick to allow any changes in gene expression.

Analysis of phosphorylation profile of the main protein kinases of LAD2 cells after exposure to S. aureus was critical to highlight possible mechanisms behind the apparent inhibitory effect of S. aureus on IgE/allergen-derived mast cell activation. The global phosphorylation pattern for 11 protein kinases in sensitised-infected cells was reduced, with marked effect on the expression of Akt2, ERK2, GSK-3α/β and GSK-3β. It has been reported that antigen stimulation of IgE-primed murine bone marrow derived mast cells resulted in enzymatic activation of Akt, which enhanced the transcriptional activity of IL-2 and TNFα. It has been also reported that induction of Akt activity by FcεRI cross-linking or treatment with growth factors promoted cell survival and proliferation (289). In addition, it was found that FcεRI stimulation can result in activation of ERK and that pharmacological inhibition of Akt and ERK can lead to a significant reduction in both β-hexosaminidase release and intracellular calcium mobilization (290). It has been shown that GSK-3α/β is constitutively expressed in cells and its activation is involved in various downstream regulatory mechanisms including those for cell growth, apoptosis, and cytokine production (291). Knockdown studies of GSK-3β expression have shown that GSK-3β activation has a priming effect on FcεRI-mediated cytokine production in human mast cells (292). In addition, GSK-3β knockdown has been shown to have an impact on human mast cell survival, with induction of cell apoptosis in cell culture studies (293).. Therefore, bacterial infection might negatively influence the phosphorylation of aforementioned protein kinases during mast cell activation, resulting in reduced release and expression of mast cell mediators.

The effects of bacterial exposure on mast cell degranulation might be regulated through stimulation of Toll-like receptors (TLRs). These are pathogen-recognition receptors that play a major role in innate immunity through identification of invading bacteria. Mast cells express TLRs, and specifically TLR2 and TLR4, which recognise bacterial ligands (294, 295). It has been reported that administration of bacterial lipopolysaccharide can, via a TLR4-dependent pathway, suppress IgE-mediated early and late allergic responses in a murine asthma model (296). Moreover, a significant decrease in eosinophilia, total serum IgE, and airway hyper-responsiveness has been reported in ovalbumin-sensitised mice following administration of TLR2 or TLR 4 agonists (297). Furthermore, it has been shown that treatment of IgE-sensitised human mast cells with TLR2 agonists can suppress degranulation, intracellular calcium mobilization and ERK phosphorylation (298).

Taken together, bacterial infection might interfere with the mast cell responses through interrupting intracellular signalling downstream of FcεRI cross-linking and affect their vital role in both innate and adaptive immunity. Bacteria would have a better chance of survival within mast cells and at the same time prevent further immune responses triggered by the release of mast cell mediators. Patients with *S. aureus* infection might have less risk of mast cell activation and the

development of anaphylaxis. Targeting the pathway through which *S. aureus* influences mast cell responses could be used as a therapeutic approach to prevent or reduce the risk development of severe allergic reactions.

7.4 The subcellular localization of mast cell proteases

The current study has described for the first time the presence of DPPI in the granules of human mast cells and its co-storage with tryptase, CPA3 and chymase. To demonstrate this, we have developed a technique for attaching non-adherent LAD2 cells onto glass slides and have characterized cells according to the distribution and co-localization of proteases by multi-immunostaining. We have also separated subcellular contents of LAD2 cells through differential pelleting and specifically defined the granular contents.

We found that centrifugation could lead to activation of LAD2 cells, and that more gentle centrifugation conditions could markedly improve staining results. This finding suggested that LAD2 cells were sensitive to centrifugation and a modification of the original protocol was necessary for successful immunostaining of the cells.

The present study indicates that tryptase may be abundantly expressed within immunostained LAD2 cells. This is in agreement with the reported characteristics of LAD2 cells, which have the phenotype of mature mast cells (230). Surprisingly, we noticed for the first time the apparent presence of tryptase in the nuclei of LAD2 cells. This requires further study, but may indicate a role of tryptase in regulation of nuclear events. This finding was consistent with a report suggesting that tryptase may cleave histones in apoptotic as well as in viable cells (299).

The finding of a highly variable degree of immunostaining intensity for chymase in LAD2 cells might reflect different stages of granule maturation within the cells. Analysis of granule maturation in rat peritoneal mast cells has indicated that there is an increase in the granule size as the quantity of mediators is increased (300). It may be difficult to detect mast cell proteases at early stages of maturation, but they become more readily detectable as the granules mature.

Ours is the first study to investigate the CPA3 and DPPI content of LAD2 cells. The presence of these proteases supports the idea of LAD2 cells having characteristics of mature mast cells and supports their use in studies of the biology of human mast cells. Interestingly, we also found DPPI nuclear staining which has not been reported before. Though this needs confirmation, one might consider the possibility that DPPI has additional functions in the nucleus. The other possibility is

non-specific binding of the antibody but the results from the antibody development and validation have confirmed its specificity (215).

In the present study with triple immunostaining of LAD2 cells we found co-localization of DPPI with tryptase, CPA3 and chymase. The detection of DPPI in the granules suggests that DPPI might be released in association with the other proteases upon degranulation and hence can be used as a novel marker for mast cell activation. In support of our finding, Wolters and his colleagues reported the presence of DPPI in the granules and its relation to chymase in mast cells of dog lung tissue (133). They also reported that DPPI cleaves extracellular matrix proteins and postulated a role in airway remodelling.

The findings of triple immunostaining of LAD2 cells also shows heterogeneous staining patterns for tryptase, chymase, CPA3 and DPPI with tryptase being the most abundant protease. As mentioned above, this might be related to granule size and maturation. However, this finding can also reflect the variable composition of mast cell granules. Early immunohistochemical studies of human tissue mast cells indicated the presence of tryptase, CPA3 and chymase in one subset of cells (MC_{TC}) and the presence of tryptase only in the other subset (MC_T) (51, 53). A heterogeneous granular composition has also been described in mouse bone marrow-derived mast cells where the granules were subdivided into two subtypes; granules containing serotonin and cathepsin D, and others contained histamine and TNF (301).

Our finding that tryptase was the most predominant protease in stained preparations is strongly associated with its abundance in mast cells. It has been reported that human foreskin mast cells contain 35 pg tryptase and 4.5 pg chymase per cell (302), and the CPA3 content of mast cells has been estimated to be 16 pg per cell (303). The DPPI content of mast cells has not been investigated to date. Our findings also showed that CPA3 and chymase were co-localised in most of the cells, which was consistent with the theory of chymase and CPA3 forming a complex with heparin that is distinct from a tryptase and heparin complex (122). This would be in keeping with our observation of a close association between chymase and CPA3.

In the present study we found that DPPI was partially co-localised with avidin-sulforhodamine, a finding that further indicates the presence of DPPI within mast cell granules, though does not exclude its presence within other cellular compartments. A complete co-localization seen between chymase-CPA3 and avidin-sulforhodamine agrees with what has been mentioned earlier about the close association of chymase and CPA3 within mast cell granules.

The present study employed fractionation through differential pelleting to determine the subcellular localization of DPPI within mast cells. This proteomics technique, accompanied by

localization of organelle proteins by isotope tagging (LOPIT), has been developed to generate a global map for subcellular protein distribution within *Arabidopsis thaliana* (304) and HeLa cells (241). Though complete analysis of subcellular contents of LAD2 cells could not be achieved without subjecting the resulting fractions to mass spectrometry, preliminary analysis by dot blot indicates that the signal for DPPI coincided with that for tryptase within the cytosolic fraction, which is likely to contain mast cell granules. This strongly suggests localization of DPPI within the same compartment of tryptase, that is in the mast cell granules. The presence of lower quantities of chymase and CPA3 within mast cells (as explained above) could explain the lack of antibody reaction to chymase and CPA3 in the cytosolic fraction. On the other hand, the signal detected from the other fractions could be due to cross-contamination, which is a common problem in centrifugal separation (305). The finding that all of the four antibodies reacted with the first fraction may indicate an incomplete fractionation and that some of the organelles, including granules, were precipitated in the first fraction. Further optimisation of cell lysis process and sucrose density gradient should be applied in the future.

In the current study we used LAD2 cells as a model to investigate the localization of four mast cell proteases. Using human tissue mast cells would provide stronger evidence for the relationship between these proteases, but LAD2 cells have been validated as models in research topics including mast cell degranulation, receptors, cell signalling and genetic markers (273), and they offer the advantage of being free from contamination from other cell types.

In summary, DPPI is present inside mast cell granules in association with tryptase, chymase and CPA3. Therefore, DPPI could be released during mast cell degranulation and contribute to immune modulation. DPPI has also the potential to be a novel marker for mast cell activation.

7.5 Conclusions

We have shown for the first time a statistically significant association between baseline levels of CPA3 and the severity of allergic reactions. Our findings suggest that CPA3 baseline levels higher than the cutoff values devised may predict moderate to severe allergic reactions to drugs or food. Concomitant atopic diseases can increase the susceptibility to severe forms of allergic reactions because of the strong association observed between these atopic illnesses and high baseline levels of CPA3, and with severity of allergic reactions. We have also identified a strong connection between high levels of IL-13 and female gender that might influence the risk to drug or food allergic reactions.

We have identified four novel clinico-immunological endotypes of allergic reactions and replicated them in an independent data set using assays we have developed and optimised for mast cell proteases and assays for pro-inflammatory cytokines combined with clinical features. These endotypes have highlighted the patients at risk of severe allergic reactions and therefore their characteristics can be applied in clinical practice to facilitate proper management. Being aware of the potential risks would increase the compliance of the patients to strict avoidance measurements and encourage them to carry adrenaline auto-injectors at all times. For those who may be less susceptible to life-threatening symptoms, that knowledge may help to reduce anxiety.

We have found that exposure to *S. aureus* can inhibit IgE- and non-IgE-dependent mast cell activation and could influence the gene expression of major cytokines known for their bacterial interactions. The underlying mechanism of bacterial inhibition of mast cell responses involves alteration of phosphorylation of protein kinases downstream FcεRI crosslink. These findings indicate that bacterial infection could reduce the risk of development of severe allergic reactions by affecting the release and expression of mast cell mediators, which are responsible for initiation of signs and symptoms of allergic reactions.

At the subcellular level, we have shown that DPPI, a relatively little investigated protease of mast cell, was co-localized with tryptase, CPA3 and chymase. Therefore, DPPI could be released from mast cells in association with other mediators during mast cell degranulation. This raises the prospect of DPPI having a role in extracellular immune modulation. In addition, an assay developed for measurement of DPPI in body fluids is thus likely to provide an indication of the extent of mast cell activation and this could aid the diagnosis of allergic reactions.

In conclusion, this study emphasises the importance of CPA3 as a predictive biomarker of severity of allergic reactions to drugs or food. The incorporation of CPA3 measurements with a panel of pro-inflammatory cytokines together with the clinical characteristics of allergic reactions led to identification of four endotypes that can be applied in clinical practice to define those at greater risk of anaphylaxis. Bacterial infection with *S. aureus* through the inhibition of release and expression of mast cell mediators might reduce the susceptibility to allergic reactions. This could provide the basis for development of a new therapeutic approach by targeting the pathway for bacterial interaction with mast cells. The subcellular presence of DPPI in association with other mast cell proteases calls attention to this protease as a new biomarker of allergic reactions and as a mediator of the disease.

7.6 Future work

The ability to develop sandwich ELISAs for chymase and DPPI gives the potential for establishment of a multiplex assay using our reagents for measurement of the four mediators. For example, an electro chemiluminescent procedure (U-Plex, Meso scale discovery) could be employed that will not only allow the flexibility of running the assays but also enhance the sensitivity of the assays.

Many of our participants had low or even undetectable serum tryptase levels. Further work should be directed towards improving tryptase assay using the more stable recombinant tryptase as a standard for the assay. A preliminary work has been set by the current study to use the recombinant tryptase in the assay, but optimization is required to measure serum levels as well as levels in other body fluids.

In the present studies serum levels of CPA3 that can predict severe allergic reaction were determined successfully. In future it will be important to establish in more detail what are the normal serum levels of CPA3. We were not able to detect changes in serum levels of mast cell proteases and other biomarkers during drug or food challenge, but the challenges were designed to cause only a superficial reaction rather than a systemic one. Therefore, future research should focus on increasing the sample size to include more control subjects, which has been a weakness in our study, and more patients who are acutely ill such as those with anaphylaxis. These patients can be recruited from the accident and emergency unit of the hospital. Detailed information should be collected on the allergic reactions provoked, including the time required for development of allergic signs and symptoms, response to medication, type of medication given for treatment and the presence of any aggravating factors. Being able to determine serum levels of CPA3 and tryptase in a well-defined control group of subjects should allow the determination of normal values. In addition, taking multiple measurements of these proteases during an acute allergic reaction would allow any rise in levels to be detected and the time-course monitored. There have been many investigations of tryptase levels in serum but not of CPA3 levels. Our laboratory has the advantage of having the only optimised assay of CPA3 levels and future research studies should be directed to recruit more participants and to analyse their biomarkers.

We have focused on development, optimization and applying assays on serum samples, but during the course of these studies we were able to collect also plasma, saliva and urine samples from the same patients, as well as DNA, and these are awaiting analysis. It will be valuable to genotype the DNA samples collected from our recruited patients. By adding additional measurements, topological data analysis could enhance the understanding of the four endotypes

distinguished and allow a more precise definition of each cluster. To our knowledge, ours represents the largest study comprising clinical, immunological and possible genotypic features of allergic reactions, and the information obtained could be valuable in clinical practice for predicting of the severity of a reaction.

Our study has identified an inhibitory effect of *S. aureus* on the release and expression of mast cell mediators. However, investigation on the effect of *S. aureus* on the gene expression of mast cell mediators can be improved by increasing the stimulation time to 6, 12 and 24 hours to allow any changes on the gene expression to be detected. Research work can be extended to include other kinds of bacterial strains like *non-pathogenic E.coli*. This would allow comparison between the effect of *S. aureus* and the other bacteria on mast cell responses. Further research could be also directed to investigate the underlying mechanism of bacterial inhibition. Mast cell recognise pathogen through TLR, therefore targeting TLR would be a starting point to explore a potential therapeutic tool for allergic reactions.

Appendices

Appendix A

PATIENT INFORMATION SHEET

(DRUG CHALLENGE – ADULT)

Title of Project: *New biomarkers of allergic reactions and susceptibility to anaphylaxis*

LREC ref: 08/H0501/17

You are being asked to consider whether you would like to contribute to a research study. Before you decide it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?
To evaluate new tests for the diagnosis of severe allergic reactions and to identify people who may be at risk from such reactions. This involves measuring levels of blood, urine and saliva components which may be increased during allergic reactions or in people who may be susceptible to severe reactions, and/or investigating if there may be a genetic susceptibility.

Why have I been chosen?
You have been chosen as you are due to attend the hospital for a challenge to a medicine or anaesthetic to see if you have an allergy to it.

What would happen if I take part?
When you attend the hospital for the drug challenge, we would like you to give us several blood samples: at the beginning of your visit, the end of your visit, and if a cannula is fitted for medical reasons then perhaps also at other times during your visit. These samples may be needed for diagnosis anyway, but we would like to use leftover blood for our research. We would also like to ask you to give us several saliva samples and urine samples for the same purpose. We are also very interested in whether the drug challenge affects your breathing. It would also be helpful if we can measure your temperature in the mouth, under the arm or in the ear several times during the challenge.

Finally, if you are willing we may also ask you to return to the hospital at one or two later dates (to be agreed as convenient for you) in order to provide additional blood samples. The total volume of blood we will collect will be at most 30ml.

Should you prefer not to return for additional visits to the hospital, you can still contribute to the study and your participation would be very welcome.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This would not affect the standard of care you receive. You can also choose to take part but not to provide additional blood samples, or not to provide urine, saliva or breath samples.

What is the procedure that is being tested?

Blood, urine, and saliva will be taken for studies to evaluate new tests for allergic reaction or anaphylactic shock, or to investigate if there may be a genetic reason for being susceptible.

What are the side effects of taking part?
Having blood taken for a blood test can cause some discomfort or some local bruising.

What are the possible benefits of taking part?
It is possible that this study will provide information that will help in clinical assessment of your condition. Otherwise, there are no direct medical benefits from taking part in this study. However, should you make a later visit(s) to the hospital to provide a further blood sample for research, a payment will be made to cover travel expenses, etc. The research may lead to developments in the understanding of the mechanisms of allergic reactions, and to the development of improved methods of diagnosis.

What if something goes wrong?
In the unlikely event that you become ill or are injured as a result of taking part in this study then the normal NHS and University complaints procedures will be available. In this case please contact Dr Efrem Eren (tel: 023 **8120** 6650, Department of Immunology, Level C, MP8, Southampton General Hospital.

Would my taking part in this study be kept confidential?
Any information which is collected about you during the course of the research would be kept strictly confidential. Persons carrying out the research will not be able to identify you from your samples.

What will happen to the information you collect?
Any information we collect by asking you questions about your health or by looking at your hospital notes will be stored in a way that does not identify you. Only data directly helpful to the research will be collected. It will be processed and used to investigate the causes and conditions that can lead to allergic reactions and the ways by which such reactions can be recognised.

What will happen to the samples?
Samples will be kept for further analysis but will be unidentifiable. Several different tests will be performed on the samples within the laboratory. Your samples will be stored and may be used in future research if ethical approval is given for this.

What will happen to the results of the research?
The results of the research will be prepared for publication in a scientific journal, and may be presented at a scientific research meeting or included in a thesis that is examined as part of a medical student's training. You will not be identified in any such publication or presentation.

Who is organising and funding the research?
The research is funded by the Department of Health, the Foundation for the Study of Infant Deaths, the Food Standards Agency and Wessex Medical Trust under grants to the University of Southampton, which is responsible for its organisation and management.

Who has reviewed the study?
The Isle of Wight, Portsmouth and South East Hampshire Research Ethics Committee.

Contact for further information:
Dr Andrew Walls, Tel 023 **8120** 6151, Email: afw1@soton.ac.uk
or Dr Efrem Eren, Tel 023 **8120** 6650, Email: Efrem.Eren@suht.swest.nhs.uk . Thank you for taking the time to read this.

If you decide to take part in the study, you will be given a copy of the information sheet and a signed consent form to keep.

Appendix B

Study Number: 08/H0501/17
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: *New biomarkers of allergic reactions and susceptibility to anaphylaxis*

Name of Researcher: Dr Andrew F Walls

PLEASE **INITIAL** THE BOXES IF YOU AGREE WITH EACH SECTION:

PART A *Consent for the main study*

1. I confirm that I have read the information sheet dated 20/10/2014 for the above study, have had the opportunity to ask questions, understand why the research is being done and any possible risks which have been explained to me. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time by contacting **Dr Andrew Walls** or another member of staff involved in the study without giving any reason and without my medical care or legal rights being affected. If I withdraw I understand that any unused donated blood, urine or saliva will be disposed of. ☐
3. I understand that sections of my medical notes may be looked at by responsible individuals from the University of Southampton or from regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to my records. ☐
4. I understand that data for this research study may be collected from my medical notes or by asking me questions about my health. I give my consent for this information to be stored in anonymous form and to be processed and used for the purpose of advancing scientific understanding. ☐
5. I give my consent for samples including saliva samples, urine samples and two or more blood samples to be taken during my current hospital visit (if not already required for medical reasons), for the purposes of furthering this research study. ☐
6. I agree that any blood, urine or saliva sample taken during my current or previous hospital visit may also be used for the purposes of furthering this research study. ☐
7. I agree to return if I am able to the hospital for one or two later visits in order to provide further blood samples. I agree that any later sample may also be used to advance the above research study. ☐
8. I understand that the results of this research project may be published in the form of scientific papers in which I will not be identified. ☐

Samples gifted for storage and use in future studies

PART B *Linked Anonymised Samples*

9. Provided that specific study protocols have been reviewed and approved by the Local Research Ethics Committee, I indicate my consent for the samples and their derived cells to be stored (potentially for many years) for the following types of studies. I understand that these studies are not for the purpose of directly benefiting my health:

   a) I give permission for the samples to be used for treatments/investigations of medical conditions relating to allergic and anaphylactic disorders. ☐

   b) I give permission for the samples to be stored for use in other unrelated research studies the precise nature of which will depend upon future scientific advances, but excluding genetic engineering and germ-line research. ☐

   c) I understand that future research using the samples I give may include genetic research aimed at understanding the genetic influences on allergy, inflammation and susceptibility to anaphylaxis, but that the results of these investigations are unlikely to have any implications for me personally. ☐

   d) In the case of linked anonymised samples, I give permission for a member of the research team to look at my medical records, to obtain information relating to allergic disease and susceptibility to anaphylaxis. I understand that the information will be kept confidential. ☐

10. I understand that the samples may be used for commercial development, without financial or other benefit to myself, for the investigation and treatment of medical conditions, potentially leading to new preventative measures against such conditions in keeping with the gift nature of my samples. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

Appendix C

UNIVERSITY OF Southampton School of Medicine

New biomarkers of anaphylaxis drug challenge record

Subject number	CRA
Date	/ /
Age	
Gender	☐ Male ☐ Female

Observations

Time		**° C**		**mmHg**		**BPM**		**%**		**L/min**
	Temp 1		**BP 1**		**HR 1**		**SATS 1**		**PEFR 1**	
	Temp 2		**BP 2**		**HR 2**		**SATS 2**		**PEFR 2**	
	Temp 3		**BP 3**		**HR 3**		**SATS 3**		**PEFR 3**	
	Temp 4		**BP 4**		**HR 4**		**SATS 4**		**PEFR 4**	
	Temp 5		**BP 5**		**HR 5**		**SATS 5**		**PEFR 5**	

Challenges

Drug 1…….

SPT	**Time**						☐ +ve control ☐-ve control
	Dose	1/	1/	1/	1/	1/	
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No	
IDT	**Time**						☐-ve control
	Dose	1/	1/	1/	1/	1/	
	Reaction	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No	

Drug 2……..

SPT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No
IDT	**Time**					
	Dose	1/	1/	1/	1/	1/

	Reaction	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No

Drug 3……..

SPT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No
IDT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No

Drug 4……

SPT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No
IDT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No

Sample collection

Collection Point	Sample	Time Collected
Baseline	Saliva Blood Observations	…… …… ……
Post SPT 1	Saliva	
Post SPT 2	Saliva	
Post SPT 3	Saliva	
Pre IDT	Temp	
Post IDT 1	Saliva Temp	……... ……...
Post IDT 2	Saliva	
Post IDT 3	Saliva	
Post IDT 4	Saliva	
Post IDT 5	Saliva	
15 mins post last IDT test dose	Saliva Temp	…… ……
30 mins post last IDT test dose	Saliva	
60 mins post last IDT test dose	Bloods Saliva Urine Temp Observations	……. ……. ……. ……. …….

Results

Reaction occurred	☐ Yes ☐ No
Drug	☐ Drug 1 ☐ Drug 2 ☐ Drug 3 ☐ Drug 4
Test	☐ SPT ☐ IDT ☐ Oral ☐ Other
Time of onset of first symptoms	
Time of onset of severe symptoms*	
Wheal size drug 1	
Wheal size drug 2	
Wheal size drug 3	
Wheal size drug 4	

Symptoms

	Present		**Present**
Hives	☐	**Fever**	☐
Itchiness	☐	**Sneeze**	☐
Generalised angioedema	☐	**Cough***	☐
Headache	☐	**Shortness of breath***	☐
Postnasal drip	☐	**Wheeze (expiratory) ***	☐
Abdominal pain	☐	**Stridor (inspiratory) ***	☐
Vomiting	☐	**Faintness***	☐
Diarrhoea	☐	**Collapse***	☐

Hypotension (< 100/60)	☐	**Bradycardia (< 60)**	☐
Tachycardia (> 120)	☐	**Desaturation (<90-95)**	☐
Urticarial rash	☐		

Reaction observations i.e any additional symptoms or observations taken.	

Allergy history

Previous reaction	☐ Yes ☐ No ☐ Uncertain			
Presumed drug	☐ Drug 1 ☐ Drug 2 ☐ Drug 3 ☐ Drug 4 ☐ Other			
Reason for referral				
Existing allergies (number accordingly)	Drugs	Foods	Insects	Pollens
	Other			

Allergy Symptoms

	Allergen						**Allergen**				
	1	**2**	**3**	**4**	**5**		**1**	**2**	**3**	**4**	**5**
Hives	☐	☐	☐	☐	☐	**Fever**	☐	☐	☐	☐	☐
Itchiness	☐	☐	☐	☐	☐	**Sneeze**	☐	☐	☐	☐	☐
Generalised angioedema	☐	☐	☐	☐	☐	**Cough**	☐	☐	☐	☐	☐
Headache	☐	☐	☐	☐	☐	**Shortness of breath**	☐	☐	☐	☐	☐
Postnasal drip	☐	☐	☐	☐	☐	**Wheeze (expiratory)**	☐	☐	☐	☐	☐
Abdominal pain	☐	☐	☐	☐	☐	**Stridor (inspiratory)**	☐	☐	☐	☐	☐
Vomiting	☐	☐	☐	☐	☐	**Faintness**	☐	☐	☐	☐	☐
Diarrhoea	☐	☐	☐	☐	☐	**Collapse**	☐	☐	☐	☐	☐

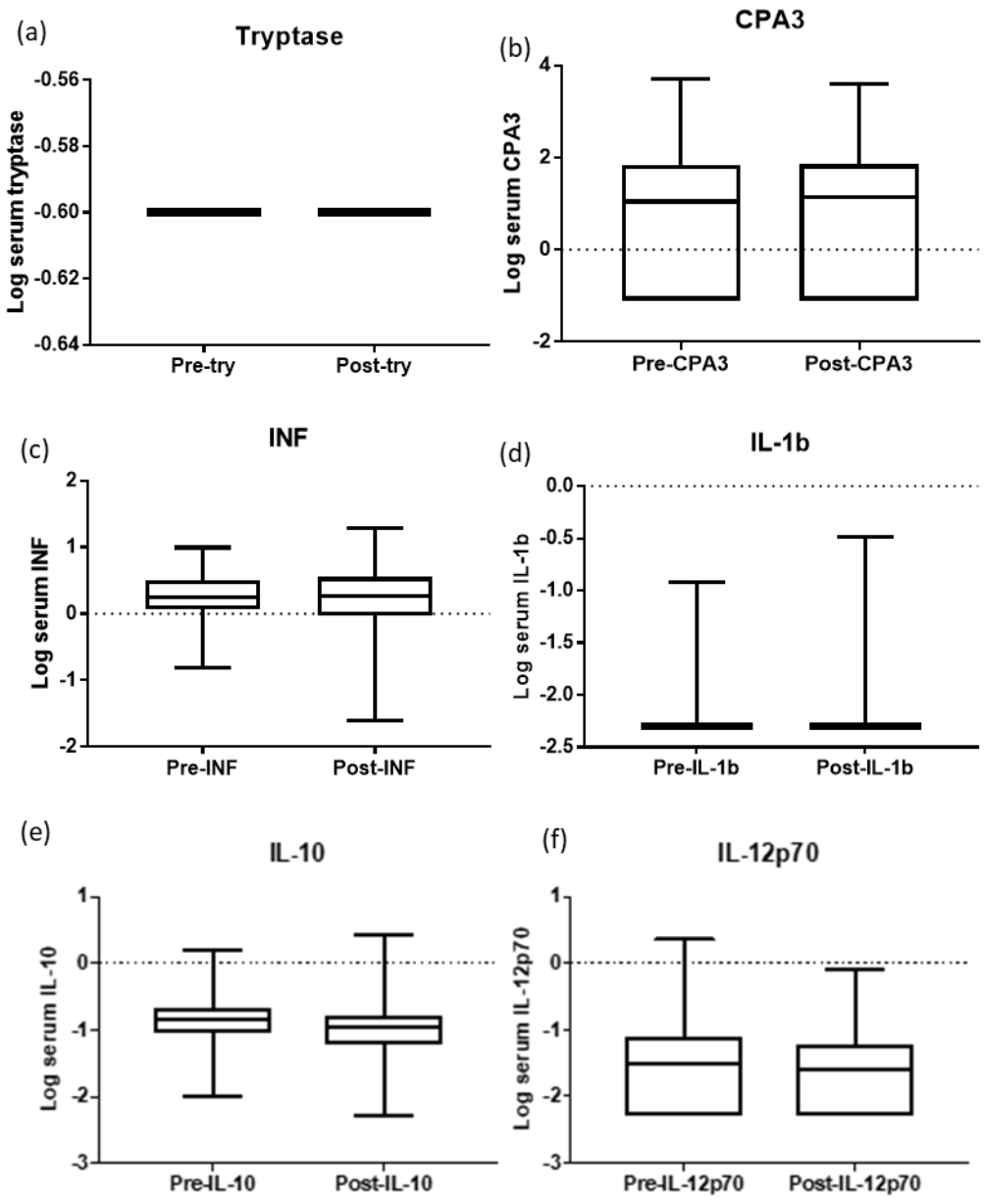
Atopy	☐ Hayfever ☐ Asthma ☐ Eczema

Medical history

Current medication list.	
Family history of allergy	
Additional notes	

Appendix D

This appendix includes figures 4.2 and 4.3 in a larger size than that presented in Chapter 4.



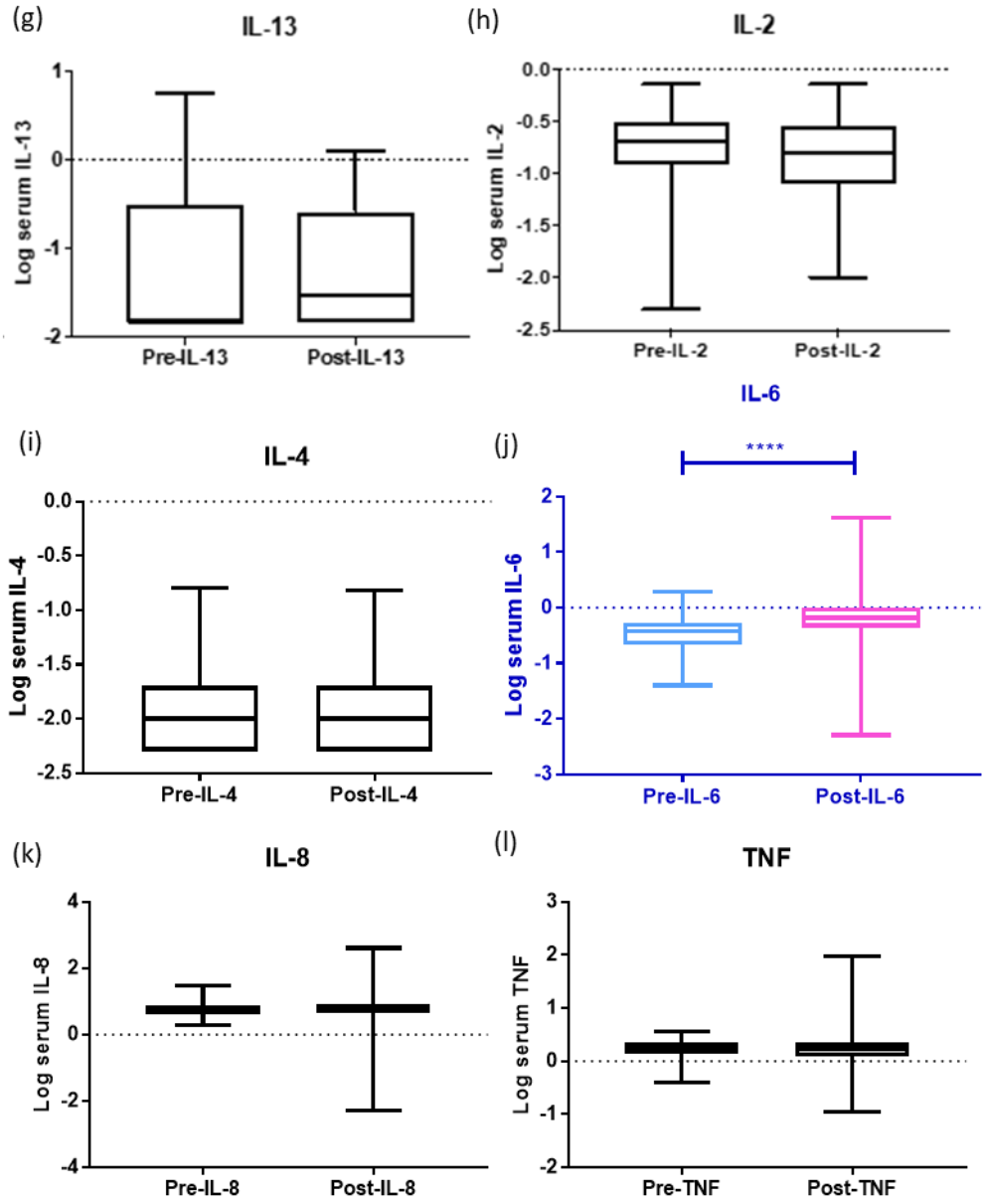
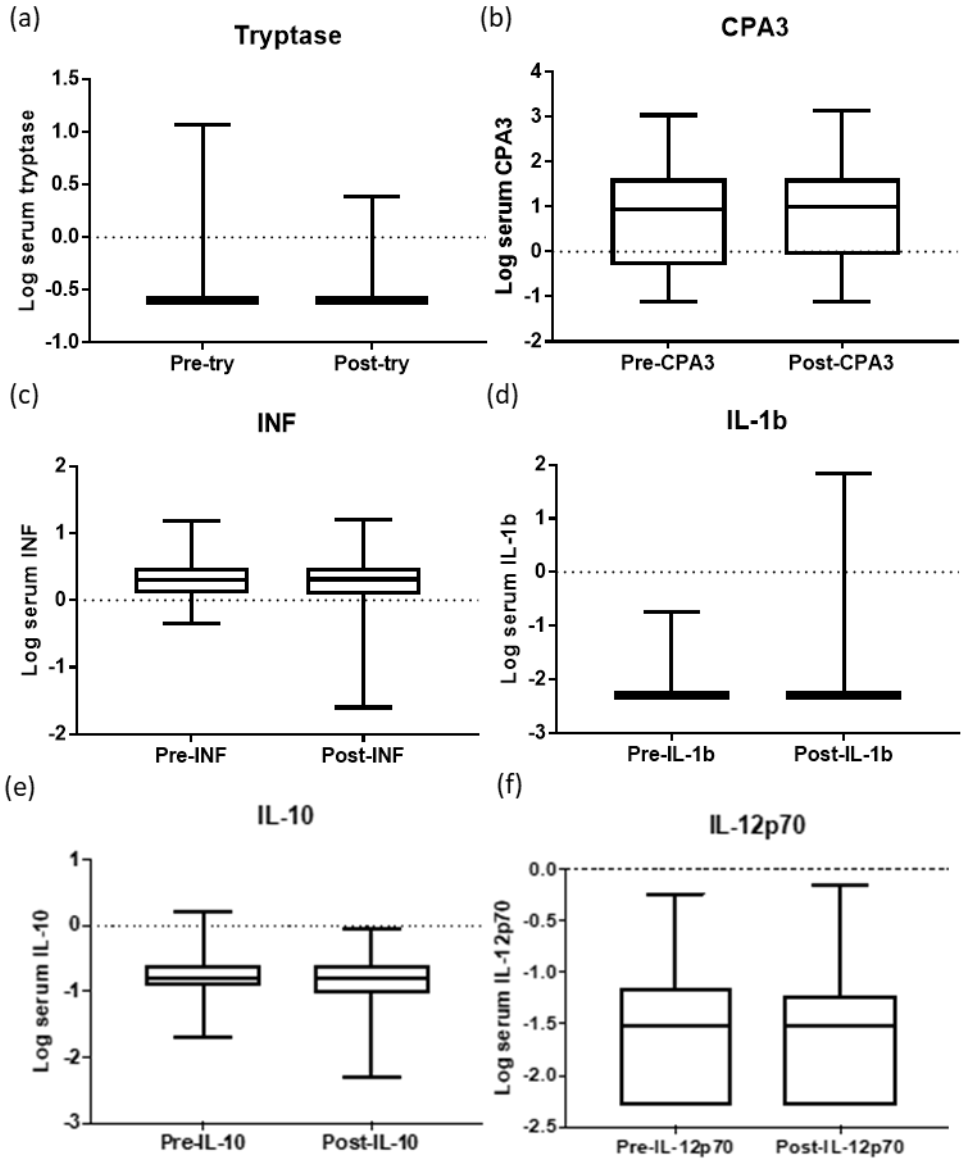


Figure 4.2 **Serum levels of mediators at baseline and post-challenge for the patients with positive outcome.**

Levels for (a) tryptase, (b) CPA3, (c) INF, (d) IL-1β, (e) IL-10, (f) IL-12p70, (g) IL-13, (h) IL-2, (i) IL-4, (j) IL-6, (k) IL-8 and (l) TNFα are shown. Statistical analysis was determined by Wilcoxon Signed Ranks test on log-transformed data (****p<0.0001).


(a)
Tryptase
Log serum tryptase
1.5
1.0
0.5
0.0
-0.5
-1.0
Pre-try
Post-try
(b)
CPA3
Log serum CPA3
4
3
2
1
0
-1
-2
Pre-CPA3
Post-CPA3
(c)
INF
Log serum INF
2
1
0
-1
-2
Pre-INF
Post-INF
(d)
IL-1b
Log serum IL-1b
2
1
0
-1
-2
-3
Pre-IL-1b
Post-IL-1b
(e)
IL-10
Log serum IL-10
1
0
-1
-2
-3
Pre-IL-10
Post-IL-10
(f)
IL-12p70
Log serum IL-12p70
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Pre-IL-12p70
Post-IL-12p70

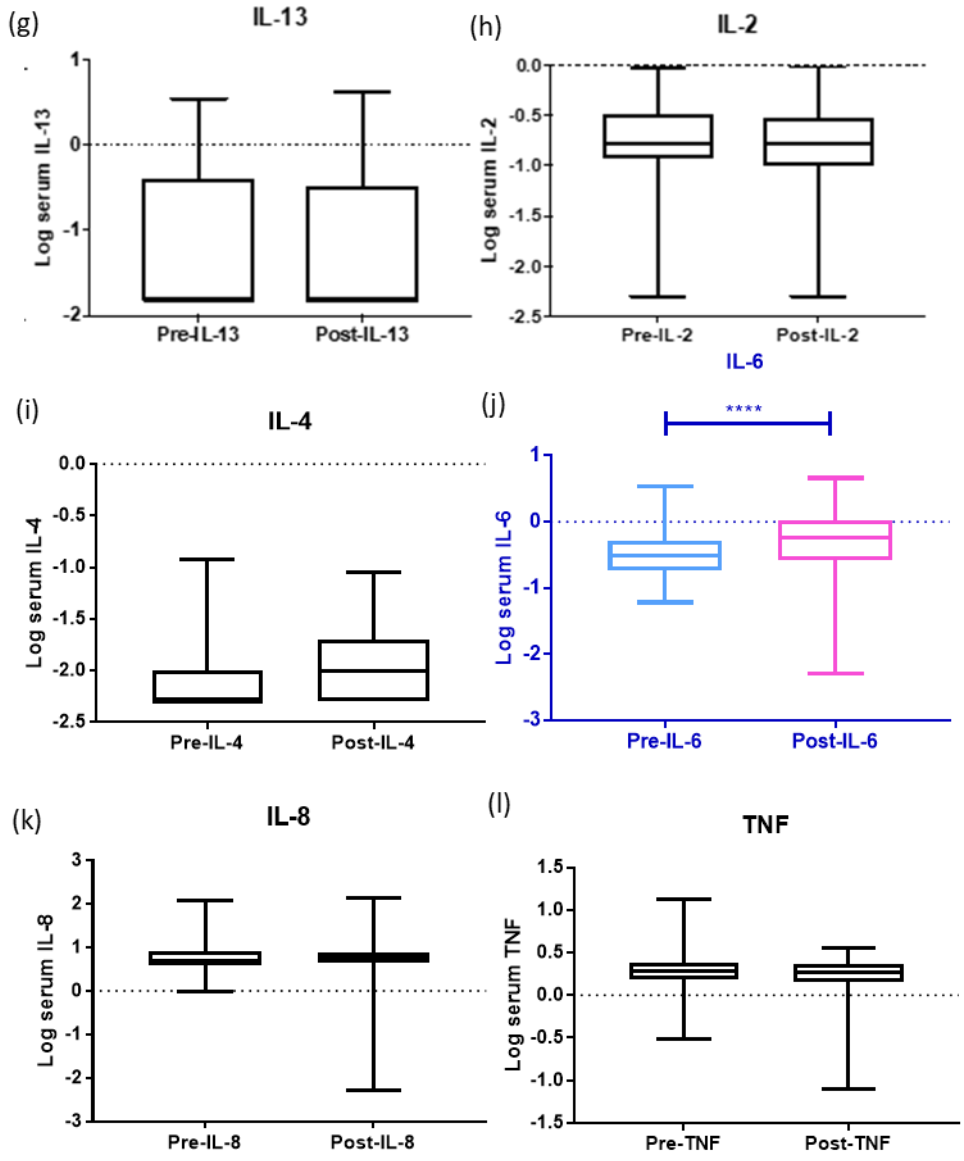
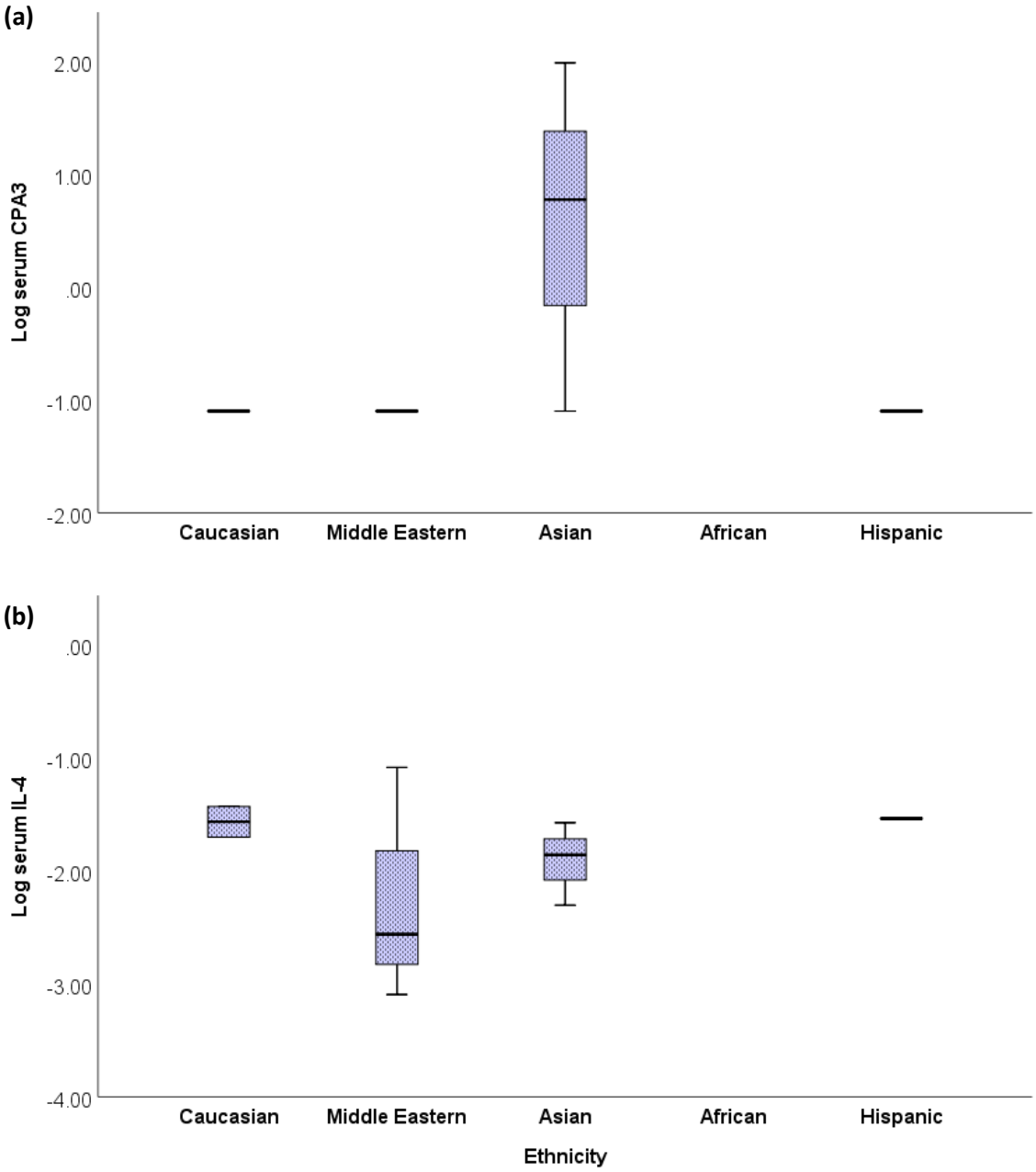


Figure 0.1 Serum levels of mast cell mediators at baseline and post-challenge for the patients with negative outcome.

Levels for (a) tryptase, (b) CPA3, (c) INF, (d) IL-1β, (e) IL-10, (f) IL-12p70, (g) IL-13, (h) IL-2, (i) IL-4, (j) IL-6, (k) IL-8 and (l) TNFα are shown. Statistical analysis was determined by Wilcoxon Signed Ranks test on log-transformed data (****p<0.0001).

Appendix E

This appendix includes the levels of CPA3, IL-4, IL-6 and IL-8 in relation to ethnicity (Figure 1). The analysis was performed in an age-, gender-, and clinically matched group of patients. Female patients (aged 20-40 years) who have had anaphylaxis as the diagnosis in the validation cohort were selected.



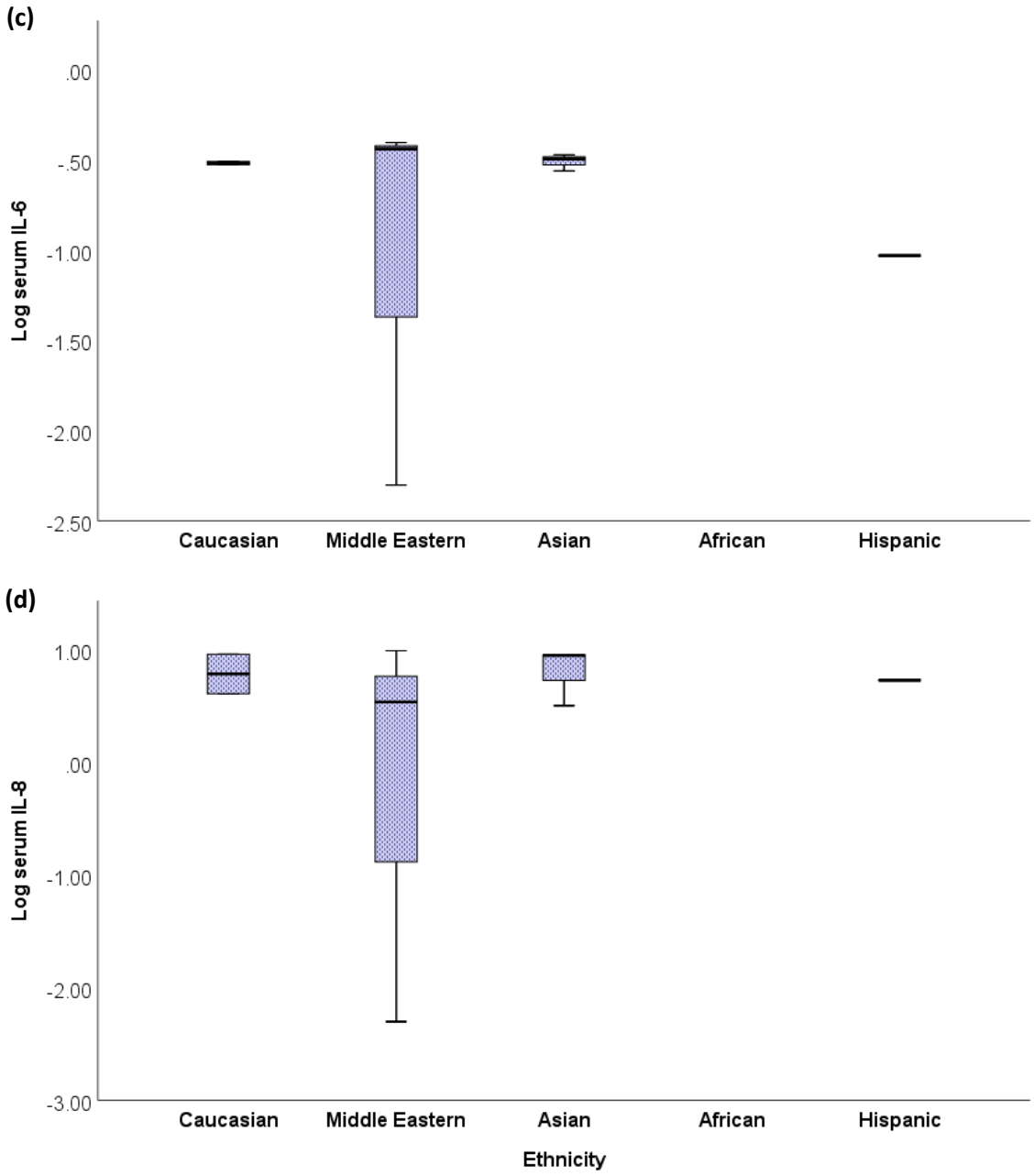


Figure 1 Lack of effect of ethnicity on levels of (a) CPA3, (b) IL-4, (c) IL-6 and (d) IL-8. Levels were compared in patients with different ethnic backgrounds. Statistical analysis was determined by the Kruskal-Wallis test on log-transformed data.

Appendix F

The work in this appendix, the isolation of *S. aureus* from tissue samples, *S. aureus* culture, RNA extraction and polymerase chain reaction, measurements of pro-inflammatory cytokines, was undertaken by my colleague Mr Tim Biggs, ENT specialist registrar.

S. aureus is a facultatively anaerobic, Gram-positive coccus and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates.

S. aureus is catalase-positive (meaning that it can produce the enzyme "catalase") and able to convert hydrogen peroxide (H_2O_2) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci.

A small percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test: *S. aureus* is primarily coagulase-positive (meaning that it can produce the enzyme "coagulase") that causes clot formation, whereas most other *Staphylococcus* species are coagulase-negative. However, while the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase.

Equipment & Chemicals

- Blood agar (Oxoid)
- Baird-Parker agar (Sigma)
- Egg yolk tellurite as supplement for Baird-Parker agar (Oxoid/Sigma)
- Trypticase soy broth (Oxoid/Sigma)
- Brain Heart Infusion (Oxoid)
- Hydrogen Peroxide (Sigma)
- Hydrochloric acid (Sigma)
- DNase agar (Sigma)
- Coagulase slide test (Sigma)
- Toluidine blue (Sigma)
- Incubator set at 37 °C
- Glass slides
- Bunsen burner & lighter
- Inoculation loops
- Universal tubes

Culture and Identification of *S. aureus*

Primary isolation media

- Inoculate DNase agar and blood agar plates and grow at 37 °C for 18-24 h.
- Examine for colonial morphology (cream or golden colour up to 3mm in diameter)
- Baird-Parker agar (Sigma), supplemented with egg yolk tellurite (Sigma) can be used to identify *S. aureus* as shown in the Table 1 when incubated for 48 h at 37 °C.

Table 1. Colonial morphology of *S. aureus* and other organisms on Baird-Parker agar.

Organisms	Colour of colony/comments
S. aureus	Grey-black, shiny colony due to reduction of tellurite. Surrounded by a zone of clearing
S. epidermidis	Not shiny black colony. Seldom produce clearing
S. saprophyticus	Irregular colony and may produce clearing/ Wide opaque zones may be produced in 24 h
Micrococcus	Very small colony in shades of brown and black. No clearing
Bacillus sp.	Dark matt brown colony with occasional clearing after 48 h
E. coli	Large brown-black colony
Proteus	Brown-black colony. No clearing

DNase production

This test is used to determine the ability of an organism to produce DNase. The test is used primarily to distinguish pathogenic Staphylcocci which produce large quantities of extracellular DNase. It reacts with medium containing DNA with the resulting hydrolysis of the DNA. The oligonucleotides liberated by the hydrolysis are soluble in acid and in a positive reaction, the addition of HCL results in a clear zone around the inoculum. A hazy zone is produced in a negative reaction, due the precipitation of DNA by HCL. In contrast to HCL, toluidine blue produces much more delineated zones of DNase activity.

Most strains of *S. aureus* hydrolyse DNA and give positive reactions in this test. However, some MRSA strains do not and some strains of coagulase-negative staphylocci may give weak reactions.

- Flood a DNase agar plate containing plated organism with Toluidine blue O solution (TBO/TBS @ 0.01-0.05 % [w/v] concentration) or 1M HCL.
- After 2 min, discard excess reagent
- TBO-positive reaction – TBO forms a complex with hydrolysed DNA to produce colonies surrounded by pink zone against a blue background
- HCL-positive reaction – colonies demonstrate a defined zone of clearing
- A negative result for both solutions equates to no zone of clearing.

Coagulase Production

Members of the genus Staphylococcus are differentiated by the ability to clot plasma by the action of the enzyme coagulase.

Coagulase exists in two forms: “bound coagulase” (or clumping factor) which is bound to the cell wall and “free coagulase” which is liberated by the cell wall. Bound coagulase is detected by the slide coagulase test, whereas free coagulase is detected by the tube coagulase test.

Bound coagulase absorbs fibrinogen from the plasma and alters it so it precipitates on the Staphylococci causing them to clump resulting in cell agglutination. The tube coagulase test detects both bound and free coagulase. Free coagulase reacts with a substance in plasma to form a fibrin clot.

Slide Coagulase test

- Place a drop of distilled water on a slide
- Emulsify the test strain to obtain and homogenous thick suspension. False negative reactions will occur if the bacterial suspension is not heavy enough
- Observe for auto-agglutination
- Dip a loop in the plasma and mix gently with the homogenous suspension
- A positive result will produce clumping within 10 seconds. Conversely, an negative control produces no visible clumping.
- Ensure you perform an auto-agglutination negative control test

Tube Coagulase Test

- Place approx. 1 ml of commercially available plasma suitable for tube coagulase in a sterile eppendorf
- Emulsify test colonies in the plasma and incubate for 4 h at 37 °C
- Examine for a clot which gels the whole contents of the tube or forms a loose web of fibrin
- If negative, incubate overnight at 22-25 °C and re-examine at 24 h.
- A positive result will produce a formation of a clot at 4 h following 37 °C incubation or following overnight incubation at 22-25 °C. A negative result will produce no clot at either time point.

Table 2. Tube and Slide Coagulase test results for a series of Staphylococci

Species	Tube Coagulase test	Slide Coagulase test
Staphylococcus aureus Subspecies aureus	+	+
Staphylococcus aureus Subspecies anerobius	+	-
Staphylococcus schleiferi Subspecies coagulans	+	-
Staphylococcus lugdunensis	-	(+)
Staphylococcus schleiferi Subspecies shleiferi	-	+
Staphylococcus delphini	+	-
Staphylococcus intermedius	+	d
Staphylococcus hyicus	d	-

*rare clinical isolates

d = 11-89 % of strains positive

(+) = delayed reaction

Catalase Test

This test is to detect the catalase enzyme present in most cytochrome-containing aerobic and facultatively anaerobic bacteria. Streptococcus and Enterococcus sp. are exceptions.

The catalase test is used to detect the presence of catalase enzymes by the decomposition of hydrogen peroxide to release oxygen and water. Hydrogen peroxide is formed by some bacteria as

an oxidative end product of the aerobic breakdown of sugars. If allowed to accumulate it is highly toxic to bacteria and can result in cell death. Catalase either decomposes hydrogen peroxide or oxidises secondary substrates, but it has no effect on other peroxides.

Media containing whole red blood cells will contain catalase and could give a false positive. Colonies taken from chocolate agar may be tested. Hydrogen peroxide is unstable and should be stored in a spark proof fridge. Avoid undue exposure to light. Cultures of anaerobic bacteria should be exposed to air for 30 min prior to testing.

- Place approx. 0.2 ml of hydrogen peroxide solution in a test tube
- Carefully pick a colony and rub the colony on the inside wall of the test tube above the surface of the hydrogen peroxide solution
- Cap the tube or bottle and tilt it to allow the hydrogen peroxide solution to cover the colony.
- Look for vigorous bubbling occurring within 10 sec.

Table 3. FINAL *S. aureus* confirmation results

Test	Result
Culture	Grows on DNase and blood agar plates to produce cream/golden colonies Baird-Parker agar produces grey-black, shiny colonies surrounded by zone of clearing
DNase test	Positive reaction (identified by zone of clearing)
Coagulase test	Positive reaction (identified by agglutination)
Catalase test	Positive (identified by bubbling around colony)
Further test suggested: PCR	

***S. aureus* Biofilm Growth**

S. aureus biofilms can be grown *in vitro* using brain heart infusion, Mueller-Hinton broth medium or Trypticase Soy broth for 24 h at 37 °C.

S. aureus bacterial culture experiments

S. aureus was isolated from CRS tissues by Mr Stephen Hayes. *S. aureus* stored in the mid-log growth phase as a glycerol stock, was streaked on a Colombia Blood Agar (CBA) plate (Life Technologies Ltd, Carlsbad, CA, USA) prior to overnight culture (37°C, 5% CO_2). Following incubation, 8 colony forming units were placed in 20mls of RPMI media and the bacteria grown to the mid-log phase. Serial optical density (OD) measurements were taken and plotted against CFU counts (**Figures 1**). These experiments were repeated separately 4 times, with linear correlation calculated (**Figure 2**).

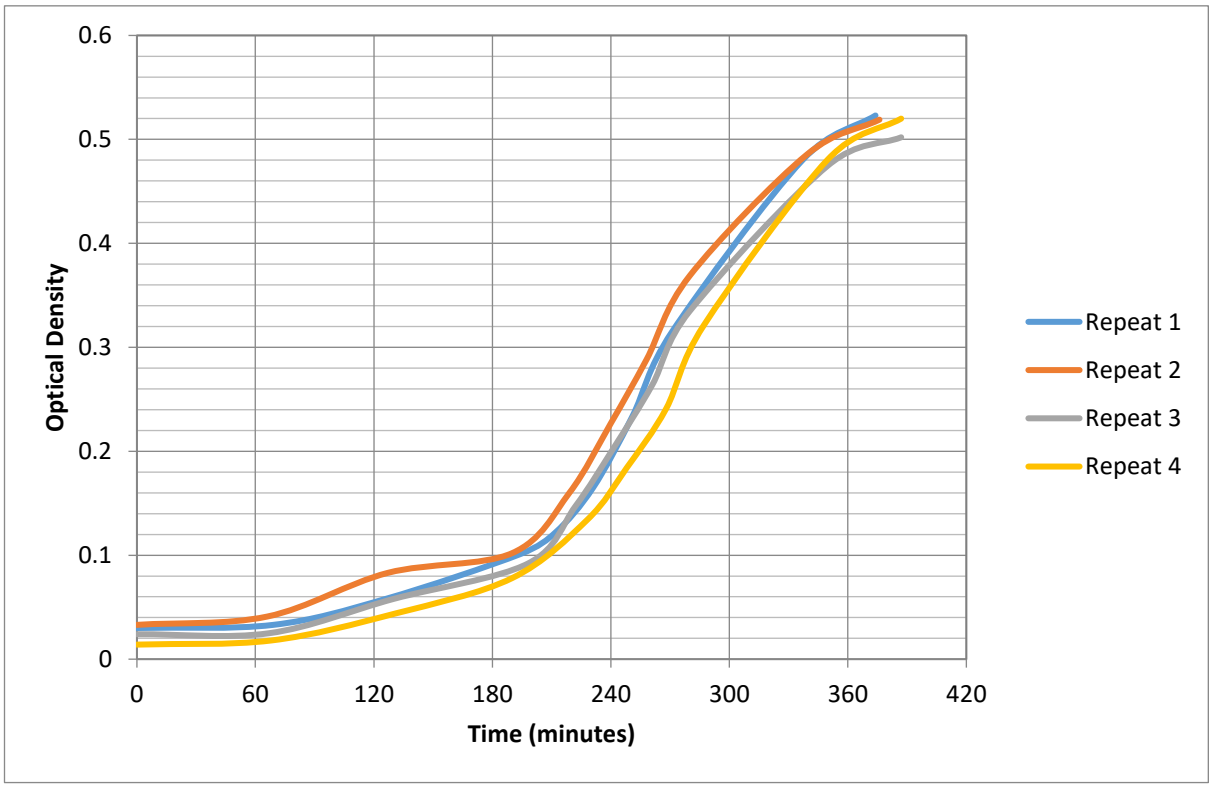


Figure 1 *S. aureus* optical density time course. *S. aureus* (P3 strain) was grown within RPMI media (37°C, 5% CO_2) and optical density measurements taken every 30 mins over 6 hours.

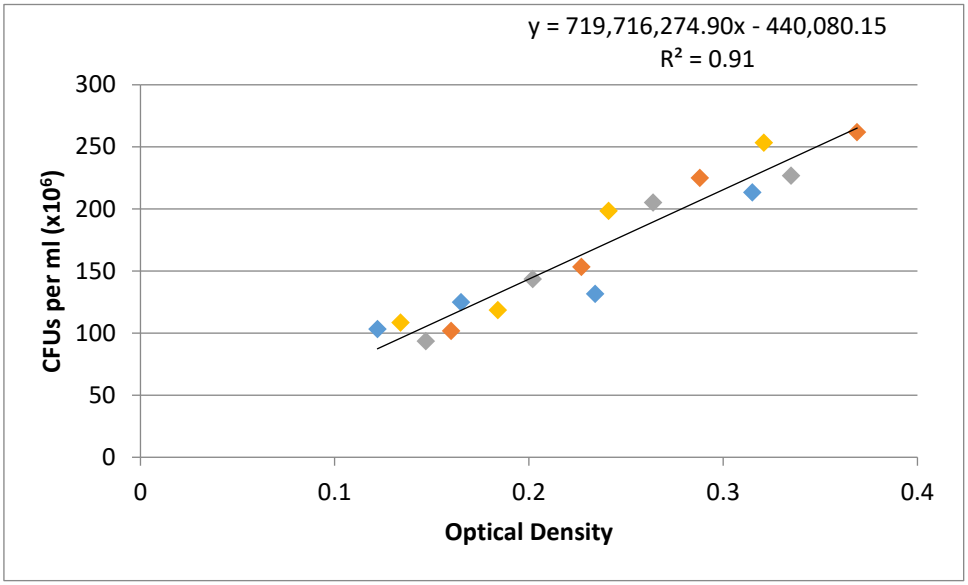


Figure 2 *S. aureus* optical density vs CFUs per ml (x10^6). *S. aureus* (P3 strain) was grown within RPMI media (37°C, 5% CO_2) to the log-phase, with optical density measurements and CFU enumeration undertaken every 30 mins over 6 hours.

S. aureus co-infection model

S. aureus was cultured with a number of cell lines to further our understanding of the uptake of intracellular bacteria and the associated immune response. *S. aureus* was grown to the mid-log phase with the optical density measured, and the number of viable bacteria calculated using the equation y = mx + c (**Figure 2**). *S. aureus* was washed two times and resuspended in HBSS. A known quantity of *S. aureus* was then added to mast cells (LAD2), at a multiplicity of infection ratio (MOI) of 1:1 over 4 hours.

Real-time quantitative polymerase chain reaction

This was conducted in an RNA/RNase and DNA/DNAase free environment.

RNA extraction and quantification

Column extraction with Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), was chosen over phenol extraction using TRIzol® (Life Technologies Ltd, Carlsbad, CA, USA), due to superior RNA quantity and quality. **Table 4** reveals the NanoDrop® ND-1000 spectrophotometer (Life Technologies Ltd, Carlsbad, CA, USA) results of the phenol (TRIzol®) extraction in comparison to column extraction (Qiagen RNeasy Mini Kit). **Figure 3** shows the spectrophotometer absorbance trace for a column extraction sample, revealing high purity and low levels of contamination.

Table 4 Comparison of phenol vs column RNA extraction.

	Mean (range) RNA yield	Mean 260/280nm absorbance ratio	Mean 260/230nm absorbance ratio
Phenol extraction (TRIzol®) n=12	41 (17-86) ng/µL	1.47	0.31
Column extraction (Qiagen RNeasy Mini Kit) n=24	62 (21-126) ng/µL	1.97	1.42

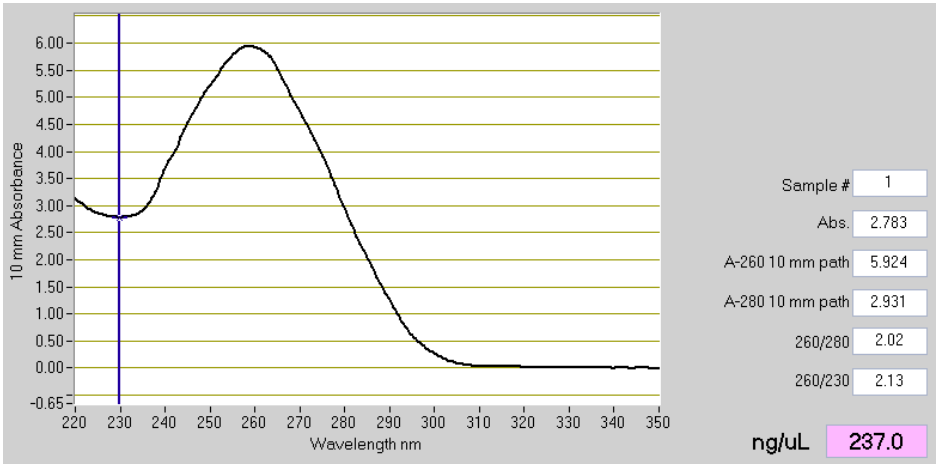


Figure 3 Column extraction spectrophotometer trace

In 350µL of buffer RLT, ribonucleic acid (RNA) extraction was undertaken using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Following a spin step (3 minutes at >8000 x g), and transfer of the supernatant into a fresh tube, 350µL of 70% ethanol was added to the lysate and mixed by pipetting. The resultant mixture, 700µL, was then transferred to a RNeasy Mini spin column, placed within a 2mL collection tube and centrifuged for 15 seconds at >8000 x g. The flow-through was discarded and the process repeated with RW1 and RPE buffers. These steps culminated in a drying step, where the column was air-spun for 1 minute, followed by an RNase-free water eluting spin. The eluting spin was repeated with the flow-through of the final step to increase RNA yield.

RNA was quantified, and assessed for purity, using the NanoDrop® ND-1000 spectrophotometer (Life Technologies Ltd, Carlsbad, CA, USA). Based on these results, some samples with low yield or purity were excluded. This also served to standardise the RNA volumes for the reverse transcription stage.

Reverse transcription

Reverse transcription was performed using the Thermofisher Scientific high capacity RNA-to-cDNA kit following the manufacturer's instructions (Life Technologies Ltd, Carlsbad, CA, USA). In brief,

500ng of RNA template was added to RNase-free 0.5mL microfuge tubes (Life Technologies Ltd, Carlsbad, CA, USA) and made up to a volume of 9µL and kept on ice. Following this, 10µL of RT Buffer Mix was added together with 1µL RT Enzyme Mix to each tube, making 20µL per reaction in total. Tubes were centrifuged for 30 seconds (2000rpm) and then placed within a thermal cycler for 60 minutes at 37°C, 5 minutes at 95°C, then held at 4°C. cDNA was stored at -20°C for later use.

Quantitative polymerase chain reaction

Due to standardisation of the volume of RNA template, all samples contained the same amount of cDNA, but differing ratios of target genes depending on the experimental conditions. cDNA samples were diluted by 1/20 in RNase-free water prior to plating.

Using a MicroAmp Optical 384-well reaction plate (Life Technologies Ltd, Carlsbad, CA, USA) 4.5µL of diluted cDNA was placed into each well in duplicate. 5µL of SYBR Green PCR Master Mix (Primerdesign Ltd, Southampton, Hampshire, UK) was added to 0.5µL of primer (Primerdesign Ltd, Southampton, Hampshire, UK), making a final reaction volume of 10µL. Once complete, the PCR plate was firmly sealed with a plastic cover (Life Technologies Ltd, Carlsbad, CA, USA), centrifuged (2 minutes at 1,500rpm) and the reaction undertaken (ABI 7900HT, Applied Biosystems, Foster City, CA, USA). The PCR protocol consisted of 2 mins at 95°C, 40 cycles of 95°C for 15 seconds and 60°C for 1 min, followed finally by a melting curve of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds. The expression of a housekeeping gene, Beta-actin (β-actin), was used to normalise for transcription and amplification variations among samples (306).

Real time quantitative polymerase chain reaction analysis

Results were analysed using the SDS v2.4 software (Applied Biosystems, Foster City, CA, USA). Prior to calculating the absolute quantification values, and C_T values, the baseline and threshold were set to account for any variations in the data. The dissociation curve of each primer was analysed prior to exporting the data, examining for the presence of primer dimers. **Figure 4** highlights the formation of a primer dimer, indicating the potential for non-specific binding. Such data was excluded, and the experiments repeated with new primers. Following export of the C_T values from

the SDS 2.4 software into excel, the comparative C_T method ($2^{-\Delta\Delta C_T}$) was used to calculate normalised relative fold change compared to controls (307).

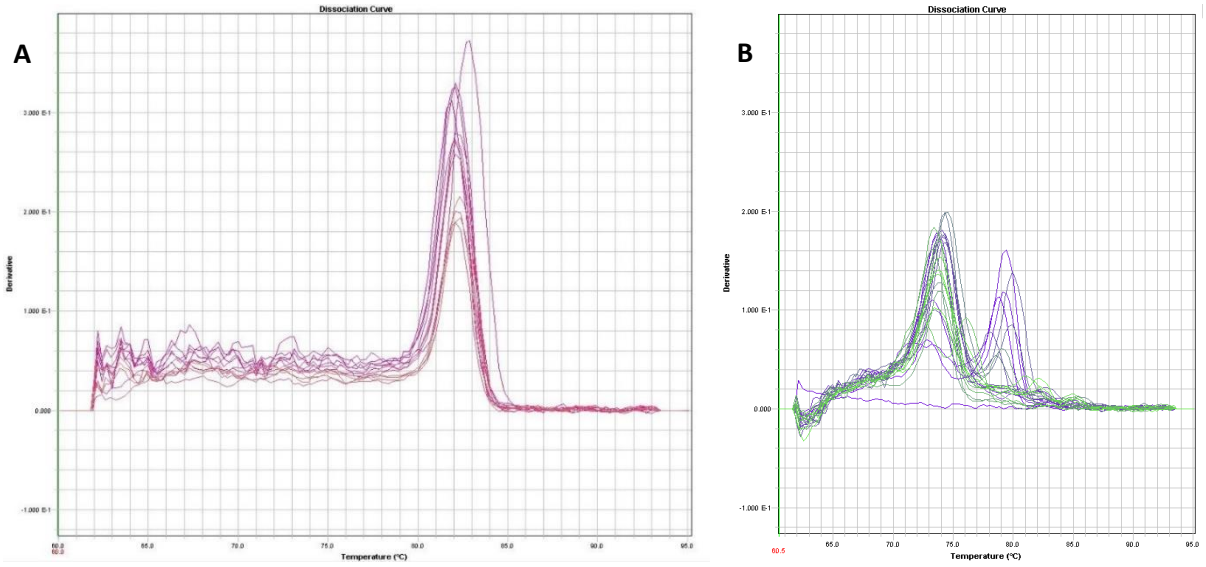


Figure 4 Primer dissociation curves. **A**, Normal melting curve. **B**, Abnormal melting curve indicating the formation of primer dimers.

Primer selection

Primer targets were selected in order to cover all of the transcriptional factors and effector cytokines of the main T-cell pathways. They were also selected to study the immune response to bacteria, together with remodelling and general inflammatory responses. Primer targets, and their main functions, are outlined in **Table 5.**

Gene name	Function
IL-1β	Anti-bacterial pro-inflammatory response
IL-8	Chemotaxis of immune cells in response to infection (Mainly Neutrophils)
TNFα	General immune response

Table 5 Prime targets and their main functions.

Measurement of pro-inflammatory cytokines released from LAD2 cells

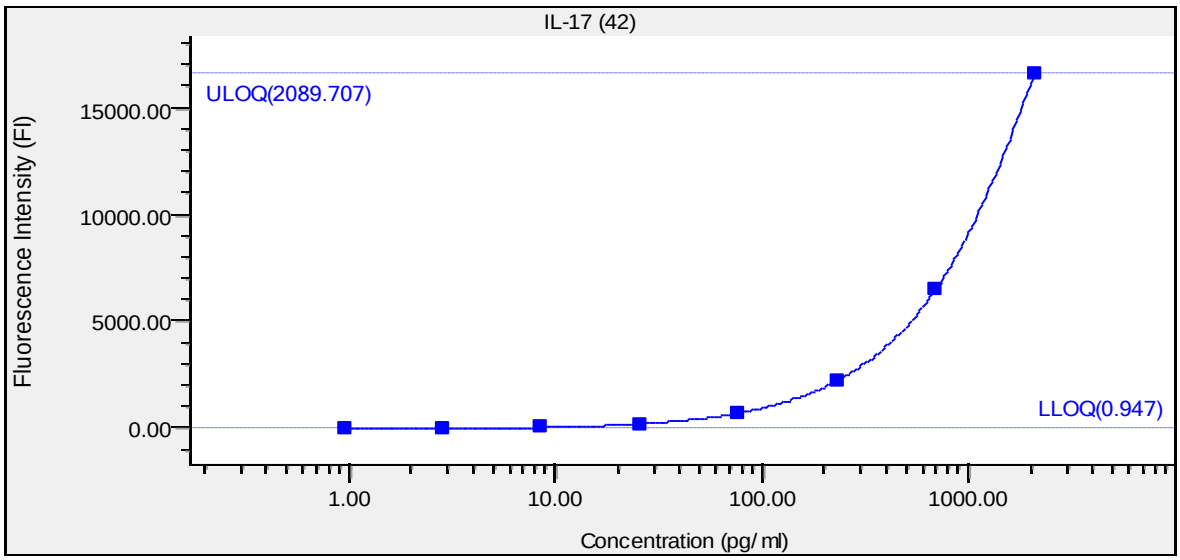
Culture supernatant protein analytes (IFNγ, IL-1β, IL-5, IL-6, MMP-7, TNFα and IL-17A) were quantified using Luminex (R&D systems Inc, Minneapolis, MN, USA) as per the manufacturer's instructions. Within a 96 well microplate, 50µL of the microparticle cocktail was added to each well. Following this, 50µL of each standard and experimental sample (in duplicate) was added. The plate was securely covered with a foil plate sealer and incubated at room temperature for 2 hours on a horizontal orbital microplate shaker (800rpm). Wells were washed three times with wash buffer (100µL), whilst utilising a microplate magnetic device attached to the bottom. 50µL of diluted Biotin Antibody Cocktail was added to each well, sealed, incubated and shaken (800rpm) for a further 1 hour. Each well was washed, as previously detailed, prior to the addition of 50µL diluted Steptavidin-PE. The plate was sealed, incubated and shaken (800rpm) for 30 minutes at room temperature. Wash steps were repeated, prior to plate reading using a Luminex analyser (Bio-Plex® 200 System, Bio-Rad Laboratories Ltd, Hercules, CA, USA). Total protein was measured for tissue samples, to standardise results, as detailed in section 2.6.2. **Table 6** outlines the cytokine concentration range within the standard curve, with **Figure 5** representing an example of a standard curve for IL-17A.

Cytokine	Lowest detectable concentration within the standard curve (pg/ml)	Highest detectable concentration within the standard curve (pg/ml)
IFNγ	4.66	11,322
IL-1β	1.54	3,922
IL-5	0.66	476
IL-6	1.64	1,176
MMP-7	44.11	33,136
TNFα	0.86	2,149
IL-17A	2.47	2,093

Table 6

Range of Luminex cytokine concentrations within the standard curve.

Figure 5 IL-17 Luminex standard curve.



Supplementary materials

https://doi.org/10.5258/SOTON/D0947

READ ME File For 'Data for Mast cell mediators: their sub cellular localisation, bacterial influence on their release and expression and their potential value as markers for predicting the severity of allergic reactions'

Dataset DOI: 10.5258/SOTON/D0947

ReadMe Author: Rana S. Abadalkareem, University of Southampton

This dataset supports the thesis entitled "Mast cell mediators: their sub cellular localisation, bacterial influence on their release and expression and their potential value as markers for predicting the severity of allergic reactions"

AWARDED BY: Faculty of Medicine, University of Southampton

DATE OF AWARD: 1st May 2018

DESCRIPTION OF THE DATA

This PhD project focused on the role of mast cell proteases in predicting the severity of allergic reactions to drugs or food. The project involved analysis of serum levels of mast cell proteases and a panel of pro-inflammatory cytokines in patients with allergic reactions provoked by drugs or food. The clinical and immunological data were combined and analysed by topological data analysis. In addition, factors that can modulate the release and expression of mast cell mediators and their subcellular localization were investigated.

We found that carboxypeptidase A3 (CPA3), a mast cell protease, have the potential to predict the severity of allergic reactions. Topological data analysis identified four novel multidimensional endotypes. Studies with LAD2 mast cell line culture showed that S. aureus could have an inhibitory effect on release and expression of mast cell mediators.

Supplementary materials

The potential for bacterial infection to interfere with mast cell responses could reduce the susceptibility to allergic reactions and as the mechanisms involved deserve consideration as a novel therapeutic approach to prevent development of severe reactions. Serum levels of CPA3 have the potential to predict the severity of allergic reactions to drugs or food. Identification of four multidimensional endotypes underlines the connection between levels of mast cell proteases and pro-inflammatory cytokines and their association with clinical features of patients who have drug or food allergies. The application of the characteristics of these endotypes clinically can help to identify those at particular risk of allergic reactions and allow optimal interventions to be undertaken

This dataset contains:

Spreadsheets and SPSS files of clinical and immunological parameters (log transformed) for two cohort studies (Southampton and Qatar). It also contains prism files of the effect of Staph aureus infection on the sensitization of LAD2 mast cell line, including levels of b-hexasominidase and cytokines and gene expression results. The results of MAPK analysis is also included (spreadsheet and prism file)

Date of data collection: Clinical data was collected from January 2015 to December 2016

Information about geographic location of data collection: University of Southampton, U.K.

Ethical approval: The study was approved by the Isle of Wight, Portsmouth and South East Hampshire Region Ethics Committee (registration number 08-H0501-17), and by the University Hospital Southampton NHS Foundation Trust Research and Development Department

Related projects/Funders:

The project was entirely funded by Iraqi Ministry of Higher Education and Scientific Research

Date that the file was created: May, 2019

List of References

1. Schnyder B, Pichler WJ. Mechanisms of drug-induced allergy. Mayo Clin Proc. 2009;84(3):268-72.

2. Caughey GH. Mast Cell Proteases as Protective and Inflammatory Mediators. Adv Exp Med Biol. 2011;716:212-34.

3. Pejler G, Ronnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. Blood. 2010;115(24):4981-90.

4. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol. 2014;14(7):478-94.

5. NICE. Anaphylaxis: assessment and referral after emergency treatment. NICE Guidlines: https://www.nice.org.uk/guidance/cg134/chapter/1-Recommendations; 2011.

6. Sahiner UM, Yavuz ST, Buyuktiryaki B, Cavkaytar O, Yilmaz EA, Tuncer A, et al. Serum basal tryptase may be a good marker for predicting the risk of anaphylaxis in children with food allergy. Allergy. 2014;69(2):265-8.

7. Yavuz ST, Sackesen C, Sahiner UM, Buyuktiryaki B, Arik Yilmaz E, Sekerel BE, et al. Importance of serum basal tryptase levels in children with insect venom allergy. Allergy. 2013;68(3):386-91.

8. Panesar SS, Javad S, de Silva D, Nwaru BI, Hickstein L, Muraro A, et al. The epidemiology of anaphylaxis in Europe: a systematic review. Allergy. 2013;68(11):1353-61.

9. Neugut AI, Ghatak AT, Miller RL. Anaphylaxis in the United States: an investigation into its epidemiology. Arch Intern Med. 2001;161(1):15-21.

10. Turner PJ, Gowland MH, Sharma V, Ierodiakonou D, Harper N, Garcez T, et al. Increase in anaphylaxis-related hospitalizations but no increase in fatalities: an analysis of United Kingdom national anaphylaxis data, 1992-2012. The Journal of allergy and clinical immunology. 2015;135(4):956-63 e1.

11. Motosue MS, Bellolio MF, Van Houten HK, Shah ND, Campbell RL. Increasing Emergency Department Visits for Anaphylaxis, 2005-2014. The journal of allergy and clinical immunology In practice. 2017;5(1):171-5 e3.

12. Gonzalez-Perez A, Aponte Z, Vidaurre CF, Rodriguez LA. Anaphylaxis epidemiology in patients with and patients without asthma: a United Kingdom database review. The Journal of allergy and clinical immunology. 2010;125(5):1098-104 e1.

13. Sheikh A, Alves B. Hospital admissions for acute anaphylaxis: time trend study. BMJ. 2000;320(7247):1441.

14. Liew WK, Williamson E, Tang ML. Anaphylaxis fatalities and admissions in Australia. The Journal of allergy and clinical immunology. 2009;123(2):434-42.

15. Jerschow E, Lin RY, Scaperotti MM, McGinn AP. Fatal anaphylaxis in the United States, 1999-2010: temporal patterns and demographic associations. The Journal of allergy and clinical immunology. 2014;134(6):1318-28 e7.

16. Fleischer DM, Perry TT, Atkins D, Wood RA, Burks AW, Jones SM, et al. Allergic reactions to foods in preschool-aged children in a prospective observational food allergy study. Pediatrics. 2012;130(1):e25-32.

17. Sampson MA, Munoz-Furlong A, Sicherer SH. Risk-taking and coping strategies of adolescents and young adults with food allergy. The Journal of allergy and clinical immunology. 2006;117(6):1440-5.

18. Gruchalla RS. Antibiotic drug allergy: challenges of diagnosis and treatment. Arbeiten aus dem Paul-Ehrlich-Institut. 1997(91):237-49.

19. Gibbison B, Sheikh A, McShane P, Haddow C, Soar J. Anaphylaxis admissions to UK critical care units between 2005 and 2009. Anaesthesia. 2012;67(8):833-9.

20. Tejedor Alonso MA, Moro Moro M, Mugica Garcia MV, Esteban Hernandez J, Rosado Ingelmo A, Vila Albelda C, et al. Incidence of anaphylaxis in the city of Alcorcon (Spain): a population-based study. Clin Exp Allergy. 2012;42(4):578-89.

21. Gaspar A, Santos N, Piedade S, Santa-Marta C, Pires G, Sampaio G, et al. One-year survey of paediatric anaphylaxis in an allergy department. European annals of allergy and clinical immunology. 2015;47(6):197-205.

22. Gomes E, Cardoso MF, Praca F, Gomes L, Marino E, Demoly P. Self-reported drug allergy in a general adult Portuguese population. Clin Exp Allergy. 2004;34(10):1597-601.

23. Worm M, Edenharter G, Rueff F, Scherer K, Pfohler C, Mahler V, et al. Symptom profile and risk factors of anaphylaxis in Central Europe. Allergy. 2012;67(5):691-8.

24. Brown SG. Clinical features and severity grading of anaphylaxis. The Journal of allergy and clinical immunology. 2004;114(2):371-6.

25. Buka RJ, Crossman RJ, Melchior CL, Huissoon AP, Hackett S, Dorrian S, et al. Anaphylaxis and ethnicity: higher incidence in British South Asians. Allergy. 2015;70(12):1580-7.

26. Wang Y, Allen KJ, Suaini NHA, Peters RL, Ponsonby AL, Koplin JJ. Asian children living in Australia have a different profile of allergy and anaphylaxis than Australian-born children: A State-wide survey. Clin Exp Allergy. 2018.

27. ISAAC Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Lancet. 1998;351(9111):1225-32.

28. Lai CK, Beasley R, Crane J, Foliaki S, Shah J, Weiland S, et al. Global variation in the prevalence and severity of asthma symptoms: phase three of the International Study of Asthma and Allergies in Childhood (ISAAC). Thorax. 2009;64(6):476-83.

29. Ait-Khaled N, Pearce N, Anderson HR, Ellwood P, Montefort S, Shah J, et al. Global map of the prevalence of symptoms of rhinoconjunctivitis in children: The International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three. Allergy. 2009;64(1):123-48.

30. Sampson HA, Munoz-Furlong A, Campbell RL, Adkinson NF, Jr., Bock SA, Branum A, et al. Second symposium on the definition and management of anaphylaxis: summary report--Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. The Journal of allergy and clinical immunology. 2006;117(2):391-7.

31. Muraro A, Roberts G, Worm M, Bilo MB, Brockow K, Fernandez Rivas M, et al. Anaphylaxis: guidelines from the European Academy of Allergy and Clinical Immunology. Allergy. 2014;69(8):1026-45.

32. Campbell RL, Hagan JB, Manivannan V, Decker WW, Kanthala AR, Bellolio MF, et al. Evaluation of national institute of allergy and infectious diseases/food allergy and anaphylaxis network criteria for the diagnosis of anaphylaxis in emergency department patients. The Journal of allergy and clinical immunology. 2012;129(3):748-52.

33. Kim H, Fischer D. Anaphylaxis. Allergy Asthma Clin Immunol. 2011;7 Suppl 1:S6.

34. de Silva IL, Mehr SS, Tey D, Tang ML. Paediatric anaphylaxis: a 5 year retrospective review. Allergy. 2008;63(8):1071-6.

35. Pumphrey RS. Lessons for management of anaphylaxis from a study of fatal reactions. Clin Exp Allergy. 2000;30(8):1144-50.

36. Treudler R, Kozovska Y, Simon JC. Severe immediate type hypersensitivity reactions in 105 German adults: when to diagnose anaphylaxis. Journal of investigational allergology & clinical immunology. 2008;18(1):52-8.

37. Stark BJ, Sullivan TJ. Biphasic and protracted anaphylaxis. The Journal of allergy and clinical immunology. 1986;78(1 Pt 1):76-83.

38. Khan BQ, Kemp SF. Pathophysiology of anaphylaxis. Curr Opin Allergy Clin Immunol. 2011;11(4):319-25.

39. Kalesnikoff J, Galli SJ. Anaphylaxis: mechanisms of mast cell activation. Chem Immunol Allergy. 2010;95:45-66.

40. Yu Y, Blokhuis BR, Garssen J, Redegeld FA. Non-IgE mediated mast cell activation. Eur J Pharmacol. 2016;778:33-43.

41. Johansson SG, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, et al. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. The Journal of allergy and clinical immunology. 2004;113(5):832-6.

42. Mondino A, Jenkins MK. Surface proteins involved in T cell costimulation. J Leukoc Biol. 1994;55(6):805-15.

43. Nakayama T, Yamashita M. The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. Semin Immunol. 2010;22(5):303-9.

44. Rautajoki KJ, Kylaniemi MK, Raghav SK, Rao K, Lahesmaa R. An insight into molecular mechanisms of human T helper cell differentiation. Ann Med. 2008;40(5):322-35.

45. Poulsen LK, Hummelshoj L. Triggers of IgE class switching and allergy development. Ann Med. 2007;39(6):440-56.

46. Ishizaka T, Hirata F, Ishizaka K, Axelrod J. Stimulation of phospholipid methylation, Ca2+ influx, and histamine release by bridging of IgE receptors on rat mast cells. Proceedings of the National Academy of Sciences of the United States of America. 1980;77(4):1903-6.

47. Yong LC. The mast cell: origin, morphology, distribution, and function. Exp Toxicol Pathol. 1997;49(6):409-24.

48. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol. 2010;10(6):440-52.

49. Urb M, Sheppard DC. The role of mast cells in the defence against pathogens. PLoS pathogens. 2012;8(4):e1002619.

50. Schwartz LB, Foley JV, Austen KF, Soter NA, Shepard R, Murphy GF. Localization of tryptase to human cutaneous mast cells and keratinocytes by immunofluorescence and immunoperoxidase cytochemistry with monoclonal antitryptase antibody. The Journal of allergy and clinical immunology. 1985;76(2 Pt 1):182-8.

51. Irani AA, Schechter NM, Craig S, Deblois G, Schwartz LB. 2 Human Mast-Cell Subsets with Different Neutral Protease Compositions. J Allergy Clin Immun. 1986;77(1):247-.

52. Irani AM, Bradford TR, Kepley CL, Schechter NM, Schwartz LB. Detection of MCT and MCTC types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies. J Histochem Cytochem. 1989;37(10):1509-15.

53. Irani AM, Goldstein SM, Wintroub BU, Bradford T, Schwartz LB. Human mast cell carboxypeptidase. Selective localization to MCTC cells. Journal of immunology. 1991;147(1):247-53.

54. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nature immunology. 2005;6(2):135-42.

55. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. Immunol Rev. 2007;217:141-54.

56. Trivedi NN, Caughey GH. Mast Cell Peptidases Chameleons of Innate Immunity and Host Defense. Am J Resp Cell Mol. 2010;42(3):257-67.

57. Fukuishi N, Murakami S, Ohno A, Yamanaka N, Matsui N, Fukutsuji K, et al. Does beta-hexosaminidase function only as a degranulation indicator in mast cells? The primary role of beta-hexosaminidase in mast cell granules. J Immunol. 2014;193(4):1886-94.

58. Lieberman P, Garvey LH. Mast Cells and Anaphylaxis. Curr Allergy Asthma Rep. 2016;16(3):20.

59. Amin K. The role of mast cells in allergic inflammation. Respir Med. 2012;106(1):9-14.

60. Peavy RD, Metcalfe DD. Understanding the mechanisms of anaphylaxis. Curr Opin Allergy Clin Immunol. 2008;8(4):310-5.

61. Ackermann D. Über den bacteriellen Abbau des Histidins. Physiol Chem. 1910;65:504.

62. Dale HH, Laidlaw PP. The physiological action of beta-iminazolylethylamine. The Journal of physiology. 1910;41(5):318-44.

63. Dale HH, Laidlaw PP. Histamine shock. The Journal of physiology. 1919;52(5):355-90.

64. Best CH, Dale HH, Dudley HW, Thorpe WV. The nature of the vaso-dilator constituents of certain tissue extracts. The Journal of physiology. 1927;62(4):397-417.

65. Dale HH, Dudley HW. The presence of histamine and acetylcholine in the spleen of the ox and the horse. The Journal of physiology. 1929;68(2):97-123.

66. Shahid M. Histamine, Histamine receptors, and their role in immunomodulation: An updated systematic review. The Open Immunology Journal 2009;2:9-41.

67. Riley JF, West GB. The presence of histamine in tissue mast cells. The Journal of physiology. 1953;120(4):528-37.

68. Code CF, Mitchell RG. Histamine, eosinophils and basophils in the blood. The Journal of physiology. 1957;136(3):449-68.

69. Imrie PR, Marley E, Thomas DV. Metabolism and distribution of exogenous histamine in cats. Br J Pharmacol. 1978;64(1):109-22.

70. Parsons ME, Ganellin CR. Histamine and its receptors. Br J Pharmacol. 2006;147 Suppl 1:S127-35.

71. Schwartz LB, Yunginger JW, Miller J, Bokhari R, Dull D. Time course of appearance and disappearance of human mast cell tryptase in the circulation after anaphylaxis. J Clin Invest. 1989;83(5):1551-5.

72. White MV. The role of histamine in allergic diseases. The Journal of allergy and clinical immunology. 1990;86(4 Pt 2):599-605.

73. MacGlashan D, Jr. Histamine: A mediator of inflammation. The Journal of allergy and clinical immunology. 2003;112(4 Suppl):S53-9.

74. Arrang JM, Garbarg M, Schwartz JC. Autoinhibition of histamine synthesis mediated by presynaptic H3-receptors. Neuroscience. 1987;23(1):149-57.

75. Zampeli E, Tiligada E. The role of histamine H4 receptor in immune and inflammatory disorders. Br J Pharmacol. 2009;157(1):24-33.

76. Schwartz LB, Metcalfe DD, Miller JS, Earl H, Sullivan T. Tryptase Levels as an Indicator of Mast-Cell Activation in Systemic-Anaphylaxis and Mastocytosis. New Engl J Med. 1987;316(26):1622-6.

77. Ren S, Sakai K, Schwartz LB. Regulation of human mast cell beta-tryptase: conversion of inactive monomer to active tetramer at acid pH. Journal of immunology. 1998;160(9):4561-9.

78. Pereira PJ, Bergner A, Macedo-Ribeiro S, Huber R, Matschiner G, Fritz H, et al. Human beta-tryptase is a ring-like tetramer with active sites facing a central pore. Nature. 1998;392(6673):306-11.

79. Harvima RJ, Harvima IT, Dull D, Dunder UK, Schwartz LB. Identification and characterization of multiple forms of tryptase from human mast cells. Arch Dermatol Res. 1999;291(2-3):73-80.

80. Miller JS, Moxley G, Schwartz LB. Cloning and characterization of a second complementary DNA for human tryptase. J Clin Invest. 1990;86(3):864-70.

81. Schwartz LB, Sakai K, Bradford TR, Ren S, Zweiman B, Worobec AS, et al. The alpha form of human tryptase is the predominant type present in blood at baseline in normal subjects and is elevated in those with systemic mastocytosis. J Clin Invest. 1995;96(6):2702-10.

82. Lyons JJ, Yu X, Hughes JD, Le QT, Jamil A, Bai Y, et al. Elevated basal serum tryptase identifies a multisystem disorder associated with increased TPSAB1 copy number. Nat Genet. 2016;48(12):1564-9.

83. Sakai K, Ren SL, Schwartz LB. A novel heparin-dependent processing pathway for human tryptase - Autocatalysis followed by activation with dipeptidyl peptidase I. J Clin Invest. 1996;97(4):988-95.

84. Schwartz LB. Clinical utility of tryptase levels in systemic mastocytosis and associated hematologic disorders. Leukemia Res. 2001;25(7):553-62.

85. Ono S, Kuwahara S, Takeuchi M, Sakashita H, Naito Y, Kondo T. Syntheses and evaluation of amidinobenzofuran derivatives as tryptase inhibitors. Bioorg Med Chem Lett. 1999;9(23):3285-90.

86. Oh SW, Pae CI, Lee DK, Jones F, Chiang GK, Kim HO, et al. Tryptase inhibition blocks airway inflammation in a mouse asthma model. Journal of immunology. 2002;168(4):1992-2000.

87. Hamilton MJ, Sinnamon MJ, Lyng GD, Glickman JN, Wang X, Xing W, et al. Essential role for mast cell tryptase in acute experimental colitis. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(1):290-5.

88. Cairns JA, Walls AF. Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. Journal of immunology. 1996;156(1):275-83.

89. Walls AF, He SH, Teran LM, Buckley MG, Jung KS, Holgate ST, et al. Granulocyte Recruitment by Human Mast-Cell Tryptase. Int Arch Allergy Imm. 1995;107(1-3):372-3.

90. He SH, Peng Q, Walls AF. Potent induction of a neutrophil and eosinophil-rich infiltrate in vivo by human mast cell tryptase: Selective enhancement of eosinophil recruitment by histamine. Journal of immunology. 1997;159(12):6216-25.

91. Compton SJ, Cairns JA, Holgate ST, Walls AF. The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: Tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. Journal of immunology. 1998;161(4):1939-46.

92. He SH, Walls AF. Human mast cell tryptase: A stimulus of microvascular leakage and mast cell activation. Eur J Pharmacol. 1997;328(1):89-97.

93. He S, Gaca MDA, Walls AF. A role for tryptase in the activation of human mast cells: Modulation of histamine release by tryptase and inhibitors of tryptase. J Pharmacol Exp Ther. 1998;286(1):289-97.

94. He SH, Gaca MDA, Walls AF. The activation of synovial mast cells: modulation of histamine release by tryptase and chymase and their inhibitors. Eur J Pharmacol. 2001;412(3):223-9.

95. He SH, Xie H. Modulation of tryptase and histamine release from human lung mast cells by protease inhibitors. Asian Pac J Allergy. 2004;22(4):205-12.

96. Huang C, Wong GW, Ghildyal N, Gurish MF, Sali A, Matsumoto R, et al. The tryptase, mouse mast cell protease 7, exhibits anticoagulant activity in vivo and in vitro due to its ability to degrade fibrinogen in the presence of the diverse array of protease inhibitors in plasma. J Biol Chem. 1997;272(50):31885-93.

97. Schwartz LB, Bradford TR, Littman BH, Wintroub BU. The fibrinogenolytic activity of purified tryptase from human lung mast cells. Journal of immunology. 1985;135(4):2762-7.

98. Shalit M, Schwartz LB, von Allmen C, Atkins PC, Lavker RM, Zweiman B. Release of histamine and tryptase during continuous and interrupted cutaneous challenge with allergen in humans. The Journal of allergy and clinical immunology. 1990;86(1):117-25.

99. Sommerhoff CP, Bode W, Pereira PJ, Stubbs MT, Sturzebecher J, Piechottka GP, et al. The structure of the human betaII-tryptase tetramer: fo(u)r better or worse. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(20):10984-91.

100. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. Immunol Rev. 2007;217:141-54.

101. Benditt EP, Arase M. An enzyme in mast cells with properties like chymotrypsin. The Journal of experimental medicine. 1959;110:451-60.

102. Wintroub BU, Kaempfer CE, Schechter NM, Proud D. A human lung mast cell chymotrypsin-like enzyme. Identification and partial characterization. J Clin Invest. 1986;77(1):196-201.

103. Gallwitz M, Hellman L. Rapid lineage-specific diversification of the mast cell chymase locus during mammalian evolution. Immunogenetics. 2006;58(8):641-54.

104. Heuston S, Hyland NP. Chymase inhibition as a pharmacological target: a role in inflammatory and functional gastrointestinal disorders? Br J Pharmacol. 2012;167(4):732-40.

105. Wolters PJ, Pham CT, Muilenburg DJ, Ley TJ, Caughey GH. Dipeptidyl peptidase I is essential for activation of mast cell chymases, but not tryptases, in mice. J Biol Chem. 2001;276(21):18551-6.

106. Reilly CF, Tewksbury DA, Schechter NM, Travis J. Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. J Biol Chem. 1982;257(15):8619-22.

107. Reilly CF, Schechter NB, Travis J. Inactivation of bradykinin and kallidin by cathepsin G and mast cell chymase. Biochemical and biophysical research communications. 1985;127(2):443-9.

108. Li M, Liu K, Michalicek J, Angus JA, Hunt JE, Dell'Italia LJ, et al. Involvement of chymase-mediated angiotensin II generation in blood pressure regulation. J Clin Invest. 2004;114(1):112-20.

109. Rueff F, Przybilla B, Bilo MB, Muller U, Scheipl F, Aberer W, et al. Predictors of severe systemic anaphylactic reactions in patients with Hymenoptera venom allergy: importance of baseline serum tryptase-a study of the European Academy of Allergology and Clinical Immunology Interest Group on Insect Venom Hypersensitivity. The Journal of allergy and clinical immunology. 2009;124(5):1047-54.

110. Sommerhoff CP, Caughey GH, Finkbeiner WE, Lazarus SC, Basbaum CB, Nadel JA. Mast cell chymase. A potent secretagogue for airway gland serous cells. Journal of immunology. 1989;142(7):2450-6.

111. Matin R, Tam EK, Nadel JA, Caughey GH. Distribution of chymase-containing mast cells in human bronchi. J Histochem Cytochem. 1992;40(6):781-6.

112. Yu CK, Chen CL. Activation of mast cells is essential for development of house dust mite Dermatophagoides farinae-induced allergic airway inflammation in mice. Journal of immunology. 2003;171(7):3808-15.

113. Rubinstein I, Nadel JA, Graf PD, Caughey GH. Mast cell chymase potentiates histamine-induced wheal formation in the skin of ragweed-allergic dogs. J Clin Invest. 1990;86(2):555-9.

114. Lazaar AL, Plotnick MI, Kucich U, Crichton I, Lotfi S, Das SK, et al. Mast cell chymase modifies cell-matrix interactions and inhibits mitogen-induced proliferation of human airway smooth muscle cells. Journal of immunology. 2002;169(2):1014-20.

115. Tchougounova E, Lundequist A, Fajardo I, Winberg JO, Abrink M, Pejler G. A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2. J Biol Chem. 2005;280(10):9291-6.

116. Wastling JM, Knight P, Ure J, Wright S, Thornton EM, Scudamore CL, et al. Histochemical and ultrastructural modification of mucosal mast cell granules in parasitized mice lacking the beta-chymase, mouse mast cell protease-1. Am J Pathol. 1998;153(2):491-504.

117. Knight PA, Wright SH, Lawrence CE, Paterson YY, Miller HR. Delayed expulsion of the nematode Trichinella spiralis in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. The Journal of experimental medicine. 2000;192(12):1849-56.

118. Orinska Z, Maurer M, Mirghomizadeh F, Bulanova E, Metz M, Nashkevich N, et al. IL-15 constrains mast cell-dependent antibacterial defenses by suppressing chymase activities. Nat Med. 2007;13(8):927-34.

119. Goldstein SM, Kaempfer C, Proud D, Schwartz LB, Irani AM, Wintroub BU. Detection and Partial Characterization of Carboxypeptidase in Human-Skin and Lung Mast-Cells. Clin Res. 1987;35(1):A249-A.

120. Goldstein SM, Kaempfer CE, Kealey JT, Wintroub BU. Human Mast-Cell Carboxypeptidase - Purification and Characterization. J Clin Invest. 1989;83(5):1630-6.

121. Reynolds DS, Gurley DS, Austen KF. Cloning and characterization of the novel gene for mast cell carboxypeptidase A. J Clin Invest. 1992;89(1):273-82.

122. Pejler G, Knight SD, Henningsson F, Wernersson S. Novel insights into the biological function of mast cell carboxypeptidase A. Trends Immunol. 2009;30(8):401-8.

123. Henningsson F, Ledin J, Lunderius C, Wilen M, Hellman L, Pejler G. Altered storage of proteases in mast cells from mice lacking heparin: A possible role for heparin carboxypeptidase A processing. Biol Chem. 2002;383(5):793-801.

124. Goldstein SM, Leong J, Schwartz LB, Cooke D. Protease Composition of Exocytosed Human Skin Mast-Cell Protease-Proteoglycan Complexes - Tryptase Resides in a Complex Distinct from Chymase and Carboxypeptidase. Journal of immunology. 1992;148(8):2475-82.

125. Kokkonen JO, Vartiainen M, Kovanen PT. Low-Density-Lipoprotein Degradation by Secretory Granules of Rat Mast-Cells - Sequential Degradation of Apolipoprotein-B by Granule Chymase and Carboxypeptidase-A. J Biol Chem. 1986;261(34):6067-72.

126. Dikov MM, Springman EB, Yeola S, Serafin WE. Processing of Procarboxypeptidase-a and Other Zymogens in Murine Mast-Cells. J Biol Chem. 1994;269(41):25897-904.

127. Henningsson F, Yamamoto K, Saftig P, Reinheckel T, Peters C, Knight SD, et al. A role for cathepsin E in the processing of mast-cell carboxypeptidase A. J Cell Sci. 2005;118(Pt 9):2035-42.

128. Henningsson F, Wolters P, Chapman HA, Caughey GH, Pejler G. Mast cell cathepsins C and S control levels of carboxypeptidase A and the chymase, mouse mast cell protease 5. Biol Chem. 2003;384(10-11):1527-31.

129. Lundequist A, Tchougounova E, Abrink M, Pejler G. Cooperation between mast cell carboxypeptidase A and the chymase mouse mast cell protease 4 in the formation and degradation of angiotensin II. J Biol Chem. 2004;279(31):32339-44.

130. Metz M, Piliponsky AM, Chen CC, Lammel V, Abrink M, Pejler G, et al. Mast cells can enhance resistance to snake and honeybee venoms. Science. 2006;313(5786):526-30.

131. Wolters PJ, Raymond WW, Blount JL, Caughey GH. Regulated expression, processing, and secretion of dog mast cell dipeptidyl peptidase I. J Biol Chem. 1998;273(25):15514-20.

132. Turk D, Janjic V, Stern I, Podobnik M, Lamba D, Dahl SW, et al. Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases. The EMBO journal. 2001;20(23):6570-82.

133. Wolters PJ, Laig-Webster M, Caughey GH. Dipeptidyl peptidase I cleaves matrix-associated proteins and is expressed mainly by mast cells in normal dog airways. Am J Respir Cell Mol Biol. 2000;22(2):183-90.

134. McGuire MJ, Lipsky PE, Thiele DL. Generation of active myeloid and lymphoid granule serine proteases requires processing by the granule thiol protease dipeptidyl peptidase I. J Biol Chem. 1993;268(4):2458-67.

135. McEuen AR, Ashworth DM, Walls AF. The conversion of recombinant human mast cell prochymase to enzymatically active chymase by dipeptidyl peptidase I is inhibited by heparin and histamine. Eur J Biochem. 1998;253(1):300-8.

136. Pejler G, Knight SD, Henningsson F, Wernersson S. Novel insights into the biological function of mast cell carboxypeptidase A. Trends Immunol. 2009;30(8):401-8.

137. Trivedi NN, Caughey GH. Mast cell peptidases: chameleons of innate immunity and host defense. Am J Respir Cell Mol Biol. 2010;42(3):257-67.

138. Clair JMS, Pham CTN, Villalta SA, Caughey GH, Wolters PJ. Mast cell dipeptidyl peptidase I mediates survival from sepsis. J Clin Invest. 2004;113(4):628-34.

139. Sutherland RE, Barry SS, Olsen JS, Salantes DB, Caughey GH, Wolters PJ. Dipeptidyl peptidase I controls survival from Klebsiella pneumoniae lung infection by processing surfactant protein D. Biochemical and biophysical research communications. 2014;450(1):818-23.

140. Hart TC, Hart PS, Bowden DW, Michalec MD, Callison SA, Walker SJ, et al. Mutations of the cathepsin C gene are responsible for Papillon-Lefevre syndrome. J Med Genet. 1999;36(12):881-7.

141. Pham CT, Ivanovich JL, Raptis SZ, Zehnbauer B, Ley TJ. Papillon-Lefevre syndrome: correlating the molecular, cellular, and clinical consequences of cathepsin C/dipeptidyl peptidase I deficiency in humans. Journal of immunology. 2004;173(12):7277-81.

142. Sadik CD, Noack B, Schacher B, Pfeilschifter J, Muhl H, Eickholz P. Cytokine production by leukocytes of Papillon-Lefevre syndrome patients in whole blood cultures. Clin Oral Invest. 2012;16(2):591-7.

143. Wolbing F, Fischer J, Koberle M, Kaesler S, Biedermann T. About the role and underlying mechanisms of cofactors in anaphylaxis. Allergy. 2013;68(9):1085-92.

144. Mullins RJ. Anaphylaxis: risk factors for recurrence. Clin Exp Allergy. 2003;33(8):1033-40.

145. Uguz A, Lack G, Pumphrey R, Ewan P, Warner J, Dick J, et al. Allergic reactions in the community: a questionnaire survey of members of the anaphylaxis campaign. Clin Exp Allergy. 2005;35(6):746-50.

146. Cercle d'Investigations Cliniques et Biologiques en Allergologie Alimentaire, CICBAA, http://www.cicbaa.org/pages_fr/allergovigilance/index.html. Severe food anaphylaxis. 2013.

147. Magerl M, Lammel V, Siebenhaar F, Zuberbier T, Metz M, Maurer M. Non-pathogenic commensal Escherichia coli bacteria can inhibit degranulation of mast cells. Exp Dermatol. 2008;17(5):427-35.

148. Blumer N, Sel S, Virna S, Patrascan CC, Zimmermann S, Herz U, et al. Perinatal maternal application of Lactobacillus rhamnosus GG suppresses allergic airway inflammation in mouse offspring. Clin Exp Allergy. 2007;37(3):348-57.

149. Wassenberg J, Nutten S, Audran R, Barbier N, Aubert V, Moulin J, et al. Effect of Lactobacillus paracasei ST11 on a nasal provocation test with grass pollen in allergic rhinitis. Clin Exp Allergy. 2011;41(4):565-73.

150. Kulka M, Fukuishi N, Rottem M, Mekori YA, Metcalfe DD. Mast cells, which interact with Escherichia coli, up-regulate genes associated with innate immunity and become less responsive to Fc(epsilon)RI-mediated activation. J Leukoc Biol. 2006;79(2):339-50.

151. Oksaharju A, Kankainen M, Kekkonen RA, Lindstedt KA, Kovanen PT, Korpela R, et al. Probiotic Lactobacillus rhamnosus downregulates FCER1 and HRH4 expression in human mast cells. World J Gastroenterol. 2011;17(6):750-9.

152. Kawahara T. Inhibitory effect of heat-killed Lactobacillus strain on immunoglobulin E-mediated degranulation and late-phase immune reactions of mouse bone marrow-derived mast cells. Anim Sci J. 2010;81(6):714-21.

153. Maulitz RM, Pratt DS, Schocket AL. Exercise-induced anaphylactic reaction to shellfish. The Journal of allergy and clinical immunology. 1979;63(6):433-4.

154. Matsuo H, Morimoto K, Akaki T, Kaneko S, Kusatake K, Kuroda T, et al. Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis. Clin Exp Allergy. 2005;35(4):461-6.

155. Pals KL, Chang RT, Ryan AJ, Gisolfi CV. Effect of running intensity on intestinal permeability. J Appl Physiol (1985). 1997;82(2):571-6.

156. Palosuo K, Varjonen E, Nurkkala J, Kalkkinen N, Harvima R, Reunala T, et al. Transglutaminase-mediated cross-linking of a peptic fraction of omega-5 gliadin enhances IgE reactivity in wheat-dependent, exercise-induced anaphylaxis. The Journal of allergy and clinical immunology. 2003;111(6):1386-92.

157. Gonzalez-Quintela A, Vidal C, Gude F. Alcohol, IgE and allergy. Addict Biol. 2004;9(3-4):195-204.

158. Alcoceba Borras E, Botey Faraudo E, Gaig Jane P, Bartolome Zavala B. Alcohol-induced anaphylaxis to grapes. Allergol Immunopathol (Madr). 2007;35(4):159-61.

159. Robson-Ansley P, Toit GD. Pathophysiology, diagnosis and management of exercise-induced anaphylaxis. Curr Opin Allergy Clin Immunol. 2010;10(4):312-7.

160. Cant AJ, Gibson P, Dancy M. Food hypersensitivity made life threatening by ingestion of aspirin. Br Med J (Clin Res Ed). 1984;288(6419):755-6.

161. Wang H, Wang HS, Liu ZP. Agents that induce pseudo-allergic reaction. Drug Discov Ther. 2011;5(5):211-9.

162. Flemstrom G, Marsden NV, Richter W. Passive cutaneous anaphylaxis in guinea pigs elicited by gastric absorption of dextran induced by acetylsalicylic acid. Int Arch Allergy Appl Immunol. 1976;51(5):627-36.

163. Mazur N, Patterson R, Perlman D. A case of idiopathic anaphylaxis associated with respiratory infections. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 1997;79(6):546-8.

164. Bousquet J, Menardo JL, Velasquez G, Michel FB. Systemic reactions during maintenance immunotherapy with honey bee venom. Ann Allergy. 1988;61(1):63-8.

165. Staden U, Rolinck-Werninghaus C, Brewe F, Wahn U, Niggemann B, Beyer K. Specific oral tolerance induction in food allergy in children: efficacy and clinical patterns of reaction. Allergy. 2007;62(11):1261-9.

166. Grunewald SM, Hahn C, Wohlleben G, Teufel M, Major T, Moll H, et al. Infection with influenza a virus leads to flu antigen-induced cutaneous anaphylaxis in mice. J Invest Dermatol. 2002;118(4):645-51.

167. Sharma BK, Talwar KK, Bhatnagar V, Kumar L, Ganguly NK, Mahajan RC. Recurrent anaphylaxis due to Plasmodium vivax infection. Lancet. 1979;1(8130):1340-1.

168. Vuitton DA. Echinococcosis and allergy. Clin Rev Allergy Immunol. 2004;26(2):93-104.

169. Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet. 2001;357(9262):1076-9.

170. Kalliomaki M, Salminen S, Poussa T, Arvilommi H, Isolauri E. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. Lancet. 2003;361(9372):1869-71.

171. Schiffer C, Lalanne AI, Cassard L, Mancardi DA, Malbec O, Bruhns P, et al. A strain of Lactobacillus casei inhibits the effector phase of immune inflammation. J Immunol. 2011;187(5):2646-55.

172. Kulka M FN, Rottem M, Mekori Y, Metacalfe D. Mast cells, which interact with Escherichia coli, up-regulate genes associated with innate immunity and become less responsive to Fc RI-mediated activation. Journal of leukocyte biology. 2006;79:339-50.

173. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis. 2005;5(12):751-62.

174. Valour F, Chebib N, Gillet Y, Reix P, Laurent F, Chidiac C, et al. [Staphylococcus aureus broncho-pulmonary infections]. Rev Pneumol Clin. 2013;69(6):368-82.

175. Foreman A, Boase S, Psaltis A, Wormald PJ. Role of bacterial and fungal biofilms in chronic rhinosinusitis. Curr Allergy Asthma Rep. 2012;12(2):127-35.

176. Abel J, Goldmann O, Ziegler C, Holtje C, Smeltzer MS, Cheung AL, et al. Staphylococcus aureus evades the extracellular antimicrobial activity of mast cells by promoting its own uptake. J Innate Immun. 2011;3(5):495-507.

177. Goldmann O, Tuchscherr L, Rohde M, Medina E. alpha-Hemolysin enhances Staphylococcus aureus internalization and survival within mast cells by modulating the expression of beta1 integrin. Cell Microbiol. 2016;18(6):807-19.

178. Matsui K, Kanai S, Ikuta M, Horikawa S. Mast Cells Stimulated with Peptidoglycan from Staphylococcus aureus Augment the Development of Th1 Cells. J Pharm Pharm Sci. 2018;21(1):296-304.

179. Wierzbicki M, Brzezinska-Blaszczyk E. Diverse effects of bacterial cell wall components on mast cell degranulation, cysteinyl leukotriene generation and migration. Microbiol Immunol. 2009;53(12):694-703.

180. Wide L, Bennich H, Johansson SG. Diagnosis of allergy by an in-vitro test for allergen antibodies. Lancet. 1967;2(7526):1105-7.

181. Celik-Bilgili S, Mehl A, Verstege A, Staden U, Nocon M, Beyer K, et al. The predictive value of specific immunoglobulin E levels in serum for the outcome of oral food challenges. Clin Exp Allergy. 2005;35(3):268-73.

182. Pastorello EA, Incorvaia C, Ortolani C, Bonini S, Canonica GW, Romagnani S, et al. Studies on the relationship between the level of specific IgE antibodies and the clinical expression of allergy: I. Definition of levels distinguishing patients with symptomatic from patients with asymptomatic allergy to common aeroallergens. The Journal of allergy and clinical immunology. 1995;96(5 Pt 1):580-7.

183. physicians Rco. Allergy: the umet need. Allergy: a brief guide to causes, diagnosis and management. London: Royal college of physicians of London; 2003.

184. Kranke B, Aberer W. Skin testing for IgE-mediated drug allergy. Immunol Allergy Clin North Am. 2009;29(3):503-16.

185. Heinzerling L, Mari A, Bergmann KC, Bresciani M, Burbach G, Darsow U, et al. The skin prick test - European standards. Clin Transl Allergy. 2013;3(1):3.

186. Eigenmann PA, Sampson HA. Interpreting skin prick tests in the evaluation of food allergy in children. Pediatr Allergy Immunol. 1998;9(4):186-91.

187. Salkind AR, Cuddy PG, Foxworth JW. The rational clinical examination. Is this patient allergic to penicillin? An evidence-based analysis of the likelihood of penicillin allergy. Jama. 2001;285(19):2498-505.

188. NICE. Drug Allergy: Diagnosis and Management of Drug Allergy in Adults, Children and Young People. NICE Guidlines: https://www.nice.org.uk/guidance/cg183/chapter/1-Recommendations; 2014.

189. Hamilton RG. Diagnosis and treatment of allergy to hymenoptera venoms. Curr Opin Allergy Clin Immunol. 2010;10(4):323-9.

190. Mirakian R, Ewan PW, Durham SR, Youlten LJ, Dugue P, Friedmann PS, et al. BSACI guidelines for the management of drug allergy. Clin Exp Allergy. 2009;39(1):43-61.

191. Knol EF, Mul FP, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. The Journal of allergy and clinical immunology. 1991;88(3 Pt 1):328-38.

192. Buhring HJ, Simmons PJ, Pudney M, Muller R, Jarrossay D, van Agthoven A, et al. The monoclonal antibody 97A6 defines a novel surface antigen expressed on human basophils and their multipotent and unipotent progenitors. Blood. 1999;94(7):2343-56.

193. Ocmant A, Mulier S, Hanssens L, Goldman M, Casimir G, Mascart F, et al. Basophil activation tests for the diagnosis of food allergy in children. Clin Exp Allergy. 2009;39(8):1234-45.

194. De Week AL, Sanz ML, Gamboa PM, Aberer W, Sturm G, Bilo MB, et al. Diagnosis of immediate-type beta-lactam allergy in vitro by flow-cytometric basophil activation test and sulfidoleukotriene production: a multicenter study. Journal of investigational allergology & clinical immunology. 2009;19(2):91-109.

195. Eberlein B, Leon Suarez I, Darsow U, Rueff F, Behrendt H, Ring J. A new basophil activation test using CD63 and CCR3 in allergy to antibiotics. Clin Exp Allergy. 2010;40(3):411-8.

196. Kim MS, Cho YJ. Flow Cytometry-Assisted Basophil Activation Test as a Safe Diagnostic Tool for Aspirin/NSAID Hypersenstivity. Allergy, asthma & immunology research. 2012;4(3):137-42.

197. Monneret G, Benoit Y, Debard AL, Gutowski MC, Topenot I, Bienvenu J. Monitoring of basophil activation using CD63 and CCR3 in allergy to muscle relaxant drugs. Clinical immunology. 2002;102(2):192-9.

198. Boumiza R, Monneret G, Forissier MF, Savoye J, Gutowski MC, Powell WS, et al. Marked improvement of the basophil activation test by detecting CD203c instead of CD63. Clin Exp Allergy. 2003;33(2):259-65.

199. Khan FM, Ueno-Yamanouchi A, Serushago B, Bowen T, Lyon AW, Lu C, et al. Basophil activation test compared to skin prick test and fluorescence enzyme immunoassay for aeroallergen-specific Immunoglobulin-E. Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology. 2012;8(1):1.

200. Gomez E, Blanca-Lopez N, Torres MJ, Requena G, Rondon C, Canto G, et al. Immunoglobulin E-mediated immediate allergic reactions to dipyrone: value of basophil activation test in the identification of patients. Clin Exp Allergy. 2009;39(8):1217-24.

201. Wolanczyk-Medrala A, Gogolewski G, Liebhart J, Gomulka K, Litwa M, Panaszek B, et al. A new variant of the basophil activation test for allergen-induced basophil CD63 upregulation. The effect of cetirizine. Journal of investigational allergology & clinical immunology. 2009;19(6):465-73.

202. Ozdemir SK, Guloglu D, Sin BA, Elhan AH, Ikinciogullari A, Misirligil Z. Reliability of basophil activation test using CD203c expression in diagnosis of pollen allergy. Am J Rhinol Allergy. 2011;25(6):e225-31.

203. Peternelj A, Silar M, Bajrovic N, Adamic K, Music E, Kosnik M, et al. Diagnostic value of the basophil activation test in evaluating Hymenoptera venom sensitization. Wien Klin Wochenschr. 2009;121(9-10):344-8.

204. De Weck A, Sanz Maria L, Gamboa P, Aberer W, Bienvenu J, Blanca M, et al. Diagnostic Tests Based on Human Basophils: More Potentials and Perspectives Than Pitfalls. II. Technical Issues. J Invest Allerg Clin. 2008;18(3):143-55.

205. Larsen LF, Juel-Berg N, Hansen KS, Clare Mills EN, van Ree R, Poulsen LK, et al. A comparative study on basophil activation test, histamine release assay, and passive sensitization histamine release assay in the diagnosis of peanut allergy. Allergy. 2018;73(1):137-44.

206. Srivastava S, Huissoon AP, Barrett V, Hackett S, Dorrian S, Cooke MW, et al. Systemic reactions and anaphylaxis with an acute serum tryptase >/=14 mug/L: retrospective characterisation of aetiology, severity and adherence to National Institute of Health and Care Excellence (NICE) guidelines for serial tryptase measurements and specialist referral. J Clin Pathol. 2014;67(7):614-9.

207. Ordoqui E, Zubeldia JM, Aranzabal A, Rubio M, Herrero T, Tornero P, et al. Serum tryptase levels in adverse drug reactions. Allergy. 1997;52(11):1102-5.

208. Zhou X, Buckley MG, Lau LC, Sumrners C, Pumphrey RSH, Walls AF. Mast cell carboxypeptidase as a new clinical marker for anaphylaxis. J Allergy Clin Immun. 2006;117(2):S85-S.

209. A R, Baranwal AK. Child with Allergies or Allergic Reactions. Indian J Pediatr. 2018;85(1):60-5.

210. Simons FE. Anaphylaxis pathogenesis and treatment. Allergy. 2011;66 Suppl 95:31-4.

211. Lieberman P, Nicklas RA, Randolph C, Oppenheimer J, Bernstein D, Bernstein J, et al. Anaphylaxis--a practice parameter update 2015. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2015;115(5):341-84.

212. Lieberman P, Nicklas RA, Oppenheimer J, Kemp SF, Lang DM, Bernstein DI, et al. The diagnosis and management of anaphylaxis practice parameter: 2010 update. The Journal of allergy and clinical immunology. 2010;126(3):477-80 e1-42.

213. Du Toit G, Roberts G, Sayre PH, Bahnson HT, Radulovic S, Santos AF, et al. Randomized trial of peanut consumption in infants at risk for peanut allergy. The New England journal of medicine. 2015;372(9):803-13.

214. Pajno GB. Oral desensitization for milk allergy in children: state of the art. Curr Opin Allergy Clin Immunol. 2011;11(6):560-4.

215. Whitworth H. Markers of allergic reactions to food based on activation of mast cells and basophils. Southampton: Southampton; 2011.

216. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676-82.

217. Niles AL, Maffitt M, Haak-Frendscho M, Wheeless CJ, Johnson DA. Recombinant human mast cell tryptase beta: stable expression in Pichia pastoris and purification of fully active enzyme. Biotechnology and applied biochemistry. 1998;28 (Pt 2):125-31.

218. Carlsson G. Topology and data. AMS Bulletin. 2009;46(2):255-308.

219. Offroy M, Duponchel L. Topological data analysis: A promising big data exploration tool in biology, analytical chemistry and physical chemistry. Anal Chim Acta. 2016;910:1-11.

220. Hinks TS, Brown T, Lau LC, Rupani H, Barber C, Elliott S, et al. Multidimensional endotyping in patients with severe asthma reveals inflammatory heterogeneity in matrix metalloproteinases and chitinase 3-like protein 1. The Journal of allergy and clinical immunology. 2016;138(1):61-75.

221. Rossi-deVries J, Pedoia V, Samaan MA, Ferguson AR, Souza RB, Majumdar S. Using multidimensional topological data analysis to identify traits of hip osteoarthritis. J Magn Reson Imaging. 2018;48(4):1046-58.

222. Banerjee S, Tian T, Wei Z, Peck KN, Shih N, Chalian AA, et al. Microbial Signatures Associated with Oropharyngeal and Oral Squamous Cell Carcinomas. Scientific reports. 2017;7(1):4036.

223. Banerjee S, Tian T, Wei Z, Shih N, Feldman MD, Alwine JC, et al. The ovarian cancer oncobiome. Oncotarget. 2017;8(22):36225-45.

224. Bruno JL, Romano D, Mazaika P, Lightbody AA, Hazlett HC, Piven J, et al. Longitudinal identification of clinically distinct neurophenotypes in young children with fragile X syndrome. Proceedings of the National Academy of Sciences of the United States of America. 2017;114(40):10767-72.

225. Lin TJ, Gao Z, Arock M, Abraham SN. Internalization of FimH+ Escherichia coli by the human mast cell line (HMC-1 5C6) involves protein kinase C. J Leukoc Biol. 1999;66(6):1031-8.

226. Malaviya R, Ross EA, MacGregor JI, Ikeda T, Little JR, Jakschik BA, et al. Mast cell phagocytosis of FimH-expressing enterobacteria. J Immunol. 1994;152(4):1907-14.

227. Lin TJ, Garduno R, Boudreau RT, Issekutz AC. Pseudomonas aeruginosa activates human mast cells to induce neutrophil transendothelial migration via mast cell-derived IL-1 alpha and beta. J Immunol. 2002;169(8):4522-30.

228. Munoz S, Rivas-Santiago B, Enciso JA. Mycobacterium tuberculosis entry into mast cells through cholesterol-rich membrane microdomains. Scand J Immunol. 2009;70(3):256-63.

229. Butterfield JH, Weiler D, Dewald G, Gleich GJ. Establishment of an immature mast cell line from a patient with mast cell leukemia. Leuk Res. 1988;12(4):345-55.

230. Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. Leuk Res. 2003;27(8):677-82.

231. Laidlaw TM, Steinke JW, Tinana AM, Feng C, Xing W, Lam BK, et al. Characterization of a novel human mast cell line that responds to stem cell factor and expresses functional FcepsilonRI. The Journal of allergy and clinical immunology. 2011;127(3):815-22 e1-5.

232. Guhl S, Babina M, Neou A, Zuberbier T, Artuc M. Mast cell lines HMC-1 and LAD2 in comparison with mature human skin mast cells--drastically reduced levels of tryptase and chymase in mast cell lines. Exp Dermatol. 2010;19(9):845-7.

233. Hill PB, MacDonald AJ, Thornton EM, Newlands GF, Galli SJ, Miller HR. Stem cell factor enhances immunoglobulin E-dependent mediator release from cultured rat bone marrow-derived mast cells: activation of previously unresponsive cells demonstrated by a novel ELISPOT assay. Immunology. 1996;87(2):326-33.

234. Hundley TR, Gilfillan AM, Tkaczyk C, Andrade MV, Metcalfe DD, Beaven MA. Kit and FcepsilonRI mediate unique and convergent signals for release of inflammatory mediators from human mast cells. Blood. 2004;104(8):2410-7.

235. Tkaczyk C, Horejsi V, Iwaki S, Draber P, Samelson LE, Satterthwaite AB, et al. NTAL phosphorylation is a pivotal link between the signaling cascades leading to human mast cell degranulation following Kit activation and Fc epsilon RI aggregation. Blood. 2004;104(1):207-14.

236. Wallace PK. Fixing suspension cells to slides for confocal microscopy. 2011.

237. Manders EM, Stap J, Brakenhoff GJ, van Driel R, Aten JA. Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. J Cell Sci. 1992;103 (Pt 3):857-62.

238. Manders E, Verbeek F, Aten J. Measurement of co-localisation of objects in dual-colour confocal images. Journal of Microscopy. 1993;169(3):375-82.

239. Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, Lockett S. Automatic and quantitative measurement of protein-protein colocalization in live cells. Biophys J. 2004;86(6):3993-4003.

240. Tharp MD, Seelig LL, Jr., Tigelaar RE, Bergstresser PR. Conjugated avidin binds to mast cell granules. J Histochem Cytochem. 1985;33(1):27-32.

241. Itzhak DN, Tyanova S, Cox J, Borner GH. Global, quantitative and dynamic mapping of protein subcellular localization. Elife. 2016;5.

242. U.S. Department of Health and Human Services FDA. Guidance for industry: Bioanalytical method validation. United states of America:

http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070107.pdf; 2001.

243. Castillo FJ, Mullen LJ, Grant BC, DeLeon J, Thrift JC, Chang LW, et al. Hybridoma stability. Developments in biological standardization. 1994;83:55-64.

244. Rupa P, Mine Y. Immunological comparison of native and recombinant egg allergen, ovalbumin, expressed in Escherichia coli. Biotechnology letters. 2003;25(22):1917-24.

245. Furger E, Fedosov SN, Lildballe DL, Waibel R, Schibli R, Nexo E, et al. Comparison of recombinant human haptocorrin expressed in human embryonic kidney cells and native haptocorrin. PloS one. 2012;7(5):e37421.

246. Chandra S, Latt N, Jariwala U, Palabindala V, Thapa R, Alamelumangapuram C, et al. A cohort study for the derivation and validation of a clinical prediction scale for hospital-onset Clostridium difficile infection. Can J Gastroenterol. 2012;26:885-8.

247. Sengupta N, Tapper EB. Derivation and Internal Validation of a Clinical Prediction Tool for 30-Day Mortality in Lower Gastrointestinal Bleeding. Am J Med. 2017;130(5):601 e1- e8.

248. Kelly C, Gangur V. Sex Disparity in Food Allergy: Evidence from the PubMed Database. J Allergy (Cairo). 2009;2009:159845.

249. Brown SG, Blackman KE, Heddle RJ. Can serum mast cell tryptase help diagnose anaphylaxis? Emerg Med Australas. 2004;16(2):120-4.

250. Steptoe A, Hamer M, Chida Y. The effects of acute psychological stress on circulating inflammatory factors in humans: a review and meta-analysis. Brain Behav Immun. 2007;21(7):901-12.

251. Rohleder N, Aringer M, Boentert M. Role of interleukin-6 in stress, sleep, and fatigue. Ann N Y Acad Sci. 2012;1261:88-96.

252. Petersen AM, Pedersen BK. The anti-inflammatory effect of exercise. J Appl Physiol (1985). 2005;98(4):1154-62.

253. Serafin WE, Dayton ET, Gravallese PM, Austen KR, Stevens RL. Carboxypeptidase-a in Mouse Mast-Cells - Identification, Characterization, and Use as a Differentiation Marker. Journal of immunology. 1987;139(11):3771-6.

254. Ribbing C, Engblom C, Lappalainen J, Lindstedt K, Kovanen PT, Karlsson MA, et al. Mast cells generated from patients with atopic eczema have enhanced levels of granule mediators and an impaired Dectin-1 expression. Allergy. 2011;66(1):110-9.

255. Schwartz LB. Cellular inflammation in asthma: neutral proteases of mast cells. Am Rev Respir Dis. 1992;145(2 Pt 2):S18-21.

256. Mahmood M, Rashid B, Amin K, Tofiq D, Nore B. Correlation between serum tryptase level and disease severity in asthmatic patients in the Sulaimani Governorate. International Journal of Medical Research and Health Science. 2016;5(6):34-41.

257. Tunon de Lara JM, Okayama Y, McEuen AR, Heusser CH, Church MK, Walls AF. Release and inactivation of interleukin-4 by mast cells. Ann N Y Acad Sci. 1994;725:50-8.

258. Waern I, Karlsson I, Pejler G, Wernersson S. IL-6 and IL-17A degradation by mast cells is mediated by a serglycin:serine protease axis. Immun Inflamm Dis. 2016;4(1):70-9.

259. Zhao W, Oskeritzian CA, Pozez AL, Schwartz LB. Cytokine production by skin-derived mast cells: endogenous proteases are responsible for degradation of cytokines. Journal of immunology. 2005;175(4):2635-42.

260. Mullan CS, Riley M, Clarke D, Tatler A, Sutcliffe A, Knox AJ, et al. Beta-tryptase regulates IL-8 expression in airway smooth muscle cells by a PAR-2-independent mechanism. Am J Respir Cell Mol Biol. 2008;38(5):600-8.

261. Romagnani S. Cytokines and chemoattractants in allergic inflammation. Mol Immunol. 2002;38(12-13):881-5.

262. Theoharides TC, Kalogeromitros D. The critical role of mast cells in allergy and inflammation. Ann N Y Acad Sci. 2006;1088:78-99.

263. Alvarez-Rodriguez L, Lopez-Hoyos M, Munoz-Cacho P, Martinez-Taboada VM. Aging is associated with circulating cytokine dysregulation. Cell Immunol. 2012;273(2):124-32.

264. Forsey RJ, Thompson JM, Ernerudh J, Hurst TL, Strindhall J, Johansson B, et al. Plasma cytokine profiles in elderly humans. Mech Ageing Dev. 2003;124(4):487-93.

265. Giuliani N, Sansoni P, Girasole G, Vescovini R, Passeri G, Passeri M, et al. Serum interleukin-6, soluble interleukin-6 receptor and soluble gp130 exhibit different patterns of age- and menopause-related changes. Exp Gerontol. 2001;36(3):547-57.

266. Carpagnano GE, Turchiarelli V, Spanevello A, Palladino GP, Barbaro MP. Aging and airway inflammation. Aging Clin Exp Res. 2013;25(3):239-45.

267. Bermudez DM, Canning DA, Liechty KW. Age and pro-inflammatory cytokine production: wound-healing implications for scar-formation and the timing of genital surgery in boys. J Pediatr Urol. 2011;7(3):324-31.

268. Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(8):3730-4.

269. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest. 1999;103(6):779-88.

270. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science. 1998;282(5397):2261-3.

271. Sinha S, Kaler LJ, Proctor TM, Teuscher C, Vandenbark AA, Offner H. IL-13-mediated gender difference in susceptibility to autoimmune encephalomyelitis. Journal of immunology. 2008;180(4):2679-85.

272. Davoodi P, Mahesh PA, Holla AD, Vijayakumar GS, Jayaraj BS, Chandrashekara S, et al. Serum levels of interleukin-13 and interferon-gamma from adult patients with asthma in Mysore. Cytokine. 2012;60(2):431-7.

273. Kirshenbaum AS, Petrik A, Walsh R, Kirby TL, Vepa S, Wangsa D, et al. A ten-year retrospective analysis of the distribution, use and phenotypic characteristics of the LAD2 human mast cell line. Int Arch Allergy Immunol. 2014;164(4):265-70.

274. Ronnberg E, Johnzon CF, Calounova G, Garcia Faroldi G, Grujic M, Hartmann K, et al. Mast cells are activated by Staphylococcus aureus in vitro but do not influence the outcome of intraperitoneal S. aureus infection in vivo. Immunology. 2014;143(2):155-63.

275. Rocha-de-Souza CM, Berent-Maoz B, Mankuta D, Moses AE, Levi-Schaffer F. Human mast cell activation by Staphylococcus aureus: interleukin-8 and tumor necrosis factor alpha release and the role of Toll-like receptor 2 and CD48 molecules. Infect Immun. 2008;76(10):4489-97.

276. Hoek KL, Cassell GH, Duffy LB, Atkinson TP. Mycoplasma pneumoniae-induced activation and cytokine production in rodent mast cells. J Allergy Clin Immunol. 2002;109(3):470-6.

277. Barbuti G, Moschioni M, Censini S, Covacci A, Montecucco C, Montemurro P. Streptococcus pneumoniae induces mast cell degranulation. Int J Med Microbiol. 2006;296(4-5):325-9.

278. Zhang X, Hu X, Rao X. Apoptosis induced by Staphylococcus aureus toxins. Microbiol Res. 2017;205:19-24.

279. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol. 2014;14(7):478-94.

280. Swindle EJ, Brown JM, Radinger M, DeLeo FR, Metcalfe DD. Interferon-gamma enhances both the anti-bacterial and the pro-inflammatory response of human mast cells to Staphylococcus aureus. Immunology. 2015;146(3):470-85.

281. Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JW, Joosten LA. IL-1beta processing in host defense: beyond the inflammasomes. PLoS Pathog. 2010;6(2):e1000661.

282. LaRock CN, Todd J, LaRock DL, Olson J, O'Donoghue AJ, Robertson AA, et al. IL-1beta is an innate immune sensor of microbial proteolysis. Sci Immunol. 2016;1(2).

283. Ali A, Na M, Svensson MN, Magnusson M, Welin A, Schwarze JC, et al. IL-1 Receptor Antagonist Treatment Aggravates Staphylococcal Septic Arthritis and Sepsis in Mice. PLoS One. 2015;10(7):e0131645.

284. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013;13(3):159-75.

285. Nilsson G, Mikovits JA, Metcalfe DD, Taub DD. Mast cell migratory response to interleukin-8 is mediated through interaction with chemokine receptor CXCR2/Interleukin-8RB. Blood. 1999;93(9):2791-7.

286. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. Neuropeptides activate human mast cell degranulation and chemokine production. Immunology. 2008;123(3):398-410.

287. Yu Y, Huang Z, Mao Z, Zhang Y, Jin M, Chen W, et al. Go is required for the release of IL-8 and TNF-alpha, but not degranulation in human mast cells. Eur J Pharmacol. 2016;780:115-21.

288. Nagai K, Fukushima T, Oike H, Kobori M. High glucose increases the expression of proinflammatory cytokines and secretion of TNFalpha and beta-hexosaminidase in human mast cells. Eur J Pharmacol. 2012;687(1-3):39-45.

289. Kitaura J, Asai K, Maeda-Yamamoto M, Kawakami Y, Kikkawa U, Kawakami T. Akt-dependent cytokine production in mast cells. J Exp Med. 2000;192(5):729-40.

290. Takayama G, Ohtani M, Minowa A, Matsuda S, Koyasu S. Class I PI3K-mediated Akt and ERK signals play a critical role in FcepsilonRI-induced degranulation in mast cells. Int Immunol. 2013;25(4):215-20.

291. Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci. 2003;116(Pt 7):1175-86.

292. Radinger M, Kuehn HS, Kim MS, Metcalfe DD, Gilfillan AM. Glycogen synthase kinase 3beta activation is a prerequisite signal for cytokine production and chemotaxis in human mast cells. J Immunol. 2010;184(2):564-72.

293. Radinger M, Smrz D, Metcalfe DD, Gilfillan AM. Glycogen synthase kinase-3beta is a prosurvival signal for the maintenance of human mast cell homeostasis. J Immunol. 2011;187(11):5587-95.

294. Chen K, Xiang Y, Yao X, Liu Y, Gong W, Yoshimura T, et al. The active contribution of Toll-like receptors to allergic airway inflammation. Int Immunopharmacol. 2011;11(10):1391-8.

295. Barton GM, Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol. 2009;9(8):535-42.

296. Rodriguez D, Keller AC, Faquim-Mauro EL, de Macedo MS, Cunha FQ, Lefort J, et al. Bacterial lipopolysaccharide signaling through Toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity. J Immunol. 2003;171(2):1001-8.

297. Velasco G, Campo M, Manrique OJ, Bellou A, He H, Arestides RS, et al. Toll-like receptor 4 or 2 agonists decrease allergic inflammation. Am J Respir Cell Mol Biol. 2005;32(3):218-24.

298. Kasakura K, Takahashi K, Aizawa T, Hosono A, Kaminogawa S. A TLR2 ligand suppresses allergic inflammatory reactions by acting directly on mast cells. Int Arch Allergy Immunol. 2009;150(4):359-69.

299. Melo FR, Vita F, Berent-Maoz B, Levi-Schaffer F, Zabucchi G, Pejler G. Proteolytic histone modification by mast cell tryptase, a serglycin proteoglycan-dependent secretory granule protease. J Biol Chem. 2014;289(11):7682-90.

300. Kruger PG, Lagunoff D. Effect of age on mast cell granules. International archives of allergy and applied immunology. 1981;65(3):291-9.

301. Puri N, Roche PA. Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(7):2580-5.

302. Schwartz LB, Irani AM, Roller K, Castells MC, Schechter NM. Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. Journal of immunology. 1987;138(8):2611-5.

303. Goldstein SM, Kaempfer CE, Kealey JT, Wintroub BU. Human mast cell carboxypeptidase. Purification and characterization. J Clin Invest. 1989;83(5):1630-6.

304. Dunkley TP, Watson R, Griffin JL, Dupree P, Lilley KS. Localization of organelle proteins by isotope tagging (LOPIT). Mol Cell Proteomics. 2004;3(11):1128-34.

305. Majekodunmi SO. A review on centrifugation in pharmaceutical industry. American journal of biochemical engineering. 2015;5(2):67-78.

306. Perez-Novo CA, Claeys C, Speleman F, Van Cauwenberge P, Bachert C, Vandesompele J. Impact of RNA quality on reference gene expression stability. Biotechniques. 2005;39(1):56.

307. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.