UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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Markers of Allergic Reactions to Food Based on Activation of Mast Cells and Basophils

by

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ABSTRACT

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EFFECTIVE DIAGNOSIS OF ALLERGIC REACTIONS TO FOOD BASED ON MAST
CELL AND BASOPHIL ACTIVATION

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Confirming an allergic reaction can be a challenge, and it is difficult to assess which patients may be at risk of anaphylaxis. Our objective has been to investigate and compare potential serum markers for diagnosis of allergic reactions and predicting susceptibility to severe reactions based on mast cell and basophil activation.

Levels of tryptase, chymase, carboxypeptidase and DPPI were measured in serum and saliva from 92 children (8-18 y), before, during and after diagnostic food challenge. Levels were measured using ELISAs developed and validated in our laboratory. Results were analyzed with respect to challenge outcome, and severity of reactions occurring in challenge and historically. Similar studies were performed using samples from 32 adults (17-72 y) undergoing diagnostic drug challenge. In addition, significant progress has been made towards development of new ELISA techniques for measurement of α-tryptase and ß-tryptase individually, and also for the basophil specific protease basogranulin.

Serum DPPI levels were increased after moderate/severe reactions occurring in food challenge (p = 0.004). Levels of chymase in serum, and carboxypeptidase and DPPI in saliva, were elevated after positive drug challenge (p = 0.02 for all). Baseline serum levels of carboxypeptidase were predictive of severity of historical reactions to foods (p = 0.009) or drugs (p = 0.008); and concentrations of carboxypeptidase (p = 0.03) and DPPI (p = 0.02) were also associated with reaction severity in food challenge.

The measurement of mast cell and basophil products should be useful not only in providing laboratory confirmation of an allergic reaction to food, but could also allow reactions to be characterized according to underlying disease mechanisms. The strong associations found between baseline levels of certain markers and the severity and nature of previous reactions raise the prospect of a test for identifying subjects who may be at particular risk of a severe reaction.

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Declaration of Authorship

I, Hilary Sîan Whitworth, declare that the thesis entitled 'Effective diagnosis of allergic reactions to food based on mast cell and basophil activation' and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;

- 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;

- where I have consulted the published work of others, this is always clearly attributed;

- 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;

- I have acknowledged all main sources of help;

- 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;

- none of this work has been published before submission

Signed: ………………………………………………………………………..

Date:…………………………………………………………………………….

List of abbreviations

ACE	Angiotensin-converting enzyme
AGP	Antibodies, glutamine and pyruvate
APC	Antigen-presenting cell
APS	Ammonium persulphate
ATI	Angiotensin I
ATII	Angiotensin II
BSA	Bovine serum albumin
DBPCFC	Double blind, placebo-controlled food challenge
D.H^{2}O	Distilled H^{2}O
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DPPI	Dipeptidyl peptidase I
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FcεRI	Fc-epsilon receptor I
FCS	Foetal calf serum
HAT	Hypoxanthine aminopterin thymidine
HRP	Horse radish peroxidise
HCl	Hydrochloric acid
HES	Hybridoma enhancing supplement
IgE	Immunoglobulin E
IgM	Immunoglobulin M
MC$_{TC}$	Connective tissue mast cells
MC$_T$	Mucosal mast cells
MHC	Major histocompatibility complex
NHS	National Health Service
PAF	Platelet-activating factor
PAFAH	PAF acetylhydrolase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PBS-T	Tween in PBS
PEG	Polyethylene glycol
PKC	Protein kinase C
R&D	Research and Development
SDS	Sodium dodecyl sulphate
SGM	Standard growth medium
SPT	Skin prick test
SUHT	Southampton University Hospital Trust
TEMED	Tetramethylethylenediamine
TMB	Tetramethylbenzidine
VEGF	Vascular endothelial growth factor

Unit abbreviations

°C	Degrees Celsius
g	Gram
kDa	Kilodaltons
M	Molar
µg	Microgram
µl	Microlitre
mg	Milligram
ml	Millilitre
mM	Millimolar
ng	Nanogram
nm	Nanometres
OD	Optical density
%	Per cent
pg	Picogram
RCF (x g)	Relative centrifugal force
V	Volts
w/v	Weight per volume

CHAPTER 1: INTRODUCTION

The prevalence of allergic disease and incidence of anaphylaxis are an increasing problem in the United Kingdom, as well as other westernized countries. However, diagnosis of reactions, particularly to food, can be difficult; and there is little means of assessing risk of severe symptoms (Simons *et al.*, 2011). These issues are worsened by the lack of reliable supporting laboratory tools. Within this study we aimed to develop novel tests for diagnosis and predicting severity of allergic reactions to foods and other allergens, based on mast cell and basophil activation.

1.1 Allergy and Anaphylaxis

Allergy has been defined by the Nomenclature Review Committee of the World Allergy Organisation as '*a hypersensitivity reaction initiated by specific immunologic mechanisms*', whereby hypersensitivity describes '*objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons*' (Johansson *et al.*, 2004). Non-allergic hypersensitivity has also been described. Allergy can be Immunoglobulin E (IgE) or non-IgE mediated, the latter of which involves allergen-specific lymphocytes. Types of hypersensitivity reactions are illustrated in Fig 1.1. Within this study we have focused on IgE-mediated allergic hypersensitivity and, in particular, food allergy. Food allergy was recently defined in guidelines produced by the National Institute of Allergy and Infectious Disease (NIAID) as '*an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food*' (NIAID-Sponsored Expert Panel *et al.*, 2010).

Anaphylaxis is the most severe manifestation of allergy. It was previously described by Johansson *et al.* (2004) as '*a severe, life-threatening generalised or systemic hypersensitivity reaction*'; and it was proposed that the condition is divided into allergic and non-allergic anaphylaxis. However, during a more recent symposium held between the NIAID and the Food Allergy and Anaphylaxis Network (FAAN), anaphylaxis was defined as '*a serious allergic reaction that is rapid in onset and may cause death*' (Sampson *et al.*, 2006), thus encompassing

only reactions involving an immunological mechanism. The focus of this study has been on IgE-mediated anaphylaxis.

Hypersensitivity

Allergy

Non-allergic
hypersensitivity

Cell-mediated
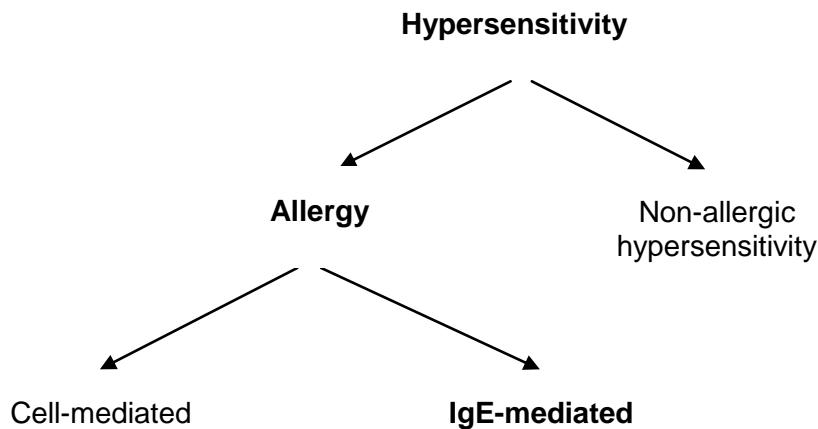
IgE-mediated

Fig 1.1 Types of hypersensitivity. The focus of this study is IgE-mediated allergic hypersensitivity (highlighted in bold), particularly food allergy.

Prevalence and economic burden

Allergic disorders in the UK are common, with reported diagnosis in 30% of adults and almost 40% of children (Gupta *et al.*, 2004). Furthermore, the incidence has increased over the past few decades, in particular for generalised/systemic conditions. Hospital admissions in England, determined using national hospital discharge statistics, increased dramatically between 1990 and 2003 for food allergy, anaphylaxis, urticaria and angioedema (Gupta *et al.,* 2003; Gupta *et al.*, 2007). The greatest rise in admissions for food allergy was observed in children up to 14 years of age (Gupta *et al.*, 2007). Within this age group, numbers increased from 16 to 107 per million of population.

There is a need for new studies of more recent trends of the incidence and prevalence of allergic conditions. An increase in hospital admissions up until 2003 has been demonstrated and published (described above; Gupta *et al.*, 2007), but there is a lack of further studies since this time. However, data on hospital

admissions for allergic conditions provided by Hospital Episode Statistics (HES) Online indicate a continuing upward trend (own observations). According to these statistics, reported inpatient admissions with a primary diagnosis of anaphylaxis have increased from 145 to 217 per million total admissions between 2003 and 2009 (table 1.1). These figures are likely to be an underestimate due to the potential for vagueness and inaccuracy in diagnostic coding. The data show that inpatient admissions under a consultant in allergy and clinical immunology have also increased from 194 to 835 per million since 2003.

Table 1.1 Hospital inpatient admissions for anaphylaxis and other allergic conditions. Rates shown are obtained from HES Online, and are expressed per million of total hospital admissions.

Year	Admission with primary diagnosis of anaphylaxis	Admissions under consultant in allergy and clinical immunology
2002/2003	145	194
2003/2004	164	217
2004/2005	194	323
2005/2006	200	413
2006/2007	214	648
2007/2008	214	765
2008/2009	217	835

Not only does allergic disease put a strain on the individuals affected in terms of a restriction in lifestyle and fear of potential reactions, it also imposes a heavy burden on the NHS. Taking into account primary care costs, primary care prescribing costs, and hospital inpatient costs, allergic disorders were estimated to cost the NHS over one billion pounds per annum (Gupta *et al.*, 2004). Food allergy accounts for a significant proportion of this.

Symptoms of allergic reactions

Symptoms of allergic reactions vary considerably, and can affect the skin, oral mucosa, gastrointestinal tract, and/or the respiratory/cardiovascular systems (Sampson *et al.*, 2006; Fig 1.2). The most severe form of a reaction is anaphylaxis. Symptoms of this may include respiratory difficulties, hypotension or collapse; and the onset and development is often very rapid. Spontaneous recovery can occur with increased endogenous production of adrenaline. In other cases, however, anaphylaxis can be fatal. Until relatively recently there has been a lack of diagnostic criteria for anaphylaxis. This will almost certainly have had negative implications on selection of appropriate therapy for patients and identification of those at risk of life threatening reactions, as well as increasing knowledge on immunological mechanisms and accurate reporting of incidence rates.

In the symposium held between the NIAID and the FAAN in 2005, specific clinical criteria were established for diagnosis of anaphylaxis (Sampson *et al.*, 2006). It was recommended that the condition was highly likely if one of three criteria were met (Fig 1.3). The first of these is acute onset of illness involving the skin and/or mucosal tissue, and either respiratory compromise or hypotension (or associated symptoms of end-organ failure). The second criterion is for two or more symptoms occurring after exposure to a *likely* allergen for that patient, including skin/mucosal tissue involvement, persistent gastrointestinal symptoms, respiratory compromise or hypotension (or associated symptoms of end organ failure). The final alternative criterion is for hypotension following exposure to a *known* allergen for that patient. In each case, illness must occur within minutes to hours. These criteria have recently been re-published in guidelines of the World Allergy Organisation (Simons *et al.*, 2011) for the diagnosis and management of anaphylaxis.

Oral symptoms:
Pruritus, oedema

Respiratory symptoms:
Hoarseness, dry cough, shortness of breath,
throat/chest tightness, wheezing, congestion, sneezing

Cardiovascular symptoms:
Hypotension, chest pain, syncope, dysrhythmia

Gastrointestinal symptoms:
Nausea, abdominal pain, vomiting,
diarrhoea

Cutaneous symptoms:
Pruritus, urticaria, flushing, oedema
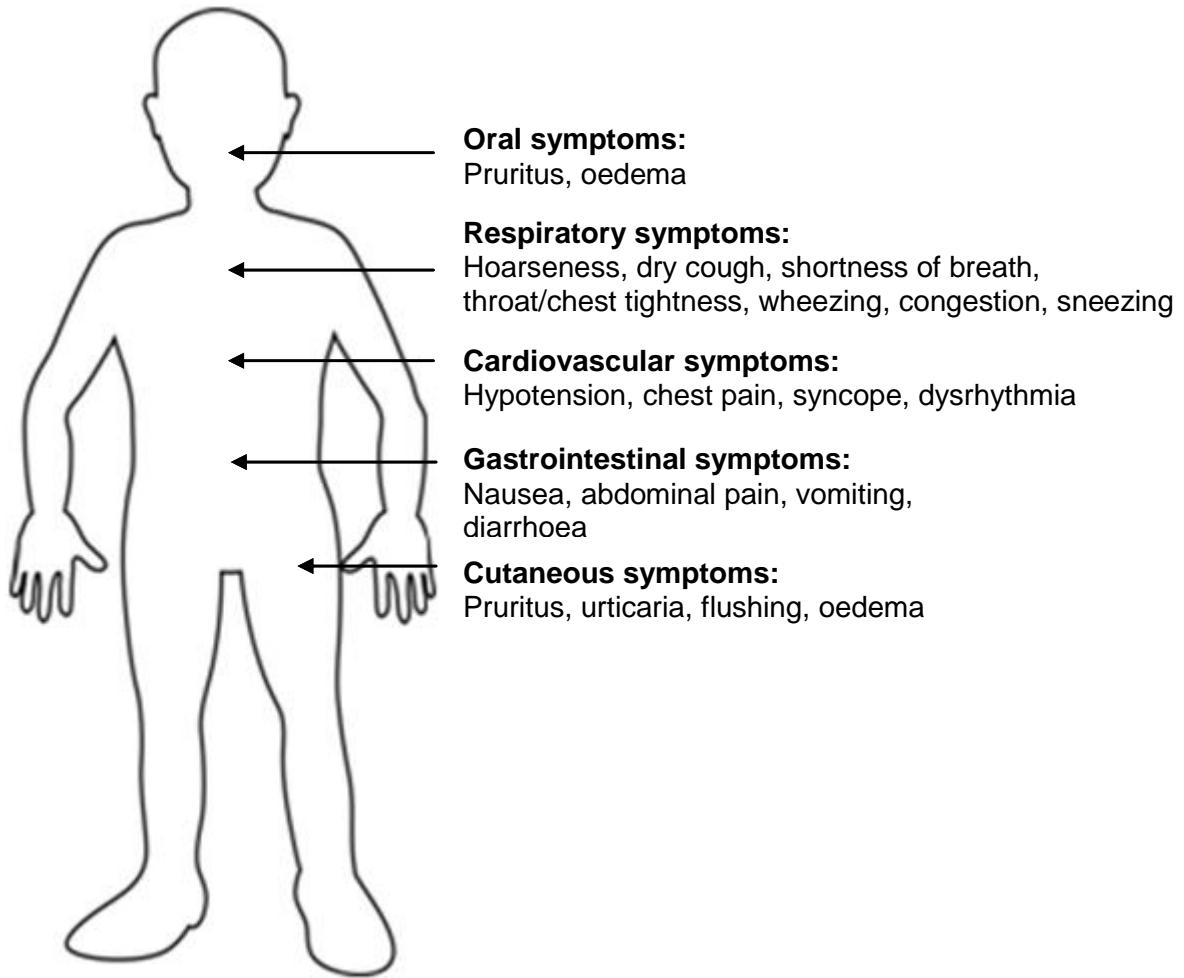
Fig 1.2 Symptoms of allergic reactions. Symptoms can range from very mild to life-threatening or even fatal. Adapted from Simons *et al.*, 2011.
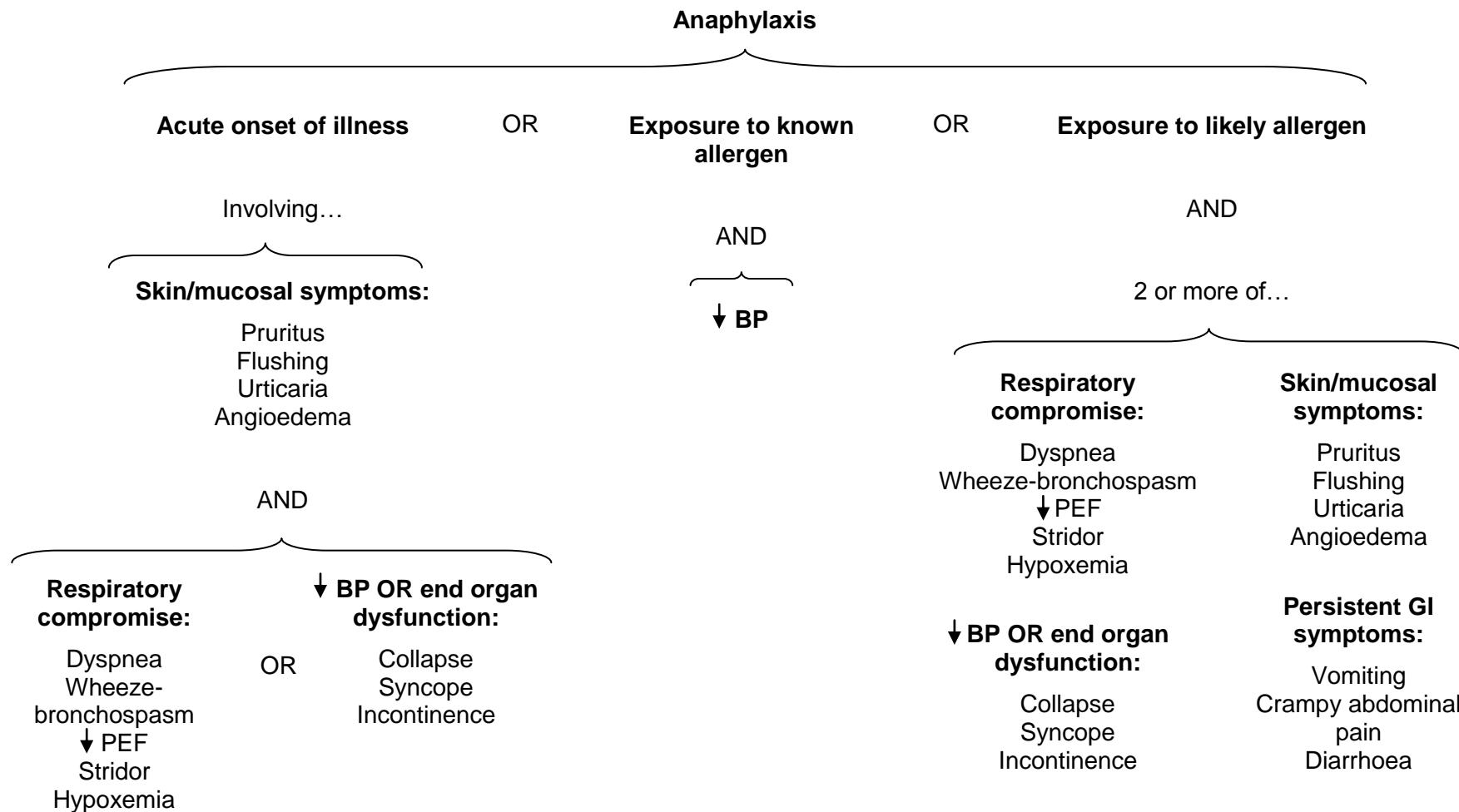
Fig 1.3 Diagnostic criteria for anaphylaxis. Criteria were established by the NIAID and FAAM (Sampson *et al.*, 2006). The diagram is adapted from a visual representation of the criteria devised by Manivannan *et al.*, 2009. (BP: blood pressure; PEF: peak expiratory flow; GI: gastrointestinal)

Aetiology

The aetiology of allergic disease is multi-factorial, involving both genetic and environmental factors. In an American study of 581 families, food allergy in the index child was found to be an independent predictor of food allergy in other siblings (Tsai *et al.*, 2009). Total IgE levels, and also IgE specific for nine major food allergens tested, were correlated between the index child and other siblings. These same associations were seen between the mother and offspring, and also the father and offspring.

As for food allergy, there is some evidence for a genetic predisposition to allergies to latex, drugs and insect venom. Promoter polymorphisms of IL-13 and IL-18 were found to be associated with development of latex allergy (Brown *et al.*, 2005), and IL-4 single nucleotide polymorphisms were linked to penicillin allergy (Apter *et al.*, 2008). In addition, expression of human leukocyte antigen class I and II antigen haplotypes differed in patients with venom allergy and non-allergic controls (Karakis *et al.*, 2010).

Studies of the role of environmental factors are more numerous, particularly in the aetiology of food allergy. Until recently, oral allergen avoidance was considered to reduce the risk of developing food allergies (Gruskay, 1982; Arshad *et al.*, 1992). Mothers were advised not to consume peanut during pregnancy, and to delay oral exposure to allergenic foods in young infants (COT report on peanut allergy, 1998). There is now evidence to the contrary; early introduction of allergens may be protective (Du Toit *et al.*, 2008).

Interestingly, a retrospective questionnaire-based study found that high levels of non-oral environmental peanut exposure promoted sensitisation to the allergen in atopic children, but this risk was reduced by early oral exposure (Fox *et al.*, 2009). This suggests that tolerance may occur via oral exposure, whereas sensitisation occurs through the skin. Indeed Lack *et al.*, (2003) had previously shown that use of skin preparations containing peanut oil in infants was independently associated

with development of peanut allergy in a cohort of preschool children. Several large scale cohort studies are now underway to investigate the effects of early oral allergen exposure on development of allergies. These include the Learning Early About Peanut Allergy (LEAP) and Enquiring About Tolerance (EAT) studies.

The biggest risk factor for development of latex allergy is thought to be exposure. Rates of sensitisation are higher than average in those with occupational exposure, and also in children with diseases such as spina bifida who are likely to have been exposed medically from a very young age (Alenius *et al.*, 2002; Deval, 2008). Similarly, bee keepers are considered to be at high risk of venom allergy (Bousquet *et al.*, 1982). The majority of reactions to drugs are likely to be upon first exposure to that agent. Thus perhaps allergies to therapeutic agents may result due to sensitisation upon exposure to similar compounds in everyday medicines, or even in cleaning or beauty products.

Problems in diagnosis

Diagnosis of allergic reactions and anaphylaxis can present a challenge (Simons, 2005). Symptoms may appear and disappear very quickly, and can be hard to determine in young patients. Many symptoms are not specific to allergy, such as wheeze, collapse, nausea and vomiting, and there has been a lack of consensus on specific diagnostic criteria. Also, an allergic condition may not be recognized when an individual suffers their first reaction. Specifically in the diagnosis of food allergy, there can be confusion between IgE-mediated allergic disease and other food-related disorders, such as food intolerance or celiac disease (NIAID-Sponsored Expert Panel *et al.*, 2010). The only test currently available to aid diagnosis of allergic reactions and anaphylaxis is measurement of serum tryptase levels. However studies investigating its usefulness have yielded contradictory results, suggesting that it may have limited use clinically (Treudler *et al.*, 2008; Lin *et al.*, 2000; Vila *et al.*, 2001). Current methods and tests for diagnosis are described in detail in Section 1.4.

Because of the difficulties in establishing a diagnosis, allergic reactions and anaphylaxis may be underreported. At post mortem, anaphylaxis as a cause of death is often rejected, despite supportive clinical records, due to a lack of specific evidence (Pumphrey and Roberts, 2000). Thus it is highly likely that fatal anaphylaxis is also more common than reported.

There are two main consequences of misdiagnosis for patients. Firstly, those who have suffered from an allergic reaction may be at greater risk of another if the reaction was not confirmed and the allergen therefore not identified. On the other hand, an individual may take unnecessary precautions, for example avoiding certain food types, if it is incorrectly thought that a reaction to a particular allergen has occurred. This may lead to lifestyle restrictions and unwarranted anxiety, and may have nutritional implications. There is a need for the development of further tests that can reliably establish a positive or negative diagnosis in cases of suspected allergic reactions.

Risk of severe reactions

Anxiety is common amongst allergic patients and their families due to uncertainty of potential consequences of accidental allergen exposure (Avery *et al.*, 2003; Kelsay, 2003). This is particularly the case for those with food allergy. Peanut-allergic children have been reported to feel safer when carrying adrenaline auto-injectors (Avery *et al.*, 2003), and recent work at the University of Southampton has found that children who have an auto-injector device report improved quality of life (Cummings *et al.,* 2010). However, patients are not always prescribed this medication.

Other than a few known risk factors (reviewed in detail in Section 1.5), there is currently little means of predicting which allergic individuals may be at risk of developing anaphylactic, and possibly fatal, reactions; some sensitized individuals are unlikely to ever have a severe reaction (Simons *et al.*, 2007). As a result, patients at greater risk may not have the necessary interaction with allergy

specialists, be prescribed appropriate rescue medication, or take adequate precautions. On the other hand, in patients who are at low risk, strict allergen avoidance may cause unnecessary lifestyle and/or dietary restrictions, leading to increased anxiety (Noimark and Cox, 2008). Development of tests that are effective in predicting susceptibility to severe allergic reactions would be of great value to allergy practitioners and their patients.

1.2 Sensitisation and the Allergic Response

Allergy is an IgE-mediated immunological condition in which exposure to a specific antigenic molecule results in a hypersensitivity reaction (Johansson *et al.*, 2004). In order for an allergic reaction to occur, an individual must be sensitized to the allergen. Exposure previous to the first reactive episode must therefore have occurred (Bleumink, 1983; Lehrer *et al.*, 2002).

The immunologic mechanisms involved in allergic disease are discussed in detail in this section and illustrated in Fig 1.4. In brief, upon initial exposure, antigen is presented by specialized antigen-presenting cells (APCs) (Banchereau and Steinman, 1998) to T lymphocytes (T cells; Mondino and Jenkins, 1994). Activated T cells stimulate B cell multiplication and maturation into plasma cells (Poulsen and Hummelshoj, 2007), which produce and secrete allergen specific antibodies of the IgE isotype (Fig 1.5). This IgE binds to mast cell and basophil membranes via the high affinity IgE receptor, FcεRI. During subsequent exposure, the allergen binds to the specific IgE present on mast cells and basophils, resulting in cell activation and granule exocytosis (Ishizaka *et al.*, 1980; Siraganian and Hazard, 1979). A range of mediators are released that cause the symptoms experienced during an allergic reaction.

Sensitisation

Allergen exposure

Allergens are antigenic molecules, often protein, that have the potential to be recognised as foreign by the body, thus initiating an immune response in atopic individuals (Johansson *et al.*, 2004). Common antigens implicated in allergy are in foods, drugs, insect venom, pollens, fungi, animal dander and dust mite excretion. The route of allergen exposure can be via skin contact, inhalation into the respiratory tract, uptake across mucous membranes, or digestion in the gastrointestinal tract.

(a)

MHC class II

IL-2, IL-4

TCR

APC

T cell

CD28

CD80/86

CD28 CD80/86

TCR

MHC class II

BCR

B cell

CD40L CD40

IL-4, IL-13

Mast cell

(b)
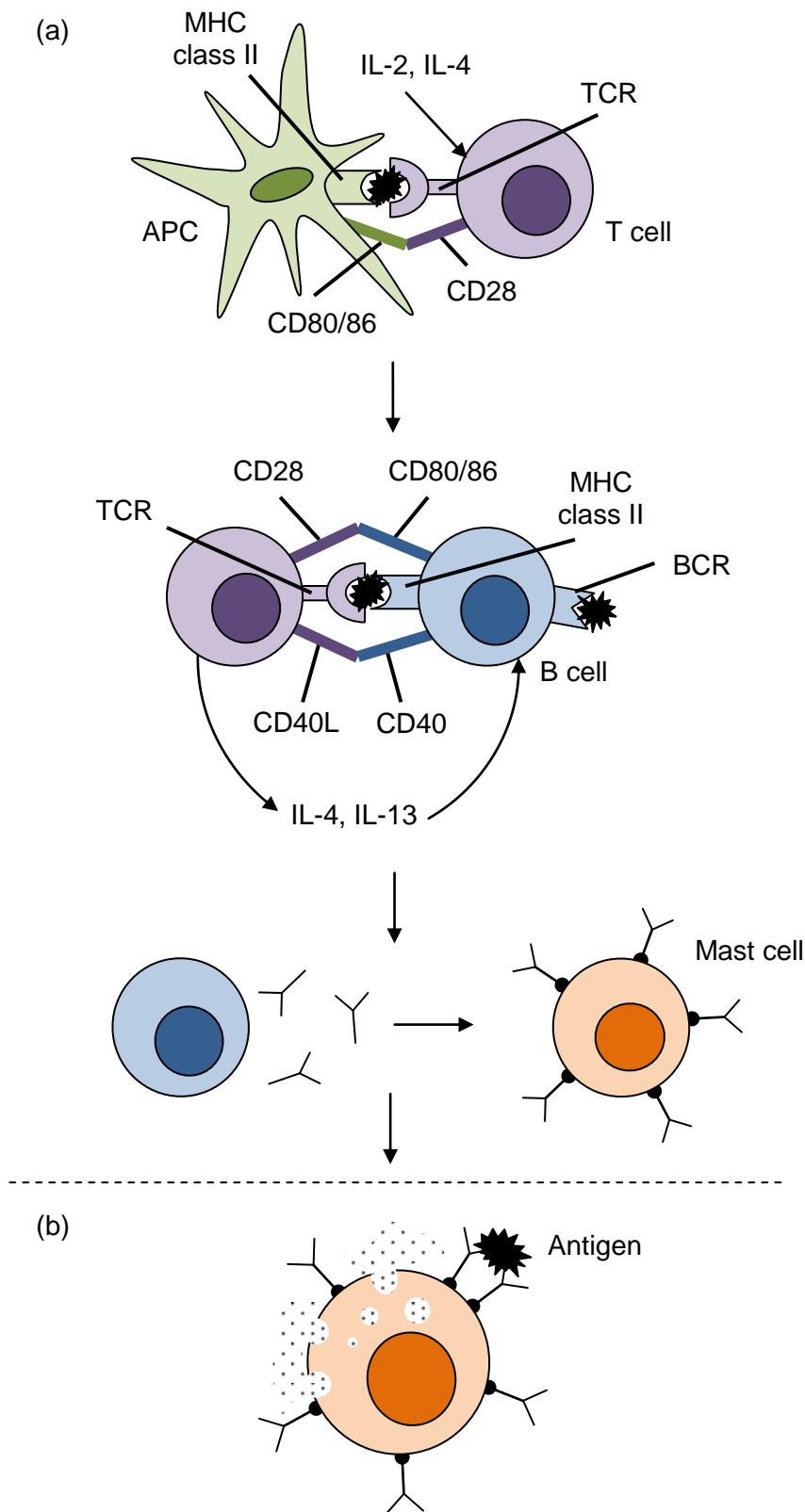
Antigen

Fig 1.4 (a) Sensitisation to an allergen and (b) the allergic response. Primary sensitisation involves antigen presentation by APCs, T cell activation, B cell activation and IgE production, and binding of IgE to mast cells. Upon subsequent exposure, antigen binding to IgE causes mast cell activation and degranulation.

38
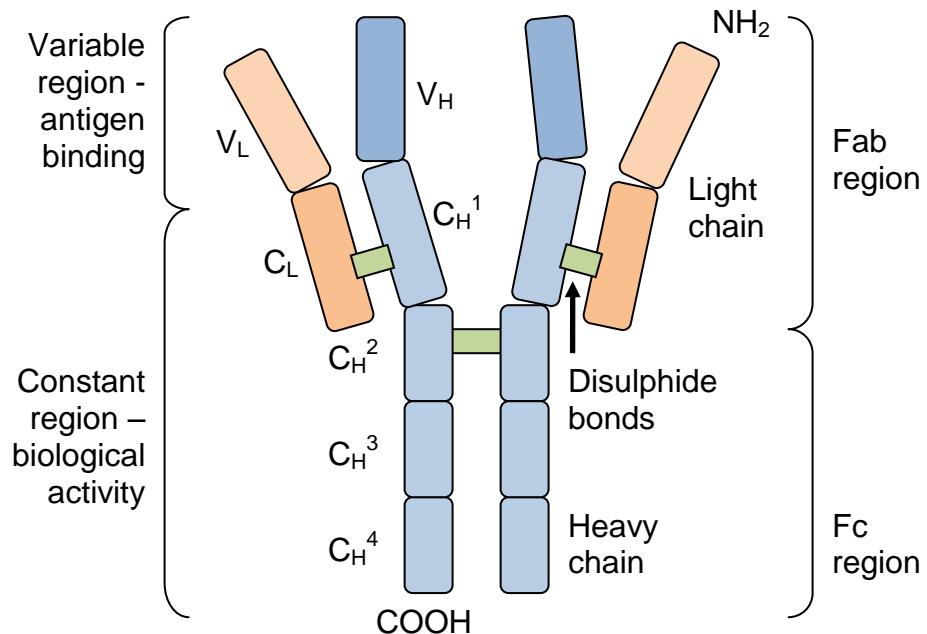
Fig 1.5 Immunoglobulin E. IgE is a monomeric antibody made up of two light chains (L) and two heavy chains (H). Within these are variable (V) regions, where antigen binding occurs, and constant (C) effector regions. Adapted from Gould and Sutton (2008) and Averbeck *et al.* (2007).

In food allergy, allergens undergo harsh digestive processes. These molecules must therefore be sufficiently stable to reach the intestinal mucosa (Astwood *et al.*, 1996). Methods of food preparation can affect the allergenicity of proteins (Lehrer *et al.*, 2002; Paschke, 2009). For example, heating of hen's egg or cow's milk makes allergens less allergenic (Cooke and Sampson, 1997; Des Roches *et al.*, 2006; Morisawa *et al.*, 2009). Cooking has been shown to alter the structure of peanut allergens, but it is controversial as to whether this changes the allergenicity (Mondoulet *et al.*, 2005; Koppelman *et al.*, 1999; Beyer *et al.*, 2001; Chung *et al.*, 2003).

Antigen presentation

Upon initial exposure, allergens are taken up by antigen presenting cells (APCs), and presented to T cells by major histocompatibility complex (MHC) class II

molecules. The dendritic cell (DC) is the major APC (Mellman and Steinman, 2001; Von Bubnoff, Geiger and Bieber, 2001; Banchereau and Steinman, 1998; Cella *et al.*, 1997).

In peripheral tissues, DCs are typically present in their immature form. These cells are competent in uptake of allergens by endocytosis, but express only low levels of MHC molecules on their surface (Mellman and Steinman, 2001; Bubnoff, Geiger and Bieber, 2001; Banchereau and Steinman, 1998; Cella *et al.*, 1997). Maturation occurs in response to microbial exposure, signals from pro-inflammatory cytokines, or other unrelated stimulation, upon migration of DCs to lymphoid organs. The ability of the cell to take up antigen is reduced (though not abolished completely), and MHC class II molecules accumulate on the cell surface. Complexes form between MHC class II molecules and antigen.

T cell activation

Within the lymph node, antigen presentation by DCs via MHC class II complexes results in activation of naïve CD4+ T cells, and maturation of these cells into T helper (T_H) cells (Mondino and Jenkins, 1994). T_H cells have the capacity to differentiate into T_H1, T_H2 or T_H17 cells. In allergic sensitisation, cells differentiate down the T_H2 pathway (Nakayama and Yamashita, 2010).

Maturation of naïve T cells into T_H cells requires two signals resulting from interaction with DCs: stimulation and co-stimulation (Mondino and Jenkins, 1994). Stimulation occurs upon recognition of MHC bound antigen on DCs by a specific T cell antigen receptor (TCR)/CD3 complex. Co-stimulation is the interaction of other cell surface molecules, including the T cell surface marker CD28 with its DC ligand B7 (CD80 and CD86). Expression of B7, and also other DC co-stimulatory molecules such as ICAM-1 (CD54), is up-regulated upon T cell binding. Without this supporting stimulation, the T cell would become anergic or apoptose.

Stimulation and co-stimulation of T cells signals for an increase in IL-2 production. IL-2 acts in an autocrine manner, causing maturation of T cells into T_H cells and proliferation of these cells. Up-regulation of the cell surface IL-2 receptor (CD25) occurs synergistically (Mondino and Jenkins, 1994).

Differentiation of proliferated T_H cells down the T_H2 effecter pathway occurs in a process called polarisation, and results from IL-4 binding to its T cell receptor IL-4R. Within the lymph node, IL-4 is secreted from CD4+ T_H cells upon initial activation, and it is therefore likely that IL-4-mediated T_H2 differentiation occurs in an autocrine manner (Rautajoki *et al.*, 2008). A three cell model for activation of T_H cells has also been proposed, whereby polarisation occurs in response to IL-4 released from a third innate immune cell type, such as the mast cell, basophil, eosinophil or natural killer (NK) T cell (Corthay, 2006).

Antibody production

B cells are bone marrow-derived lymphocytes that have the capacity to produce and secrete antibodies. Similarly to T cells, naïve B cells have the capacity to recognise specific antigens, but have never previously been exposed. In allergic sensitisation naïve B cells present in lymphoid tissues are activated by signals from T cells, resulting in antibody isotype switching and B cell differentiation into memory cells or IgE-producing plasma cells (Poulsen and Hummelshoj, 2007).

In order for this process to occur, B cells must be recognized by T_H2 cells. This occurs in a similar manner to that described above. Within lymphoid tissues, antigens bind to their specific B cell receptor (BCR). The receptor is internalized, and MHC class II molecule production is up-regulated. The antigen forms a complex with the MHC class II molecules, and the MHC class II/antigen complex is presented on the cell surface, allowing recognition by differentiated T_H2 cells (Poulsen and Hummelshoj, 2007).

Antibody class switching in naïve B cells is dependent on two signals. One signal is the secretion of IL-4 and IL-13 that occurs upon differentiation of T cells down the T_H2 pathway; and the second is the interaction between B cell surface CD40 and its ligand, CD40L (CD154), present on T cells (Poulsen and Hummelshoj, 2007). Co-stimulation between CD28 on T cells and CD80/CD86 on B cells may also play a key role (Nakajima *et al.*, 1997). Stimulation of BCR, CD40 and IL-4R causes B cell proliferation and re-arrangement of the IgE genomic locus, resulting in IgE production (Poulsen and Hummelshoj, 2007).

IgE binding to FcεRI

IgE synthesised from B cells migrates from the lymph nodes into the circulation and tissues, and binds to the high affinity IgE receptor, FcεRI, on mast cells and basophils (Poulsen and Hummelshoj, 2007). Binding occurs via an interaction between the Fc region of the antibody and the α subunit of its receptor.

The allergic response

Following sensitisation, allergen exposure results in antigen binding to mast cell and basophil-bound IgE, and thus activation and degranulation of these cells. For this to occur, the antigen must cross-link multiple antibodies, causing aggregation of FcεRI. This leads to a sequence of downstream events that result in increased intracellular calcium and protein kinase C (PKC), and consequently in cell degranulation (Ishizaka *et al.*, 1980; Siraganian and Hazard, 1979). A variety of preformed and newly synthesized mediators are released (table 1.2), causing the physiological changes that lead to symptoms of an allergic reaction (Poulsen and Hummelshoj, 2007). Mast cells and basophils, and their granular constituents, are discussed in detail in Section 1.3.

Table 1.2 Products of mast cell and basophil activation. Granular constituents released from mast cells and/or basophils are shown. Adapted from Marquardt and Wasserman (1982) and Stone *et al.*, (2010).

	Cell type		
	Mast cell	**Basophil**	**Both**
Amines			Histamine
Proteases	Plasminogen activator Renin MMP-3 MMP-9		Tryptase Carboxypeptidase Chymase Cathepsin G
Proteoglycans	Heparin	Chondroitin sulphate	
Lipid mediators	PGD_2		LTC_4 PAF
Cytokines	IL-5 IL-6 GM-CSF Fibroblast growth factor Stem cell factor	MIP-1α IgE-dependent histamine releasing factor	IL-4 IL-8 IL-13 TNF-α
Basic proteins		Basogranulin 2D7 antigen Pro form of eosinophil major basic protein Eosinophil major basic protein Eosinophil cationic protein Eosinophil-derived neurotoxin	
Other enzymes	Arylsuphatase	Eosinophil peroxidase	ß-hexosaminidase ß-glucuronidase

1.3 Mast cells and basophils

Mast cells

Structure, origin and localization

Mast cells were first identified in connective tissues in 1877. They are ovoid granular cells (Fi g 1.6(a)), measuring approximately 10 to 15 µm in diameter, and can be stained using metachromatic dyes such as toluidine blue or methylene blue (Marquardt and Wasserman, 1982). Different granular patterns have been described, including scrolls, crystals, particles and mixed (Dvorak, 2005). Mast cells originate from CD34+ bone marrow-derived progenitor cells (Kirshenbaum *et al.,* 1991) and, once matured, locate in connective and mucosal tissues. Within human lung and skin, they are estimated to be present at a concentration of 10^6 to 10^7 cells/g tissue (Marquardt and Wasserman, 1982).

(a)   (b)
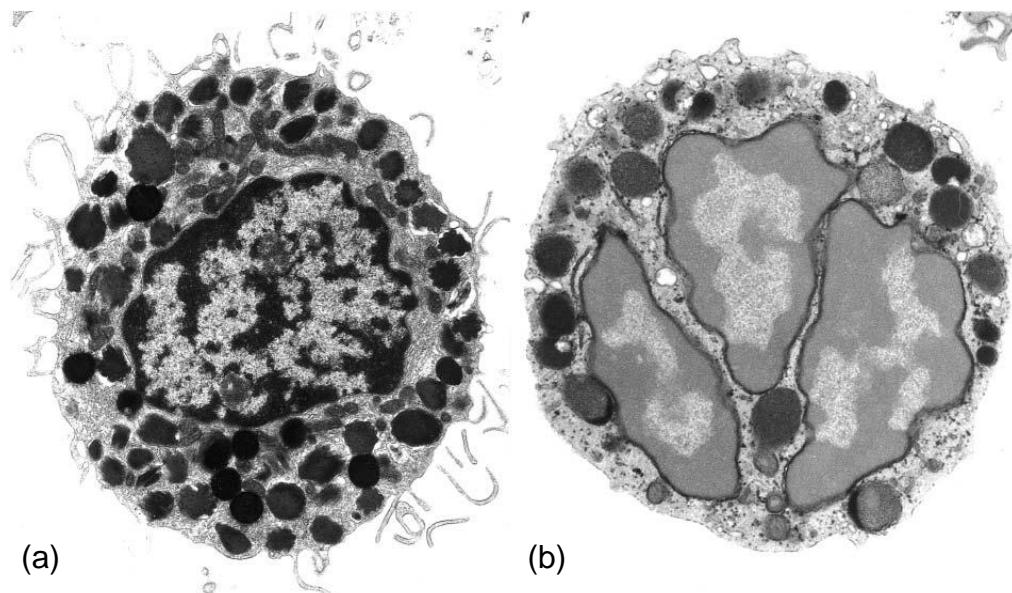
Fig 1.6 Ultrastructure of isolated human (a) lung mast cells and (b) peripheral blood basophils. Cells were imaged by electron microscopy. (a) x 10,000; (b) x 17,000 (Dvorak, 2005)

Mast cells tend to be localised specifically around nerves, lymphatics and blood vessels. Within connective tissue of the skin, localization has been shown to occur by adhesion of the cells to the laminin-containing neural and vascular membranes via the laminin receptor, α6/β1 integrin (CD49f) (Walsh *et al*., 1991). Similarly, an association between mast cells and laminin of the vascular basement membrane was identified in the oral mucosa (Zhao *et al*., 1998). In addition, *in vitro* studies suggested that, upon mast cell degranulation, the laminin receptor down-regulates allowing cell motility and migration (Walsh *et al*., 1991).

Function

Mast cells are inflammatory cells, best known for their role in allergy. However, they are also implicated in numerous other conditions, in some cases contributing to pathology and in others to defence (reviewed in Rao and Brown, 2008). Mast cells are thought to have the original purpose of protection against parasitic infection and, in addition, have been shown to aid defence against bacteria and possibly viruses. On the other hand, they have been suggested to contribute to the pathology of various inflammatory conditions and autoimmune diseases such as atherosclerosis, pulmonary fibrosis and rheumatoid arthritis. Mast cell involvement in cancer has been widely reported. However, whether the cell is playing a role that is beneficial to the patient is a matter of debate (Rao and Brown, 2008).

Allergic degranulation

Mast cell activation and degranulation result in the release of a range of mediators, including proteases, cytokines, lipid mediators and proteoglycans (listed in table 1.1, Section 1.2). This externalization of granule contents has been identified *in vitro* and *in vivo*, following stimulation of human skin explant culture with mast cell secretagogues such as anti-IgE antibody, and intra-dermal ragweed antigen challenge, respectively (Kaminer *et al*., 1991). Pre-formed mediators are released, including histamine (Ishizaka *et al*., 1991), tryptase (Walls *et al*., 1990), chymase (Wintroub *et al*., 1986) and carboxypeptidase (Goldstein *et al*., 1987), as well as

newly synthesized mediators, such as prostaglandins and leukotrienes (Walsh, 2003; Marquardt and Wasserman, 1982).

Subtypes

Immunohistochemical techniques in skin, lung and intestinal tissue identified two different subsets of mast cells; distinguishable by the proteins they contain (Irani *et al.*, 1986). One of these subsets contained both tryptase and chymase (MC_{TC}), and the other contained tryptase without chymase (MC_T). MC_{TC} were found to be on average slightly larger than MC_T, but a significant degree of overlap of size was seen.

The proportions of the two subtypes differed according to location. MC_{TC} were the more prevalent type in the skin and the sub-mucosa of the small intestine; whereas MC_T were present in higher numbers in the mucosa of the small intestine and the bronchi/bronchiole sub-epithelium and alveoli of the lung. These results were achieved using a rabbit polyclonal antibody for detection of chymase (Irani et al., 1986). Subsequently, upon production of a new mouse monoclonal anti-chymase antibody, the results were replicated: MC_{TC} predominated in human skin, small intestinal sub-mucosa and tonsils, and MC_T were predominant in small intestinal mucosa and alveoli of the lung (Irani *et al.*, 1989). Consistent with these findings, tryptase and chymase were detected in preparations of dispersed human foreskin mast cells, but chymase was detected in only minimal amounts in lung mast cells (Schwartz *et al.*, 1987).

Basophils

Basophils have similarities with mast cells in that they are granular cells (Fig 1.6(b)) arising from CD34 positive bone marrow-derived progenitor cells (Kirshenbaum *et al.,* 1991) that possess FcεRI in its complete, fully active tetrameric form, $\alpha\beta\gamma_2$ (Hempstead *et al.,* 1979). However, these cells are leukocytes, possessing poly-lobed nuclei (Dvorak, 2005). Basophil granules are

generally larger, but present in smaller numbers, than in mast cells. Granules are packed with dense particles, and can contain Charcot-Leydon crystals. Furthermore, whereas mast cells locate in tissues, basophils circulate in the bloodstream upon maturation. Similarly to mast cells, basophil activation results in release of various pre-formed and newly formed constituents upon cell degranulation (table 1.1, Section 1.2). In addition, there is also evidence that basophils are able to reconstitute their granules following degranulation (Dvorak, 2005).

Granular Constituents

The current study is primarily focused on the proteases that are stored within mast cell and basophil granules, and released upon cell activation and degranulation. Characteristics of the mast cell proteases tryptase, chymase, carboxypeptidase and DPPI, and the basophil protease basogranulin, are described in detail below. The proteases within the cell, and extracellularly upon degranulation, are illustrated in Fig 1.7. Histamine has also been addressed for comparison due to its well established role in mast cell and basophil-mediated allergic disease.

Histamine

Histamine is stored within the granules of both human mast cells and basophils, and is produced upon decarboxylation of histidine. Murine studies have shown that, *in vivo*, tissues with increased activation of histidine decarboxylase were able to produce greater quantities of histamine, and inhibitors of the enzyme were effective in blocking stomach histamine production (Reilly and Schayer, 1968). Histamine is degraded by histamine N-methyltransferase and monoamine oxidase (Imrie *et al.,* 1978).

Fig 1.7 Histamine, tryptase, chymase, carboxypeptidase, DPPI and basogranulin within mast cells and basophils, and extracellularly upon release. *Carboxypeptidase is activated from pro-carboxypepidase by cathepsin E. **Possibly intracellular actions.
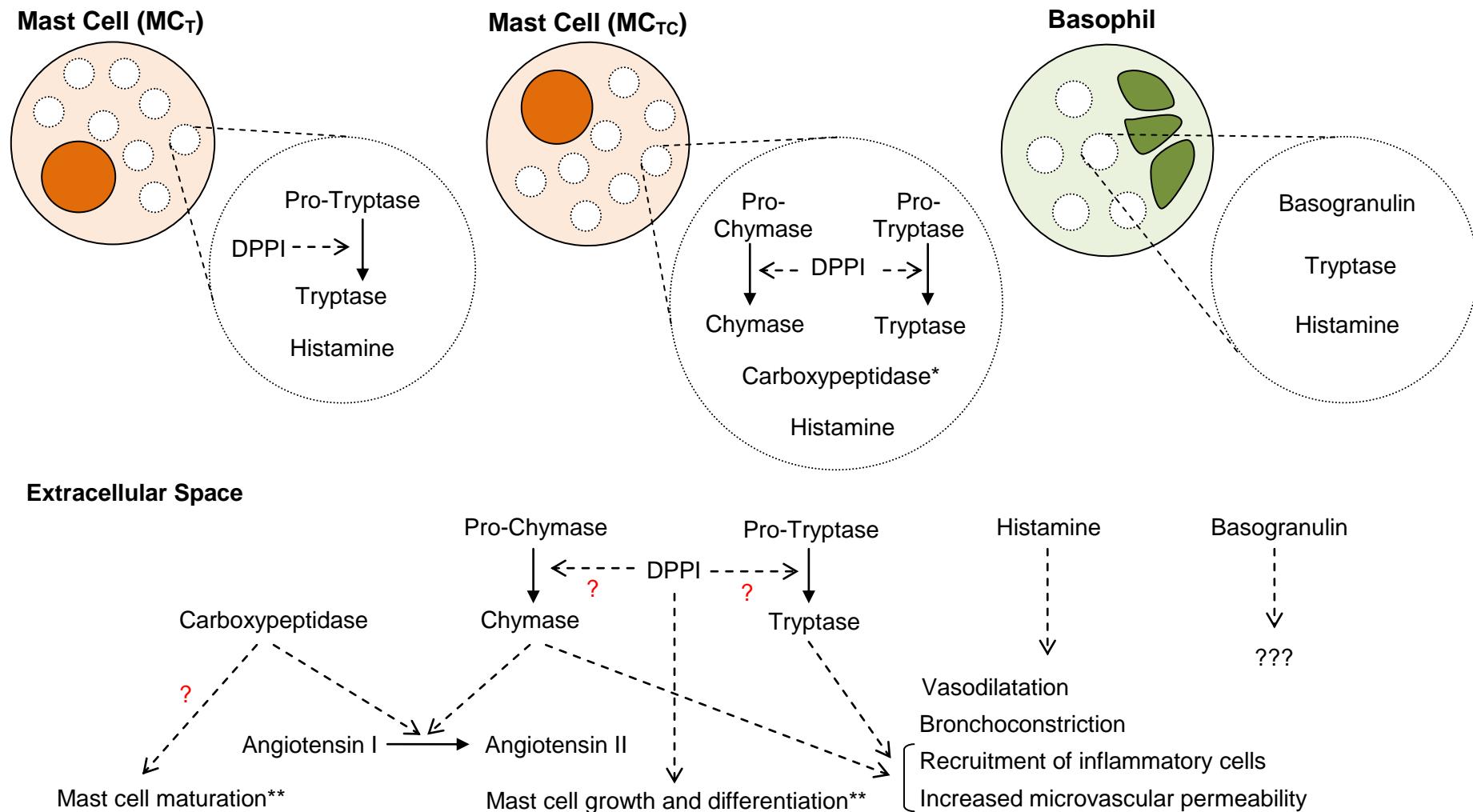
Release from mast cells and basophils

Histamine is released upon degranulation of mast cells and basophils (Ishizaka *et al.*, 1991; Mochizuki *et al.*, 2003). In one study it was found to be released from isolated rat peritoneal mast cells by calcium in the presence of phosphatidyl serine, and this occurred within 15 minutes. Concurrent with histamine release, calcium uptake into mast cell granules was increased. It was thus suggested that calcium may displace histamine (Chakravarty *et al.*, 1982). Further studies showed histamine release to be much slower than calcium uptake, supporting this hypothesis. These studies suggest that calcium may be taken up across the granule membrane, displacing histamine from the granule matrix (Chakravarty *et al.*, 1983).

Functions

Histamine causes a range of physiological effects (Reviewed in Jutel *et al.*, 2009). It's most notable effects in the allergic response include vasodilatation, increased vascular permeability and bronchoconstriction. However, it has also been shown to have involvement in numerous other processes, including haematopoiesis, cell proliferation and differentiation, regeneration and wound healing. In addition, histamine has various effects within the central nervous system.

Histamine acts via one or several of its four receptors; H_1, H_2, H_3 and H_4 (Jutel *et al*, 2009). These are all G protein-coupled receptors. H_1 and H_2 are expressed on airway and vascular smooth muscle, and are therefore most likely to be implicated in the symptoms induced by histamine in these tissues. H_3 is the primary receptor involved in histamine-mediated brain functions, and acts in an auto-regulatory fashion to control the rate of histamine synthesis and release. All four receptors are expressed on various innate immune cells, including eosinophils and dendritic cells. H_4 is also expressed on T cells, mast cells and basophils, and in numerous organs including the spleen, lung, heart, thymus, small intestine and colon. A key role of H_4 is the recruitment and activation of inflammatory cells (Jutel *et al*, 2009).

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Evidence of histamine's effects in allergy is widely reported, both in humans and animals. Histamine-mediated vasodilatation was demonstrated in dogs by intra-arterial histamine injection: an increase in carotid blood flow was observed (Saxena, 1975). Use of H_1 and H_2 blockers inhibited the response, implicating both of these receptors in the histamine-induced vasodilatation.

The anti-histaminic properties of the drug thiazinamium were found to protect against exercise-induced bronchoconstriction in asthmatic patients (Zieliński and Chodosowka, 1977); and use of H_1 and H_2-blocking agents in healthy subjects with histamine-induced bronchoconstriction suggested that this was a H_1-dependent response (Maconchie *et al.,* 1979). Increased blood vessel permeability in rat small intestine microvasculature was seen upon stimulation with histamine in a dose-dependent manner. As for bronchoconstriction, receptor antagonists demonstrated that this effect is H_1 dependent (Guth and Hirabayashi, 1983).

Tryptase

Tryptase is a serine protease that plays a key role in events mediated by mast cells. Tryptase purified from human lung was found to be tetrameric, with four non-covalently bound subunits, and had a molecular weight of 132 kDa, as shown by gel filtration (Walls *et al.*, 1990). The protease catalyzed the cleavage of tosyl L-arginine methyl esther and benzoyl DL-arginine *p*-nitroanilide, both of which are tryptic substrates. Subsequently, numerous forms of tryptase were found to exist in humans, including α-, ß-, γ-, and δ- tryptase. These are encoded by several genes and alleles (Caughey, 2006).

Alpha and beta tryptase

Following the original sequencing of tryptase, a second complementary DNA for the protease was found (Miller *et al.*, 1990). The original form was labelled α tryptase, and the new form β tryptase. Both α and β tryptase were recognized by antibodies previously produced against tryptase, and the amino acid sequence of the two forms was 90 % identical.

Beta tryptase was shown to consist of a homotetrameric structure, whereby the active sites of four monomers face in to a central pore, perhaps incurring protection against enzyme inhibitors and macromolecular substrates (Pereira *et al.*, 1998). In addition, β tryptase may be stabilized by heparin-proteoglycan. Using tryptase purified from human skin, SDS PAGE revealed major bands at 31 and 35 kDa, whereas Superose 12 gel filtration identified the relative molecular mass as 178 kDa. Similarly, molecular mass was 29 kDa on SDS PAGE but 141 kDa using Superose 12 gel filtration for purified lung tryptase. These findings support the presence of the β tryptase as a tetramer (Harvima *et al.,* 1999).

Within the mast cell granule, tryptase is stored under acidic conditions. Upon secretion during degranulation, the surrounding pH becomes neutral. Experiments that mimicked this neutral environment were found to cause dissociation of the tetramer into active monomers (Fajardo and Pejler, 2003). It was thus suggested that, upon mast cell activation and degranulation, β tryptase may dissociate into active monomers that have biological functions distinct from the tetrameric form. Upon dissociation of β tryptase from heparin, the tetramer forms inactive monomers at a neutral pH (Ren *et al.*, 1998). Addition of heparin could not reverse this; however, an acidic pH environment caused re-activation, even in the absence of heparin. It was proposed that this occurred by monomer dimerization, followed by tetramer formation and activation.

Studies using two different tryptase antibodies believed to be specific for the two subtypes suggested that α tryptase is the predominant circulating form in healthy subjects, and that levels are increased in patients with mastocytosis (Schwartz *et al.*, 1995). The antibody B12 was thought to capture both α and β tryptase, whereas the antibody G5 was thought to be mostly β tryptase specific. Levels of α tryptase were calculated using measurements of total and β tryptase.

All subjects with good health or unrelated diseases in the study had tryptase detectable with the B12 antibody, but not with the G5 antibody, suggesting that the major circulating form is α tryptase (Schwartz *et al.*, 1995). In addition, tryptase

levels measured in patients with mastocytosis were significantly higher using the B12 antibody than G5 antibody. However, later studies using polymerase chain reaction (PCR) found that almost 30 % of 274 human subjects were genetically deficient of mast cell α tryptase (Soto *et al.*, 2002).

It was subsequently identified that the G5 antibody detects the active form of tryptase, whereas the B12 antibody detects both pro and active forms (Fukuoka and Schwartz, 2006). These antibodies cannot therefore be used for measurement of α and β tryptase. However, the results of the study do suggest that the protease may be present in the circulation in its unprocessed form. Thus potential mechanisms of activation during allergic reactions may be an interesting and useful point of research.

Gamma and delta tryptase

Gamma tryptase was described in 2000 (Caughey *et al.*, 2000). It was found to be transcribed in various tissue types, including the lung and intestine, as well as in the human mast cell line, HMC-1. A C-terminal hydrophobic domain was identified, which was suggested to act as a membrane anchor. Thus, whereas the other tryptase forms are soluble, γ tryptase may be membrane bound. This form of the protease may also have a distinct mode of activation (Caughey *et al.*, 2000).

Delta tryptase was named as such in 2002, but identified previously as mouse mast cell protease 7 (mMCP-7)-like tryptase (Wang *et al.*, 2002). Delta tryptase was generated and cloned from mRNA isolated from the HMC-1 cell line, and transcripts were found in multiple human tissues, including heart, skin and stomach. Delta tryptase was suggested to be an inactive form of the protease, perhaps due to truncation of segments crucial for enzyme activity (Trivedi *et al.,* 2008).

Cell specificity

Antibody binding studies using tryptase-specific antibodies suggested that the protease was specific to mast cells; and it was therefore proposed that they can be used as a tool to identify human mast cells (Walls *et al*., 1990). In addition, tryptase detection by specific antibodies was thought to be useful in differentiating these cells from basophils. More recently, however, tryptase has been found to be present in basophils in small quantities (Foster *et al*., 2002). Staining of peripheral blood leukocytes using tryptase antibodies was selective to a population of cells that also stained positive for IgE and basophil specific granule proteins, including basogranulin. The cells did not stain positive for chymase or other mast cell markers. Studies on purified peripheral blood basophils revealed that the tryptase stored in these cells is mature and enzymatically active; and PCR demonstrated that both α and β forms of the protease may be expressed. The quantity of tryptase in basophils was suggested to be less than 1 % of that stored in mast cells (Brahim *et al*., 2004).

Functions

Functions of tryptase are not completely understood. However, injection of tryptase into the skin of guinea pigs was found to cause accumulation of eosinophils and neutrophils (He *et al.,* 1997). In addition, tryptase injection into mouse peritoneum led to accumulation of neutrophils; and, with high doses, increases in numbers of macrophages and lymphocytes were observed. Injection of histamine and tryptase enhanced accumulation of eosinophils. The protease may therefore be involved in recruitment of inflammatory cells upon degranulation from mast cells.

Injection of tryptase isolated from human lung tissue into the skin of guinea pigs also increased micro-vascular leakage in a dose-dependent fashion (He and Walls, 1997). Tryptase stimulated histamine release from skin and lung cells *in vitro*, suggesting that the increased micro-vascular leakage may involve histamine.

Tryptase has also been found to stimulate histamine release from human tonsil mast cells, and tryptase inhibitors prevented IgE-dependent histamine release (He *et al.*, 1998). Similar findings were reported in synovial fluid (He *et al.*, 2001); and inhibition of IgE-dependent histamine and tryptase release by protease inhibitors was identified in human lung mast cells (He and Xie, 2004). These results suggest that tryptase may have an amplification effect in the activation of these cells.

Chymase

Like tryptase, chymase is a serine protease. Evidence of a chymotrypsin-like enzyme was first reported in rat mast cells by Benditt and Arase in 1959; and in human lung mast cells by Wintroub *et al.* in 1986. The enzyme was found to have angiotensin I converting activity and, with increasing doses of anti-IgE, this activity correlated with histamine release (Wintroub *et al.*, 1986). It was therefore suggested to be a mast cell granule constituent. Gel filtration identified the enzyme as being distinguishable from tryptase, and indicated a molecular weight of 30 – 35 kDa.

    Release from mast cells and activation

The mast cell granule in which chymase is stored is reported to have a pH of 5.5 (McEuen *et al.*, 1998). Chymase was relatively inactive at this pH, but appeared to be immediately active upon release. This suggests that chymase may be stored within mast cell granules in its mature form, and may become instantly active when released due to the removal of the pH suppressing effect. The formation of mature chymase from its inactive form, pro-chymase, is catalyzed by the enzyme dipeptidyl peptidase I (DPPI) via the removal of a dipeptide; and this is inhibited by heparin and histamine.

Localization and subtypes

Chymase is found mainly in the subpopulation of mast cells that localise in the dermis of the skin as well as in other connective tissues (MC_{TC}); little or no chymase is expressed in the MC_T cells of mucosal tissue (Irani *et al.,* 1986; Schwartz *et al.*, 1987; Caughey, 2007). In a study by McEuen *et al.* (1998), affinity chromatography revealed several forms of human chymase. These showed differences in tissue distribution, and it was suggested that the possibility of variations in chymase action at different inflammatory sites should be investigated. The differences in forms detected may represent differences in glycosylation.

Functions

A major function of human chymase is the generation of angiotensin II, as described above (Reilly *et al.*, 1982). In addition, the protease was suggested to inactivate bradykinin (Reilly *et al.*, 1985), but this is controversial (Takai *et al.*, 1997). Chymase has been found to increase micro-vascular leakage upon injection into the skin of guinea pigs (He and Walls, 1998). The effect was less potent than that induced by similar doses of histamine, but had a much longer duration. It was not affected by pre-treatment with anti-histamines, and was therefore unlikely to be histamine-mediated. Further studies in guinea pigs showed that chymase injection into the skin caused accumulation of neutrophils and eosinophils (He and Walls, 1998). Thus chymase release upon mast cell activation may have a key role in recruitment of inflammatory cells to the area.

Carboxypeptidase

Mast cell carboxypeptidase was first described in humans in 1987, when activity resembling that of carboxypeptidase was identified in dispersed human lung and skin mast cells (Goldstein *et al.*, 1987). The enzyme was released alongside histamine upon cell activation with goat anti-human IgE, and thus suggested to be

localised to mast cell granules. Furthermore, it appeared to be present at higher concentrations in skin than lung cell granules.

The carboxypeptidase-like enzyme was purified, and suggested to be a secretory granule zinc-metalloexopeptidase (Goldstein *et al.*, 1989). It was found to have a molecular weight of approximately 34.5 kDa on SDS PAGE. Functional similarities of the protease were seen with carboxypeptidase A, for example in its ability to hydrolyze synthetic dipeptides and angiotensin; but structurally it was more similar to human pancreatic carboxypeptidase B. This novel member of the zinc-containing carboxypeptidase family is referred to as mast cell carboxypeptidase.

Localization

Immunohistochemical labelling of sections of human lung, skin and gastrointestinal tissue using mouse monoclonal antibodies specific for mast cell carboxypeptidase, tryptase and chymase, demonstrated that carboxypeptidase was selectively localised in the tryptase positive, chymase positive MC_{TC} population, but not in chymase negative MC_T (Irani *et al.*, 1991). In addition, carboxypeptidase was found to reside in protease-proteoglycan complexes released from dispersed human skin mast cells together with chymase, but distinct from tryptase (Goldstein *et al.*, 1992).

Activation

Within murine mast cell granules, carboxypeptidase is produced initially in an inactive zymogen form, known as pro-carboxypeptidase, before being activated (Dikov *et al.*, 1994). A mast cell line (KiSV-MC9) was identified that produces pro-carboxypeptidase, but lacks the capacity to activate the enzyme. This cell line can, however, activate both pro-chymase and pro-tryptase, and it was therefore suggested that carboxypeptidase is activated by separate mechanisms. Mouse mast cells deficient in cathepsin E were found to have increased levels of pro-carboxypeptidase, and, *in vitro*, recombinant cathepsin E processed recombinant

pro-carboxypeptidase in granule-like conditions (Henningsson *et al.*, 2005). Thus cathepsin E may have a key role in activation of mast cell carboxypeptidase.

Functions

The role of carboxypeptidase, alongside chymase, in the angiotensin system was studied in mice by Lundequist *et al.* (2004). Upon application of angiotensin I to peritoneal cells, and subsequent activation of mast cells, formation and degradation of angiotensin II were enhanced. Use of chymase null cells or inhibition of carboxypeptidase alone did not reduce angiotensin II generation. However, following inhibition of carboxypeptidase in chymase null cells it was completely inhibited. This implicates both proteases as important mediators in the angiotensin system. It was suggested that, whereas angiotensin-converting enzyme (ACE) plays a key role in generation of intravascular angiotensin II, the mast cell proteases are central in this task extravascularly. This role has yet to be confirmed in humans.

Other than being implicated in the generation of angiotensin II in mice, functions of carboxypeptidase are poorly understood. Mast cells of carboxypeptidase knockout mice appeared immature upon histochemical staining (Feyerabend *et al.*, 2005). Mast cell degranulation was not impaired, and the knock-out mice had the capacity to undergo experimentally-induced anaphylaxis. Thus, carboxypeptidase was suggested to play a key role, not in mast cell function, but in maturation of the cell. Other than angiotensin I, substrates of mast cell carboxypeptidase identified to date include neurotensin, endothelin I, apolipoprotein B, and interestingly venoms such as sarafotoxin (Pejler *et al.*, 2009).

Dipeptidyl peptidase I

Dipeptidyl peptidase I (DPPI), otherwise known as cathepsin C, is a cysteine protease expressed in bone marrow-derived leukocytes, such as myelomonocytes, cytotoxic T cells, neutrophils and mast cells (Wolters *et al.*, 2000). In SDS PAGE,

DPPI purified from dog mastocytomas was found to consist of four subunits, and have a molecular mass of approximately 175 kDa (Wolters *et al.*, 1998). DPPI was found to be present in dog mast cells at much greater concentrations than in other sources, including human kidney and spleen. The amount estimated per cell was comparable to that of chymase. It was thus proposed that mast cells may be the major source if the enzyme in many tissues. Indeed, in further studies using immunohistochemical techniques, mast cells were found to be the major source of the protease in the normal dog airways (Wolters *et al.*, 2000). Macrophages, on the other hand, were the major source in the alveoli.

Functions

DPPI may play a role in the growth and differentiation of mast cells, and has been implicated in asthma. Purified DPPI was found to cleave fibronectin and collagen types I, III and IV *in vitro*, suggesting involvement of the enzyme in degradation and turnover of proteins (Wolters *et al.*, 2000). In addition, DPPI has been implicated in activation of mast cell tryptase and chymase from their pro forms. Inhibition of DPPI in P815 mastocytoma cells was found to reduce tryptase activity by approximately 90 % compared to untreated cells (McGuire *et al.*, 1993), and treatment of recombinant human pro-chymase with DPPI resulted in enzymatically active chymase (McEuen *et al.*, 1998). This latter effect was inhibited by heparin or histamine.

Release from mast cells

DPPI was found to be released from the C2 dog mastocytoma cell line in a time-dependent manner following activation by ionophore or substance P, as determined by measurement of activity (Wolters *et al.*, 1998). Furthermore, DPPI was found to be active at an acidic to neutral pH. These results indicate that the enzyme may act extracellularly as well as intracellularly. Activity at an acidic pH is essential for activation of tryptase and chymase, as this likely occurs within the golgi apparatus or secretory granules where the pH is approximately 5.5 – 6.5.

Outside of the cell the pH becomes neutral. Thus, release from mast cells and continued activity at this pH would allow DPPI to act extracellularly. Release of DPPI from human mast cells has not previously been demonstrated.

Basogranulin

In 1999 a new monoclonal antibody was produced within our group that binds specifically to basophils (McEuen *et al.*, 1999). This antibody (termed BB1) stained basophils and the KU812F basophilic cell line, but not any other cell type or tissue structure, in immunocytochemistry. Staining of basophils with BB1 indicated that the antigen to which it bound was present within the cell granules, and immunoelectron microscopy confirmed this granular location. Furthermore, it was found to bind to the granule matrix. This novel basophil-specific protein identified using the antibody was named basogranulin (McEuen *et al.*, 2001).

Release from basophils

Stimulation of isolated human peripheral blood basophils with anti-IgE or calcium ionophore resulted in release of basogranulin, together with histamine, in a bell-shaped response curve (Mochizuki *et al.*, 2003). Of note, some of the secreted protein was found, by flow cytometry, to bind to the cell surface. Basogranulin release was not seen from basophils of the 'non releaser' phenotype. Basogranulin was predicted to have a large size, and its molecular mass was determined as 124 +/- 11 kDa on Hedrick-Smith gel (McEuen *et al.*, 1999). It was suggested that this may restrict the rate of diffusion of the protein upon secretion within the body. Tissue accumulated protein, i.e. protein that has left the circulation, may therefore be expected to be highly localised (McEuen *et al.*, 2001).

1.4 Diagnosis of Allergy

In this section allergy diagnosis has been split into two categories. The first of these is diagnosis of an allergic condition, i.e. confirmation that an individual is sensitized to an allergen, and has the capacity to react clinically if exposed. The second is diagnosis that an allergic reaction has occurred. These have been addressed separately below, and the requirement for new supporting laboratory tests has been discussed.

Diagnosing an allergic condition

Diagnosis of IgE-mediated allergy is largely reliant on the clinical history of a patient and performance of skin testing. *In vitro* tests for detection of total or allergen-specific IgE in serum samples are additional tools that can aid diagnosis. As a final measure, avoidance trials and/or provocation testing can be effective in confirming diagnosis. Recent guidelines produced by the NIAID recommend carrying out skin prick testing (SPT), specific IgE measurement and oral food challenge, but not intra-dermal or patch testing, in the diagnosis of food allergy (NIAID-Sponsored Expert Panel *et al.*, 2010).

Skin prick testing

Skin prick testing measures mast cell-bound allergen-specific IgE in the dermis, and is carried out by placing allergen extract onto the arm or back and then piercing the skin with a lancet. In sensitized individuals an area of red flared skin (wheal) will appear (Fig 1.6). The diameter of the wheal is measured after 15 minutes, and the result is interpreted with regard to negative (saline) and positive (histamine) control tests. Generally, a diameter of at least 3mm greater than the negative control is considered positive in epidemiological research (Eigenmann and Sampson, 1998). However, this was found to be poorly predictive of clinical reactivity to foods (Hill *et al.,* 2004).

Fig 1.8 Skin prick test. A range of common allergens were tested within our clinic. Development of a red, raised weal indicates sensitisation to the allergen. Patient consent was obtained for use of this photograph.

Cut-off values that are thought to be close to 100 % diagnostic of clinical food allergy have been reported. In children over three years these values include wheal diameters of at least 8 mm for cow's milk, 7 mm for egg and 8 mm for peanut (Hill *et al.*, 2004). In children two years and under the cut off values are 6 mm, 5 mm and 4 mm, respectively. Children with SPT measurements above these cut off values are highly likely to be allergic and generally require no further testing unless clinical suspicion is high. However, measurements below the values reported do not exclude allergy.

SPT values that are fully predictive of clinical allergy are not available for many food types, and research has not been carried out to determine those specific for adults. Wheal diameter is dependent on allergen extract concentration and technique used, and these cut-off values may therefore not be appropriate for all centres. Furthermore, all populations of patients studied are from tertiary allergy clinics, and may not be representative of other patients. There is also a possibility that factors such as geography or race may affect the results.

Skin prick testing is also performed routinely in diagnosis of IgE-mediated allergy to a range of allergens other than foods, including drugs, latex, pollens, animal dander, fungi and dust mite. However, the problems discussed above are applicable to most tests. Patch testing is another type of skin test, whereby the substance under investigation is applied to the skin for 48 hours, and resulting symptoms are examined between 48 and 72 hours (Nosbaum *et al.*, 2010). There is evidence that it has some use in diagnosis of T cell-mediated drug reactivity (Friedman and Ardern-Jones, 2010). However, it has little use in IgE-mediated immediate hypersensitivity and is generally not accepted in diagnosis of food allergy (Bahna *et al.*, 2008; NIAID-sponsored expert panel *et al.*, 2010)

IgE measurement

Serum levels of total IgE are often determined in diagnosis of allergy. This gives a measure of all circulating IgE, and is often elevated in the serum of allergic patients

(Kiellman *et al.*, 1977; Saarinen *et al.*, 1982). However, total IgE levels are perhaps more useful as an indicator of atopy rather than in diagnosis of a particular allergy.

Allergen-specific IgE measurement in serum is the most commonly utilized *in vitro* test. This is a measurement of circulating IgE in the blood that is specific to the allergen in question. Specific IgE testing is generally considered sensitive, but not always specific. Celik-Bilgili *et al* (2005) found that the sensitivity of a positive specific IgE test in predicting hen's egg allergy was 97 %, but specificity was only 51 %. For children with suspected cow's milk challenges, these values were 83 % and 53 %, respectively. Both sensitivity and specificity were relatively low for wheat and soy allergy.

Predictive decision points were established for hen's egg and cow's milk allergy using this data. Ninety five per cent predictive probabilities for hen's egg were 10.9 kU/L for children under one year of age, and 13.2 kU/L for those over one year. Decision points could not be established for cow's milk at the 95 % level. However, the 90 % predicted probability was 88.8 kU/L for all children, or 25.8 kU/L for those under one year (Celik-Bilgili *et al.*, 2005).

Previous to this study, 95 % predictive decision points had been reported for egg (6 kU_A/L), milk (32 kU_A/L), peanut (15 kU_A/L), fish (20 kU_A/L), soybean (65 kU_A/L), and wheat (100 kU_A/L) (Sampson, 2001). This study also focused on allergy in children, though the population size was smaller than that recruited by Celik-Bilgili *et al.* As for skin prick testing, these cut-off values may be useful in establishing allergy in those with higher measurements. However, a lower measurement does not exclude a positive diagnosis. Furthermore, predictive values have not been established for adults.

Specific IgE testing can be used in diagnosis of drug allergy. However its use is limited. It is reported to be less sensitive than skin testing, and assays are only available for some therapeutic agents (Kränke and Aberer, 2009). Latex-specific

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IgE can be detected in allergic subjects, but reported specificity is low. In one study 12 % of 182 Portuguese children tested positive for specific IgE, but only 0.5 % were allergic according to questionnaire (Jorge *et al.*, 2006).

Provocation testing

History, SPT diameter, and specific IgE measurement are often informative when used in conjunction. Provocation tests are generally only performed when other tools have not yielded a clear result.

Oral food challenge

Food challenges involve exposing the patient to a particular food in an incremental fashion (Taylor *et al.*, 2004; Devenney *et al.*, 2006). The procedure can be open, blind or double blind; and can confirm whether an individual is clinically reactive or tolerant to an allergen. There is a risk of systemic reaction in provocation tests; but nonetheless, the double blind, placebo controlled food challenge (DBPCFC) is considered the 'gold standard' test in allergy diagnosis (Bohle and Vieths, 2004). However, due to the expense and time consuming nature of the procedure, open challenges are usually performed within the clinical setting

Other provocation tests

The drug provocation test has been considered to be 'gold standard' for diagnosis of drug allergy. It is useful for confirming allergy, and also for excluding the diagnosis in patients with non-allergic manifestations resulting from medication (Bousquet *et al.*, 2008). Due to the high risks involved in intravenous drug administration, the usual form of provocation is the intra-dermal test (Brockow and Romano, 2008). Allergen is injected into the dermis, causing a raised area to form. A positive result is taken as an increase in this area over a period of 20 minutes, but systemic symptoms can sometimes occur. Intra-dermal tests are also carried out for diagnosis of insect venom allergies (Hamilton, 2010).

Latex challenges are similar to those for food, but can be performed by exposing the patient to the rubber in a number of different ways (e.g. cutaneous, mucous-oral, sublingual, conjunctival, nasal or bronchial). Bronchial and nasal tests were found to have the greatest sensitivity (Nucera *et al.*, 2010). However, bronchial challenge carried the highest risk. All tests had a specificity and positive predictive value of 100 %. Nasal allergen challenge has also been carried out with grass pollen in patients with seasonal allergic rhinitis. This technique can be used to examine efficacy of anti-allergic treatments and medications (Proud *et al.*, 2010).

Diagnosing a reaction

Diagnosis of a recent allergic reaction is based mainly on presenting signs and symptoms. Diagnostic criteria for anaphylaxis are outlined in Section 1.1 and illustrated in Fig 1.3. There are supporting laboratory tests available. The usefulness of these tests, and the potential for new tests, is described below.

Currently available tests

The laboratory tests that are currently available for confirming the recent occurrence of an allergic reaction are measurement of plasma histamine and serum tryptase levels. Official guidelines promote the measurement of tryptase at a series of time points in the routine diagnosis of anaphylaxis, both nationally (Project Team of The Resuscitation Council (UK), 1999) and internationally (Lieberman *et al.*, 2005). There is a lot of contradictory evidence as to how useful these tests are. This is described below and summarized in table 1.3.

Histamine

Histamine is a biogenic amine stored within the granules of human mast cells and basophils, and is released upon activation and degranulation of these cells (Ishizaka *et al.*, 1991; Mochizuki *et al.*, 2003). Histamine levels typically increase during an allergic reaction. However, levels peak very quickly after the onset of

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symptoms and usually decline to normal within an hour. In a study of patients experiencing anaphylaxis to bee sting challenge, plasma histamine levels increased, reaching a peak level about five to 10 minutes after challenge (Schwartz *et al.*, 1989). Levels then declined quickly, returning to baseline between 15 and 60 minutes.

In an emergency department-based study of almost 100 adults with suspected allergic reactions to food, drugs or latex, approximately half were found to have elevated plasma histamine levels (Lin *et al.*, 2000). The majority of these patients were not reported to have anaphylactic reactions, suggesting that histamine levels may be a marker of non-anaphylactic as well as anaphylactic reactions. However, levels did correlate with initial heart rate, and were higher in subjects with more severe symptoms such as wheezing or angioedema.

In one study looking specifically at histamine as a marker of allergic reactions to food, intra-luminal provocation in food allergic adults with food allergens resulted in an increase in intestinal histamine release (Santos *et al.*, 1999). However, plasma levels were not studied. In another study, plasma histamine levels were found to be increased in positive food challenges in children with both immediate and delayed symptoms (Ohtsuka *et al.*, 1993), and increased urinary levels of the histamine metabolite, 1-methylhistamine, were observed by one hour following reaction in another study of paediatric food challenges (Beyer *et al.*, 1994). However, in the latter study, variations were also seen in the negative challenges.

Available evidence suggests that levels of histamine in plasma are reliably increased in anaphylaxis, including reactions to foods. However, it is limited as a marker in terms of practicality due to its short half life. Measurement of histamine metabolites in urine may prove to be more useful. An additional problem is that histamine levels have been found to be increased in haemolysed plasma samples. This was a cause for false positive results (Laroche *et al.*, 1995). False negative results were obtained in pregnant women and a heparinized patient.

Table 1.3 Evidence for and against the use of histamine and/or tryptase as markers of anaphylactic reactions. Results refer to serum or plasma levels unless specified.

Allergen	Reference	Population	Results	
			Histamine	**Tryptase**
General	Treudler. *et al.*, 2008	105 anaphylactic reactions	-	Levels elevated in 8 (6 with mastocytosis)
	Shen *et al.*, 2009	14 anaphylactic deaths	-	Levels 3.3 - 200 ng/ml
	Pumphrey and Roberts, 2000	16 anaphylactic deaths	-	Levels elevated in 14
	Yunginger *et al.*, 1991	19 anaphylactic deaths	-	Levels elevated in 17
	Schwartz *et al.*, 1987	6 anaphylactic reactions	-	Levels 9 - 75 ng/ml
	Lin *et al.*, 2000	97 acute allergic reactions	Levels elevated in 42	Levels elevated in 20
Drugs	Dybendal *et al.*, 2003	18 anaphylactic reactions	-	Levels elevated in 10
	Fernandez *et al.*, 1995	20 anaphylactic or urticarial reactions	Urinary levels elevated	Levels elevated
Insect venom	Brown *et al.*, 2004	11 anaphylactic reactions	Levels elevated	Levels elevated (using cut off of 9 µg/ml)
	Schwartz *et al.*, 1989	3 anaphylactic reactions	Levels elevated in 3	Levels elevated in 3
Food	Ohtsuka *et al.*, 1993	29 open challenges in 20 children	Levels elevated	Levels elevated in immediate but not delayed reactions
	Beyer *et al.*, 1994	16 allergic reactions	Urinary 1-methylhistamine levels elevated	Levels elevated
	Bellanti *et al.*, 1981	15 allergic reactions	Levels elevated in 4	-
	Vila *et al.*, 2001	11 allergic reactions	-	Levels elevated in 2

Tryptase

Tryptase is a serine protease that is stored in and released from the granules of mast cells (Walls *et al.*, 1990), and is commonly cited as a marker of mast cell activation (Schwartz *et al.*, 1987). However, there is now evidence that small amounts are also found within basophils (Foster *et al.*, 2002). In an early study, tryptase levels were found to range from 9 to 75 µg/l in six patients who had experienced anaphylaxis in response to foods, drugs, insect venom or exercise (Schwartz *et al.*, 1987).

In the bee sting challenge study described above, levels of tryptase were found to increase in all subjects with anaphylaxis from around 30 minutes and peaked at one to two hours. After this time levels reduced, with a half life for the enzyme of approximately two hours (Schwartz *et al.*, 1989). In a study of anaphylactic deaths, serum tryptase levels were raised (greater than 12 µ/l) in all nine reactions to insect venom, both of the two reactions to therapeutic agents, and six of the eight reactions to food (Yunginger *et al.*, 1991). Of 16 anaphylactic deaths for whom serum tryptase was measured in another study, high levels were observed in 14 (Pumphrey and Roberts, 2000).

However, evidence from further studies was less supportive of tryptase measurement. Lin *et al* (2000) showed that, of 97 patients presenting at the emergency room with acute allergic reactions, only approximately 20 % had high tryptase concentrations. Similarly, only eight of 105 patients with suspected anaphylaxis in another study were found to have raised tryptase levels (Treudler *et al.*, 2008). Six of these patients reacted to insect stings and two reacted to drugs, and six of the eight patients were confirmed as having systemic mastocytosis. In a study of 14 anaphylactic deaths, serum tryptase concentrations were found to range from 3.3 to 200 µg/l. Levels were considered elevated in nine of the victims, and the lowest levels were reported to have been measured in the cases in which food was the implicated allergen (Shen *et al.*, 2009).

Research looking specifically at tryptase as a marker in food allergy is also contradictory. Vila *et al.* (2001) measured serum and saliva tryptase levels before and following food challenges in patients who had previously suffered systemic reactions to food. Only 25 % of 8 patients with a positive food challenge had elevated serum tryptase levels, and no significant differences in levels were observed between measurements before and after challenge in any of the patients. Tryptase was detected in the saliva both before and after the food challenge in one positive subject. In all other subjects, including controls, saliva tryptase was undetectable. However, Beyer *et al.* (1994) found tryptase levels to be increased following positive food challenge in sixteen children, and Ohtsuka *et al.* (1993) found levels to be increased by four hours after positive food challenge in which immediate, but not delayed, symptoms presented.

Jejunal provocation with food allergens in food allergic subjects resulted in increased intestinal release of tryptase, alongside histamine and prostaglandin D_2 (Santos *et al.*, 1999). However, no differences in plasma tryptase levels were seen after compared to before challenge. Furthermore, direct provocation of the jejunum with allergen may not accurately reflect the natural course of allergen exposure and the resulting allergic response; the allergen would not undergo the normal digestive processes.

Available evidence suggests that, although high tryptase levels are an indicator of recent anaphylaxis, low levels cannot exclude a diagnosis of this condition. In addition, tryptase levels can be raised in other conditions, for example mastocytosis (Schwartz *et al.*, 1987). It is widely agreed that further research into the use of tryptase measurement in diagnosing anaphylaxis, particularly as a result of food, is needed.

Potential new markers

Due to the contradictory data available concerning the use of currently available tests, the search for other markers for confirming diagnosis of allergic reactions,

particularly to food, has continued. As for tryptase and histamine, other potential markers are perhaps most likely to be those released from mast cells and basophils upon cell activation. These may include leukotrienes and prostaglandins, cytokines, or proteases. Coagulation alterations have been observed in anaphylaxis (Halonen and Pinckard, 1975; Lombardini *et al.*, 2009), and thus factors involved in the blood clotting process have also been studied. The evidence for use of potential new markers has been described below and summarized in table 1.4. Reasoning for and against the use of the various mediators in confirming diagnosis of allergic reactions has also been discussed.

Components of the coagulation cascade

In a study by Smith *et al* (1980), three out of 14 subjects who reacted in hymenoptera challenge following immunotherapy had systemic anaphylaxis with hypotension. Reductions in Factor V, Factor VIII, fibrinogen and high molecular weight (HMW) kininogen were observed in plasma from the two subjects with the most severe reactions. This was thought to suggest the presence of intravascular coagulation, and thus depletion of coagulation factors. In another study of insect venom challenges, HMW kininogen activation was measured in plasma in seven of 12 patients who suffered anaphylaxis (Van der Linden *et al.*, 1993). Plasma levels of C1-inhibitor complexes, including Factor XII-C1-inhibitor and kallikrein-C1-inhibitor, were also increased in seven of the anaphylactic patients. These changes were not seen in four patients who did not have anaphylactic reactions in the challenge, or in five healthy control subjects.

Increases in plasma levels of von Willebrand Factor (vWF), tissue-type plasminogen activator (tPA) and plasminogen-α2-antiplasmin complex (PAP-c) have also been observed in response to insect venom challenges in 7 patients who experienced hypotension (Van der Linden *et al.*, 1993); and levels of tPA and PAP-c showed an inverse correlation with mean arterial blood pressure.

Table 1.4 Evidence for use of new markers of anaphylaxis. Reactions are to any allergen unless specified.

Study	Population	Main findings
Smith *et al.*, 1980	3 anaphylactic reactions to insect venom	Reduced plasma factor V and VII, fibrinogen and HMW kininogen levels in 2
Van der Linden *et al.*, 1993	20 anaphylactic reactions to insect venom	Increased vWF, tPA and PAP-c levels with hypotension
Van der Linden *et al.*, 1993	12 anaphylactic reactions to insect venom	Increased plasma C1-inhibitor complex levels and HMW kininogen activation in 7
Van der Linden *et al.*, 1993	39 anaphylactic reactions to insect venom (17 with shock)	Increased epinephrine, norepinephrine and ATII levels with shock
Ono *et al.*, 2008	32 anaphylactic reactions	Increased urinary LTE4 and 9α,11ß-PGF2 levels
Van Odijk, J. *et al.*, 2006	4 reactions to food	Increased urinary LTE4 levels
Stone *et al.*, 2009	76 anaphylactic reactions	Increased serum IL-2, -4, -5, -6, -10 and -13, IFN-γ, TNF-α and TNFRI
Vadas *et al.*, 2008	41 anaphylactic reactions; 9 anaphylactic fatalities to peanut	Increased serum PAF levels in anaphylaxis, and low PAFAH activity in fatalities
Nishio, H. *et al.*, 2005	8 anaphylactic deaths	Increased serum chymase in 6

There have been few investigations into levels of components of the coagulation cascade during allergic reactions. However, the evidence that is available indicates alterations only in patients with very severe reactions; and in particular those with cardiovascular compromise. Thus a test that utilizes measurement of these markers for confirming diagnosis of an allergic reaction may be of limited value in diagnosis as it would pick up only a sub-group of patients. Available data suggests that children experience mostly respiratory difficulties, rather than cardiovascular compromise, during severe allergic reactions. Alterations in levels of clotting factors may therefore be less likely to be detected in these patients.

Leukotrienes and prostaglandins

Urinary concentrations of leukotriene E_4 (LTE_4) and 9α, 11ß-prostaglandin F2 (9α,11ß-PGF2), a degradation product of prostaglandin D_2 (PGD_2), have been reported to be significantly higher in patients presenting at a Japanese emergency department with anaphylaxis (mostly to foods and drugs) than in healthy control subjects (Ono *et al.*, 2008). LTE_4 and PGD_2 are lipid-derived mediators that are newly synthesized and released from mast cells upon cell activation and degranulation. In the same study, urinary concentrations of LTE_4 and 9α,11ß-PGF_2 were also increased compared to baseline in provocation tests in anaphylactic patients. LTE_4 reached peak levels between three and six hours, whereas 9α,11ß-PGF_2 levels peaked by three hours. In another study, levels of LTE_4 were also found to increase in urine in four patients with IgE mediated food allergy, but not in seven patients with other forms of food hypersensitivity, following double blind placebo-controlled food challenge (van Odijk *et al.*, 2005).

Urinary secretion of LTE_4 and 9α,11ß-PGF_2 are considered to be linked to mast cell activation. However, LTE_4 and PGD_2 are also produced by other cells, and changes in levels may therefore be seen in other diseases. This may limit the usefulness of these mediators as potential diagnostic biomarkers in confirming allergic reactions.

Cytokines

Various cytokines are synthesized upon mast cell activation and released during degranulation. Levels of interleukin 2 (IL-2), IL-4, IL-5, IL-6, IL-10, IL-13, Interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and TNF receptor I (TNFRI) were found to be significantly higher in serum from 36 patients presenting to emergency departments with severe allergic reactions compared to healthy control subjects (Stone *et al.*, 2009). Levels of IL-2, IL-6, IL-10 and TNFRI were also higher than in patients presenting with moderate reactions. Increased levels of the cytokines were observed in samples taken soon after presentation at the emergency departments and at 40-80 minutes after enrolment. A final sample was taken before discharge, but the cytokines were observed only occasionally in these samples.

As is the case for prostaglandins and leukotrienes, the cytokines described are not mast cell specific. Levels may therefore be altered in other disease states. Although measuring concentrations of a panel of the markers may have some value in indicating mast cell activation, this is unlikely to be specific and reliable enough for confirming a reaction in a clinical setting.

Platelet activating factor

Serum levels of PAF were found to be significantly higher in 41 subjects presenting at an accident and emergency department with anaphylaxis compared to 23 healthy control subjects (Vadas *et al.*, 2008). PAF is a glycerol-phospholipid that is released from a range of cell types, including basophils, neutrophils, and perhaps mast cells (Tencé *et al.*, 1980). In the study described, PAF levels were found to be higher with increasing grades of severity, and were negatively associated with activity levels of the enzyme that degrades it, PAF acetylhydrolase (PAFAH). PAFAH activity was also lower in serum from nine patients who died of anaphylaxis to peanut than in a range of control groups, including healthy adults and children,

cases of non-fatal anaphylaxis and non-anaphylactic fatalities, children with mild peanut allergy, and children with asthma.

PAF is very unstable, and is therefore likely to be impractical as a diagnostic biomarker. Measurement of PAFAH activity may provide a useful alternative. However, in the study described, PAFAH activity was low only in cases of *fatal* anaphylaxis to peanut. In another study, enzyme activity was not found to differ between patients with non-fatal anaphylaxis and healthy control subjects (Bansal *et al.*, 2008).

Mast cell and basophil proteases

The ideal marker (or panel of markers) for use in confirming an allergic reaction should be specific to the cells involved in the response, i.e. mast cells and basophils. Furthermore, levels of the marker(s) should be reliably altered during allergic reactions, ensuring a high sensitivity. Proteases that are mast cell and/or basophil specific, and that are stored within granules so always released upon degranulation, are perhaps likely to be most useful.

Levels of the mast cell protease, chymase, were measured in serum collected at autopsy from eight cases of fatal anaphylaxis. The protein was detected in all samples from cases of anaphylaxis, but in only two of 104 serum samples collected from non-anaphylactic fatalities (Nishio *et al.*, 2005). Levels were also found to be associated with those of tryptase. Two subjects with anaphylaxis had low levels of chymase. For one of these, the time between onset of anaphylaxis and death was 17 hours, and levels of the protease may therefore have declined. The other subject had died at the onset of the reaction, so levels may not have reached peak concentrations. Both control subjects who had detectable serum chymase died of myocardial infarction. All of the anaphylactic deaths were caused by therapeutic agents. To our knowledge this is the only study of chymase levels in anaphylaxis; and levels during reactions to food have not been investigated.

There are also other mast cell and basophil-derived proteins that may have potential use as markers of reactions, but have not yet been explored in relation to this role. Mast cell carboxypeptidase was found to be released alongside histamine upon cell activation with goat anti-human IgE (Goldstein *et al.*, 1989). Dipeptidyl peptidase I (DPPI) may also be released from mast cells upon activation (Wolters *et al.*, 2000). As described above, DPPI has been shown to be involved in the processing of tryptase (Sheth *et al.*, 2003) and chymase (Dikov *et al.*, 1994). There is evidence that tryptase is present in the circulation in its unprocessed form, and thus release of DPPI during a reaction is a potential mechanism for activation of this tryptase.

Basogranulin is a basophil-specific protein, and therefore potentially a marker of basophil involvement (McEuen *et al.*, 2001). When basophils were stimulated with both FcεRI-related and unrelated stimuli, basogranulin was released alongside histamine in a bell-shaped response curve (Mochizuki *et al.*, 2003). Peak levels occurred at 15 minutes. Basogranulin was found to be present in all granules within the basophil likely meaning that, upon basophil degranulation, basogranulin will invariably be secreted (McEuen *et al.*, 2001). Research into levels of these proteases in human bodily fluids in cases of allergic reactions and anaphylaxis is required in order to assess their usefulness as markers of the condition.

1.5 Predicting Severity of Allergic Reactions

There are a number of risk factors for anaphylaxis. Much of the research carried out in this area is specific to food-induced reactions. This is described below and summarized in tables 1.5 (intrinsic factors) and 1.6 (extrinsic factors). In addition, there have been some studies looking at biomarkers that may be predictive of anaphylaxis. These, as well as other potential markers, are also discussed in this chapter.

Risk factors for severe reactions

Age, gender, atopy and socioeconomic factors

There are a number of factors that may affect susceptibility to anaphylaxis. Age is a key factor, with most severe or fatal reactions to food occurring in adolescents and young adults (Bock *et al.*, 2001; Bock *et al.*, 2007). Fatal reactions to insect venom or drugs, on the other hand, occur most commonly in older individuals (Pumphrey, 2004). Males and females were originally reported to be affected equally (Bock *et al.*, 2001). However, subsequent studies found a higher incidence of food-induced fatal anaphylaxis in males (Bock *et al.*, 2007).

Atopy is strongly implicated in susceptibility to anaphylaxis, as is a history of previous severe reactions. Most subjects with food-induced anaphylaxis have a known allergy to the causative allergen (Bock *et al*, 2001; Bock *et al.*, 2007; Kanny *et al.*, 2001; Yunginger *et al.*, 1988). Also, socioeconomic factors may play a role; evidence from an emergency department based study suggested that individuals living in southern, rural and affluent areas of England are at greater risk (Sheikh and Alves, 2001).

Table 1.5 Intrinsic risk factors for severe or fatal allergic reactions to foods. Potential risk factors and the supporting evidence and references are provided.

Risk Factor	Reference	Main findings
Age	Bock *et al.*, 2001	Most anaphylactic fatalities occurred in patients over 10 years
	Bock *et al.*, 2007	Most anaphylactic fatalities occurred in patients between 10 and 30 years
	Kanny *et al.*, 2001	Anaphylaxis most prevalent in adults over 30 years
Gender	Bock *et al.*, 2001	No difference in numbers of anaphylactic fatalities between males and females
	Bock *et al.*, 2007	Approx. 60 % of anaphylactic fatalities occurred in males
Atopy/ History of previous severe reactions	Bock *et al*, 2001	History of food allergy in majority of anaphylactic fatalities
	Bock *et al.*, 2007	History of food allergy in majority of anaphylactic fatalities
	Kanny *et al.*, 2001	Severe symptoms to food associated with sensitisation to pollen
	Yunginger *et al.*, 1988	Atopy and history of severe reactions in all anaphylactic fatalities
Co-existing disease	Bock *et al.*, 2001	Coexisting asthma in vast majority of anaphylactic fatalities
	Bock *et al.*, 2007	Coexisting asthma in all anaphylactic fatalities
	Järvinen *et al,.* 2008	Co-existing asthma associated with administration of multiple doses of adrenaline
	Brockow *et al.*, 2008	Anaphylaxis in approx. half of adults, and one tenth of children, with mastocytosis

Table 1.6 Extrinsic risk factors for severe or fatal allergic reactions to foods. Potential risk factors and the supporting evidence and references are provided.

Risk Factor	Reference	Evidence
Socio-economic	Sheikh and Alves, 2001	Hospital emergency admissions most prevalent in southern, rural and affluent areas
Allergen	Bock *et al*, 2001	Majority of anaphylactic fatalities to peanut or tree nuts
	Bock *et al.*, 2007	Majority of anaphylactic fatalities to peanut or tree nuts
	Järvinen *et al,.* 2008	Most reactions requiring adrenaline to peanut, tree nuts or cow's milk
	Kanny *et al.*, 2001	Most anaphylactic reactions to seafood
Therapeutic agents	Kanny *et al.*, 2001	Use of non-steroidal anti-inflammatory drugs associated with increased reaction severity
	Jacobs *et al.*, 1981	Use of beta blockers associated with increased reaction severity
	Moneret-Vautrin and Latarche, 2009	Use of aspirin, non-steroidal anti-inflammatory drugs, beta blockers or ACE inhibitors associated with increased reaction severity
Exercise	Tewari *et al.*, 2006	Anaphylaxis following exercise to food normally tolerated
	Caminiti *et al.*, 2007	Anaphylaxis following exercise to food previously desensitized to
Alcohol consumption	Kanny *et al.*, 2001	Alcohol consumption associated with increased reaction severity
	Yunginger *et al.*, 1988	Alcohol consumed in seven anaphylactic fatalities
Treatment	Bock *et al*, 2001	Adrenaline delayed or not received in most anaphylactic fatalities
	Bock *et al.*, 2007	Adrenaline delayed or not received in most anaphylactic fatalities
	Yunginger *et al.*, 1988	Adrenaline delayed or not received in all anaphylactic fatalities
Location of reaction	Bock *et al*, 2001	Majority of anaphylactic fatalities occurred away from home
	Bock *et al.*, 2007	Majority of anaphylactic fatalities occurred away from home

Foods and allergens

Some foods appear to be responsible for a higher proportion of severe reactions than others. Food types believed to carry higher risks include peanut, tree nuts, seafood, and milk (Bock *et al.*, 2001; Bock *et al.*, 2007; Järvinen *et al.,* 2008). However, this is based on numbers of severe or fatal reactions to specific foods. To our knowledge, the severity of actual symptoms experienced in response to different foods has not been compared.

There is evidence that severity of reactions may depend on the individual allergens within a food that a person responds to. Most subjects with oral allergy syndrome to hazelnut were found to be sensitized to homologues of pollen allergens in the nut; whereas patients with more severe symptoms appeared to react to allergens that are homologous of those in peanuts and other tree nuts (Schocker *et al.*, 2000; Flinterman *et al.*, 2008).

Co-existing conditions and therapeutic agents

Co-existing asthma is one of the most important risk factors for fatal anaphylaxis (Bock *et al.*, 2001; Bock *et al.*, 2007). In a number of studies, the majority of patients with fatal or near fatal anaphylactic reactions to food also had asthma, albeit often well-controlled (Bock *et al.*, 2001; Bock *et al.*, 2007). Co-existing asthma was also found to be linked with administration of more than one dose of adrenaline during food-induced anaphylaxis (Järvinen *et al,.* 2008).

Other co-existing diseases such as cardiovascular disease (Mueller, 2007; Triggiani *et al.*, 2008) and mastocytosis (Brockow *et al.*, 2008), and drugs such as beta blockers (Jacobs *et al.*, 1981; Toogood, 1987), non-steroidal anti-inflammatory drugs (Kanny *et al.*, 2001) and ACE inhibitors (Stumpf *et al.*, 2006; Moneret-Vautrin and Latarche, 2009) have also been shown to contribute to severity of allergic reactions to a range of allergens, including drugs and insect venom.

Exercise

Exercise can increase the risk of a severe reaction (Moneret-Vautrin and Morisset, 2005). Some individuals can react to a certain food only if exposed before or after exercise (Caminiti *et al.*, 2007; Tewari *et al.*, 2006). This is known as food-dependent, exercise-induced anaphylaxis. Anaphylaxis to a food after exercise may be in part attributable to a shift in blood flow to splanchnic and skeletal muscle vasculature (Novey *et al.*, 1983). Drug-dependent, exercise-induced anaphylaxis has also been described (Van Wijk *et al.*, 1995)

Alcohol

Alcohol consumption has been linked with food allergy, asthma and exercise-induced anaphylaxis (Gonzalez-Quintela *et al.*, 2004). In a study of Spanish adults, consumption of at least 14 units per week was associated with increased serum total IgE levels (Gonzalez-Quintela *et al.*, 2003), and concomitant consumption has been suggested to contribute to the severity of food-induced anaphylaxis (Yunginger *et al.*, 1988). Similarly, a French population study found anaphylactic shock to be significantly associated with alcohol consumption (Kanny *et al.,* 2001). It has been suggested that alcohol can contribute to severity of reactions due to its ability to increase intestinal permeability (Moneret-Vautrin and Morisset, 2005). In addition, alcohol may reduce awareness of allergen exposure (such as foods consumed), and perhaps of symptoms experienced.

Delayed administration of adrenaline

Other than asthma, the most important risk factor for fatal anaphylaxis is untimely administration of adrenaline (Bock *et al.*, 2001; Bock *et al.*, 2007; Yunginger *et al.*, 1988). Delayed administration of adrenaline is often a result of the allergic individual being incompliant in carrying their epipen. Indeed, most cases of anaphylaxis to food occur when the patient has eaten away from home, for example at a restaurant or school (Shah and Pongracic, 2008).

Predictive markers for severe reactions

Although risk factors for severe allergic reactions have been identified, a means of recognizing those patients susceptible by way of measuring serum biomarkers would be extremely useful. Various potential markers of allergy and anaphylaxis to various allergens have been identified, some of which may be useful in predicting the risk of anaphylaxis.

IgE

Hourihane *et al.* (2005) reported that peanut-specific IgE levels were associated with severity of reactions to peanut in DBPCFC in 40 patients. This correlation was stronger in adults than in children, and also in non-asthmatics compared to asthmatics. Benhamou *et al.* (2008) reported that albumin-specific IgE levels were correlated with reaction severity in 35 children undergoing 51 reactions to raw or cooked egg. However, in the latter study, subjects with a negative challenge were included in the analysis, and thus the correlation may have been skewed by their results. Indeed a large degree of overlap was seen in IgE levels between children with mild/moderate reactions and those with severe reactions.

Angiotensin-Converting Enzyme (ACE)

Angiotensin-converting enzyme (ACE) is an enzyme involved in the renin-angiotensin system within the circulation. It is produced primarily in the epithelial cells of the kidney tubules, and the vascular endothelial cells of peripheral blood vessels and the lung. ACE acts to promote the formation of angiotensin II from angiotensin I, and is involved in the degradation and therefore inactivation of bradykinin (Erdös *et al.,* 1976; Campbell *et al.*, 2004).

In a study investigating factors that may predict patients at risk of anaphylaxis to tree nuts and peanuts, subjects with low serum angiotensin-converting enzyme (ACE) activity levels were almost ten times more likely to develop severe

pharyngeal oedema compared with other study subjects (Summers *et al.*, 2008). Summers *et al* implicated ACE's role in the degradation of bradykinin in the nut-induced angioedema. In a previous study, rises in bradykinin levels were found to occur in patients with hereditary and acquired angioedema during an attack (Nussberger *et al.*, 1998). Furthermore, in a hypertensive patient receiving an ACE-inhibitor, high levels of bradykinin accompanied an attack of angioedema. Levels were normal when the drug was withdrawn. Serum ACE concentrations (as apposed to activity levels) have not previously been investigated in relation to susceptibility to anaphylaxis.

PAF and PAF AH

Vadas *et al.* (2008) reported that serum PAF levels were higher in patients with anaphylaxis compared to controls; and concentrations were inversely correlated with PAFAH activity. Patients with anaphylaxis appeared to have lower PAFAH activity than controls (though this was not significant) and the numbers of patients with low PAFAH activity was proportional to the severity of the reaction. Patients with fatal anaphylaxis to peanuts had significantly lower PAFAH activity than a number of control groups, which included children with mild peanut allergy, patients with non-fatal anaphylaxis, deaths from non-anaphylactic causes, and children with life-threatening and non life-threatening asthma (Vadas *et al.*, 2008).

From these findings it was suggested that the severity of anaphylaxis may depend on levels of PAF, with higher levels resulting in more severe reactions. PAF levels are dependent on the degree of inactivation by PAFAH, and thus levels of this enzyme may also be associated with reaction severity (Vadas *et al.*, 2008).

Tryptase and Carboxypeptidase

Tryptase is stored within the granules of mast cells, and released upon IgE-mediated activation and degranulation of these cells (Schwartz *et al.*, 1987; Walls *et al.*, 1990). The protease may act as an amplification signal for activation of mast

cells (He *et al.*, 1998), and thus, high baseline levels of the protease may be expected to increase the extent of degranulation and the resulting release of inflammatory mediators during a reaction.

In 2001, Ludolph-Hauser *et al.* identified high baseline serum levels of the mast cell protease tryptase as a potential risk factor for severe reactions to hymenoptera venom. This work has since been supported in studies by Haeberli *et al.* (2003) and Kucharewicz *et al* (2007), in which tryptase levels were found to correlate significantly with reaction severity. In a recent study, patients with high baseline levels of serum tryptase were less likely to have suffered urticaria during venom-induced reactions, but were more likely to have experienced severe reactions (Potier *et al.*, 2009). In most of the studies described, high levels of tryptase were often associated with mastocytosis, supporting this disorder as a risk factor. To date, no evidence has been reported that supports the use of tryptase in predicting severity of reactions to food.

Development of an ELISA for measurement of the mast cell protease, carboxypeptidase, was recently reported (Zhou *et al.*, 2006). Like tryptase, carboxypeptidase is stored within mast cell granules (Goldstein *et al.*, 1987), and is therefore likely to be released upon anaphylactic degranulation. Levels of the protease were reported to be increased in subjects with anaphylaxis compared to healthy blood donors (Zhou *et al.,* 2006). Baseline levels have not been explored in relation to severity of reactions to any allergen. However, high concentrations may indicate a greater basal rate of mast cell activation, and thus perhaps an increased susceptibility to severe reactions. This is the only study previously carried out investingating carboxypeptidase levels in serum in relation to anaphylaxis. Thus further studies are required.

1.6 Hypothesis and Aims

We hypothesise that there are increased serum and saliva levels of mast cell and basophil products following activation of these cells during an allergic reaction. In addition, we predict that high baseline levels of the proteases, and low ACE concentrations, will be linked with susceptibility to anaphylaxis. Although assays have previously been developed for measurement of some of the markers of interest, many of these have not been fully validated or assessed for potential use; and evidence supporting others is contradictory. Details of available assays are provided in table 1.7.

Table 1.7 Availability of assays for measurement of allergic markers.

Marker	Assays	Comments
Tryptase	ELISA kit commercially available, and 'in-house' ELISA developed and validated	Use of assay is recommended for diagnosis of anaphylaxis, but supporting evidence is contradictory. Cannot measure subtypes of tryptase.
Chymase	'In-house' ELISA developed and validated	Potential use of assay not yet investigated.
Carboxypeptidase	'In-house' ELISA developed	Assay requires further validation for use with human biological fluids. Potential use of assay not yet fully investigated.
DPPI	'In-house' activity assay developed	Requirement for ELISA technique development, validation and assessment for potential use.
Basogranulin	'In-house' dot blot technique developed	Requirement for ELISA technique development, validation and assessment for potential use.
ACE	ELISA Duoset kit commercially available	Assay requires validation for use with human biological fluids. Potential use of assay not yet fully investigated.

The aims of this study were therefore as follows:

1. To develop and/or validate new sensitive and specific assays for measurement of mast cell and basophil products in blood and saliva;
2. To investigate alterations in levels of mediators during allergic reactions; and

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3. To examine baseline concentrations of mediators with respect to severity (and specific symptoms) of historical reactions

This work was carried out with a view to developing new tests that are useful in confirming diagnosis of an allergic reaction and that may be predictive of susceptibility to severe reactions. It was performed primarily with regard to paediatric food allergy. However, preliminary studies were also carried out in populations of adults allergic to drugs and other allergens.

CHAPTER 2: MATERIALS AND METHODS

2.1 Overview

Levels of allergic markers of inflammation were measured in blood and saliva from patients with allergic reactions, or with a history of allergy, in order to determine whether concentrations are altered during a reaction, and whether baseline levels may be predictive of reaction severity or symptoms involved. Mediators under investigation included the mast cell proteases tryptase, carboxypeptidase, chymase and DPPI; and also ACE.

The main subject population recruited for this study included children attending the Southampton University Hospital Trust (SUHT) for diagnostic food challenge. We also recruited a number of adults referred for skin testing to drugs, as well as patients presenting at the accident and emergency (A&E) department with anaphylaxis or severe exacerbations of allergic asthma. Healthy atopic and non-atopic subjects served as controls.

Specific immunoassays were employed for measurement of mediators within samples collected from recruited subjects. A commercially available assay was used for measurement of ACE concentrations; and measurement of mast cell proteases was performed using techniques developed within our laboratory. Development and validation of these assays is described in Chapters 3 and 4. The protocols finally adopted are described below.

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

2.2 Subjects and Sample Collection

Paediatric food challenges

Patients, aged eight to 18 years, undergoing diagnostic food challenge were recruited onto the study. Within the SUHT, food challenges are performed most commonly for one of three reasons. The first is when it has not been possible to establish a diagnosis based on history, SPT and specific IgE. Alternatively, a food challenge is performed when a child is suspected to have outgrown a previously confirmed food allergy on the basis of SPT or specific IgE. Finally, challenges are sometimes performed in teenagers with food allergy if they have no memory of a reaction (for example, if the reaction occurred in infancy) to demonstrate the early symptoms that may occur upon allergen exposure and to re-enforce the need for risk aversion.

Children presenting for challenge are referred from a specialist allergy clinic within the SUHT. Historically, approximately 40 % of children undergoing food challenges in our trust react. Our method of recruitment was therefore expected to provide both positive and negative outcomes to food challenge.

Food challenge procedure

Patients were given incremental doses of the index food at 15 minute intervals whilst monitoring for development of symptoms. The process took approximately one and a half hours; and a two hour observation period was implemented after completion in case of a delayed reaction. If a child reacted the challenge was immediately stopped and appropriate treatment administered.

The outcome was confirmed by the nurse performing the test. Challenges were considered positive when objective signs were seen, or significant subjective symptoms reported, during or following the procedure. A negative result was

recorded if no signs or symptoms presented; or if the patient reported minor subjective symptoms but completed the challenge.

Sample collection

Following informed consent, venous blood was collected into uncoated and heparinized tubes prior to and after challenge (approximately two hours after initial allergen exposure). Additional blood was taken two hours after challenge cessation if the patient was cannulated. When venesection was performed only two samples were obtained for ethical reasons. Saliva was obtained before, fifteen and thirty minutes into, immediately after, and two hours after the challenge by salivette (Sarstedt). Studies supporting the use of salivettes for saliva collection are described in Appendix A. The protocol for sample collection is illustrated in Fig 2.1.
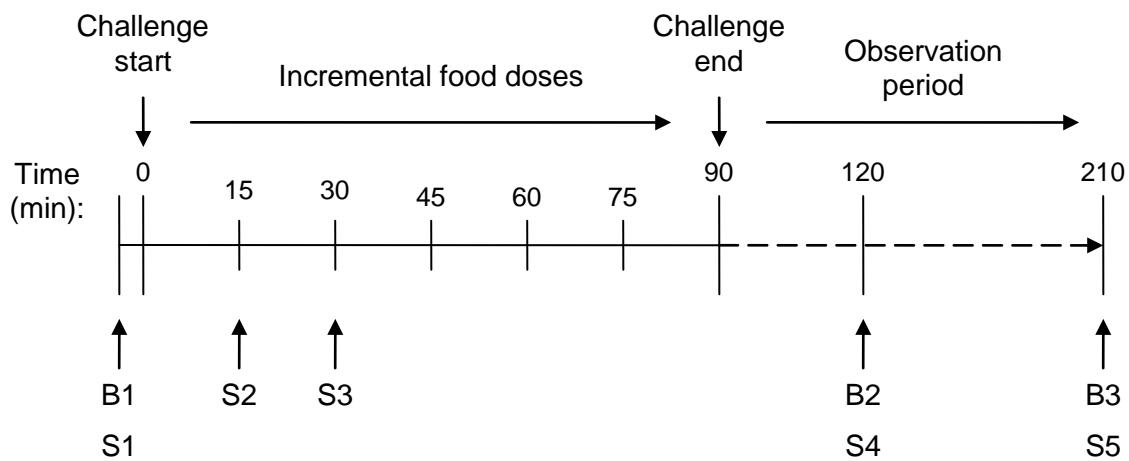
Fig 2.1 Food challenge sample collection procedure. Blood was collected prior to (B1) and post challenge (B2), and two hours after challenge (B3) in some subjects. Saliva was collected prior to, during and post challenge (S1-5).

Pro forma and Questionnaire

For each child recruited, a document was completed consisting of a pro forma and questionnaire (Appendix B). The pro forma was used to document the challenge

outcome, and previous SPT, specific IgE and food challenge results recorded in medical notes. The questionnaire was completed on the child's allergic history, including previous reactions to the index food, and to any other food, drug, medication, or insect venom.

A reaction scoring system to quantify eliciting dose and reaction severity was implemented (table 2.1). This was adapted from a system developed by Hourihane *et al.* (2005) for scoring reactions to peanut. In the current study a reaction score was given for the food challenge and previous reactions in the community to the index food. A score was also given for the most severe reaction suffered to any food allergen. For reactions occurring in the community, the degree of allergen exposure and severity of symptoms were calculated based on descriptions by the patient or their parent/guardian.

Table 2.1 Reaction scoring. Scores are based on the degree of allergen exposure and severity of reaction (adapted from Hourihane *et al.*, 2005). (1-5 = mild (blue), 6-13 = moderate (yellow), 14-25 = severe (orange))

	No reaction	Localised urticaria/ pruritus	Generalised urticaria	Abdominal pain/vomiting /angioedema	Laryngeal oedema/ respiratory problems	Anaphylaxis /systemic symptoms
Inhalation/ skin contact	0	6	12	18	20	25
Mucosal touch – no ingestion	0	4	10	16	19	24
Fragment swallowed	0	3	8	13	17	23
Small quantity ingested	0	2	7	11	15	22
Large quantity ingested	0	1	5	9	14	21

Adult drug challenges

Patients (17 to 82 years) attending an SUHT specialist adult drug challenge clinic were recruited. Allergy testing for therapeutic agents was carried out when diagnosis could not be established on the basis of clinical history and specific IgE measurement alone. Recruitment of these patients was initially started by myself; and was subsequently carried out by Thomas Brown, an undergraduate medical student, under my supervision.

Drug challenge procedure

The drug challenge process involved exposing the patient to the index agent first in SPT, and then, If no response was provoked, in intra-dermal testing (IDT). SPT was carried out as for foods: drops of the drugs implicated were placed on the forearm, and the skin below pierced using a lancet. Histamine was used as a positive control, and saline as a negative control. Increasing concentrations of the drug were tested in SPT, and a 15 minute observational period was implemented between doses. A raised wheal with a diameter of 3 mm or more greater than the negative control was considered positive. If a positive response occurred, no further doses were used. The concentrations used varied depending on the drug being tested.

If skin prick testing failed to yield a clear diagnosis, intra-dermal testing was performed. This involved injection of the drug into the dermis using a needle and syringe. A wheal was automatically formed due to localised accumulation of fluid under the epidermis, so the test was considered positive if the wheal increased in size over a twenty minute period. As for SPT, increasing doses of the drug were tested until a positive or negative result was established.

Although development of a wheal in skin prick testing, or enlargement of the wheal in intra-dermal testing, was the primary indication of a positive response, patients were also observed for clinical signs and symptoms. These included both

subjective and objective symptoms such as pruritus and urticaria, respectively. Although the protocol was designed to minimize risk, occasionally more severe symptoms occurred.

Sample collection

Following informed consent, blood was collected into uncoated and heparinized tubes through a cannula before and after challenge. Saliva was collected by salivette at the same times. A schematic diagram of the procedure is shown in Fig 2.2.
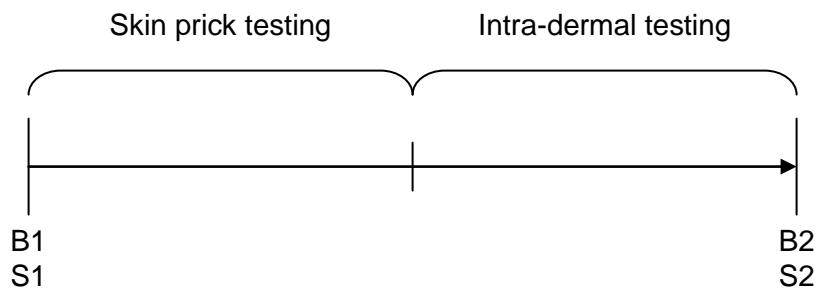
Fig 2.2 Drug challenge sample collection procedure. Blood (B) and saliva (S) were collected prior to skin prick testing and post intra-dermal testing.

Pro forma and Questionnaire

A document consisting of a pro forma and questionnaire was completed (Appendix C). Skin test results and clinical observations (temperature, peak expiratory flow rate (PEFR), heart rate and blood pressure) were recorded in the pro forma. The allergic history of patients was taken by questionnaire, including previous reactions to drugs or other allergens.

Emergency admissions for anaphylaxis or allergic asthma exacerbation

In the SUHT A&E department, blood is routinely taken from patients presenting with suspected anaphylaxis or allergic asthma exacerbations for diagnostic purposes. For this study, informed consent was obtained from adult patients for collection of remaining blood following routine laboratory testing. Diagnosis was made by the attending doctor on the ward, and blood was taken following admission into serum separator tubes (SST) and those containing EDTA via a cannula.

Control subjects

Healthy atopic and non-atopic adults were recruited to serve as controls. Atopic subjects were excluded if a history of allergy to foods, drugs or insect venom was reported, or if they had previously experience severe reactions to any allergen. Blood was taken by venesection into uncoated and heparinized tubes. Saliva was obtained by salivette.

Sample processing and storage

Upon collection, all samples were immediately stored on ice. In the laboratory blood samples (uncoated tubes) for serum were left at 22 ºC for 30 minutes; or on ice for longer periods, to allow clotting. Samples containing heparin or EDTA were placed on a roller for 30 minutes to prevent clotting.

Samples were centrifuged at 900 x g for 30 minutes at 4 °C. Serum was collected from uncoated and SST tubes, and plasma and cells from heparin/EDTA coated tubes. Saliva was aspirated using a pastette, taking care to avoid collection of cells from the bottom of the tube. Serum, plasma, blood cell and saliva samples were stored at -80 °C.

2.3 Assays

We have employed enzyme-linked immunosorbant assay (ELISA) techniques (illustrated in Fig 2.3) for measurement of markers in samples collected in these studies. ELISAs were developed within our laboratory prior to this study for tryptase, chymase and carboxypeptidase; and a commercially available ELISA Duoset kit for ACE was employed. These assays have been validated for use with human serum and saliva (described in Chapter 3). In addition, a novel ELISA procedure has been developed for measurement of DPPI, and this has also been validated for use with human biological fluids (described in Chapter 4). The final procedures used for measurement of the markers in study samples are provided below.
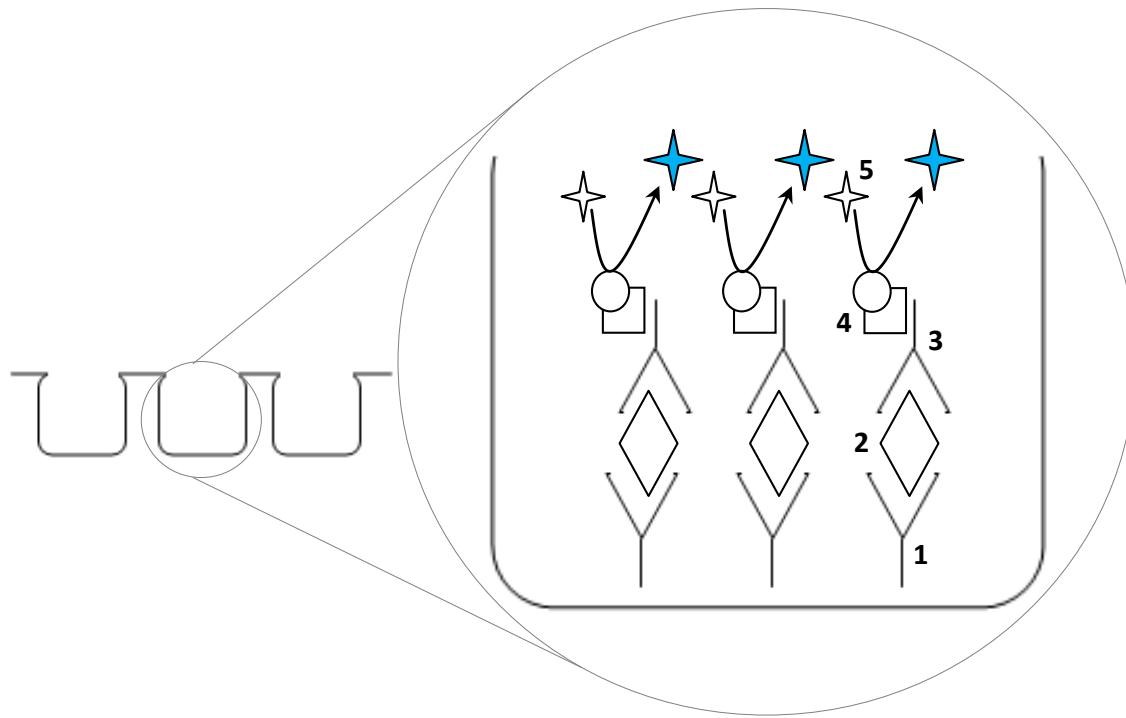
Fig 2.3 Sandwich/indirect ELISA. A plate is coated with capture antibody (1). Antigen in samples or standards (2) binds to the capture antibody, and is detected with biotinylated antibody (3). Avidin-linked HRP (4) binds to the biotin tag of the detection antibody. Addition of substrate (5) produces a visible signal such as colour change.

ELISAs for Tryptase, chymase and carboxypeptidase

For tryptase measurement, a 96 well micro-plate was coated with an in-house rabbit polyclonal anti-human tryptase antibody, termed E1, diluted 1:1000 in coating buffer (0.05 molar (M) carbonate bicarbonate buffer (pH 9.6); Sigma). Fifty ul were added per well. The plate was refrigerated at 4 °C overnight, and washed four times with PBS (Invitrogen) containing 0.05 % Tween-20 (Sigma) (T-PBS). After removing excess fluid by patting the plate on paper towels, non-specific binding sites were blocked with 2 % BSA (Sigma) in PBS (200 ul per well) for two hours at 22 ºC. The plate was washed and dried as previously, and samples were added to wells in duplicate (50 ul per well). Serum samples were diluted one in two in PBS, and saliva samples were applied neat. Double dilutions of human lung tryptase (0-32 ng/ml) were added to wells in duplicate for standards. The tryptase was purified from tissue obtained ethically from patients undergoing surgery following informed consent using methods described in McEuen and Walls (2008),.

Samples and standards were incubated on the plate shaker for two hours at 22 ºC, and the plate was washed and dried. Mouse monoclonal anti-human tryptase antibody (AA5; Walls *et al.*, 1990), diluted 1:5000 in 0.5 % BSA in PBS (dilution buffer), was added (50 ul per well) and incubated on the plate shaker for 90 minutes at 22 ºC. After washing and drying the plate, Extravidin-HRP (Sigma), 1:5000 in dilution buffer, was added (50 ul per well). The plate was incubated for 45 minutes at 22 ºC, and then washed five times.

Substrate solution, containing 6 mg/ml tetramethylbenzidine (TMB; Sigma) in dimethyl sulphoxide (DMSO; Sigma), 1:60 in sodium acetate buffer (1.5 % sodium acetate trihydrate (Sigma) in distilled H_2O (d.H_2O)), pH 5.5, with 0.01 % hydrogen peroxide (H_2O_2; Sigma), was added to the plate (50 ul per well). Colour was allowed to develop, and the reaction stopped with 50 ul sulphuric acid (H_2SO_4; Fisher Scientific). The plate was read on a Thermo-max micro-plate spectrophotometer at 450 nm, with a wavelength correction of 595 nm.

The same procedure was carried out for measurement of chymase and carboxypeptidase, but using different antibodies and standards. In the chymase ELISA, mouse monoclonal anti-human chymase antibodies, CC1 and CC3 (Buckley *et al.*, 1999), were used for capture and detection, respectively. Known concentrations of chymase purified from human skin (McEuen and Walls, 2008) were used as standards. For the carboxypeptidase assay, mouse monoclonal anti-human carboxypeptidase antibodies, CA2 and CA5 (Zhou *et al.*, 2005), were used for capture and detection, respectively. Carboxypeptidase was purified from human skin for use as standards of known concentrations (described in Chapter 3). Human skin used for purification of chymase and carboxypeptidase was obtained ethically from patients undergoing limb amputation, following informed consent. Antibodies and standards used in the tryptase, chymase and carboxypeptidase ELISAs (and the assay for DPPI) are outlined in table 2.2.

ELISA for DPPI

A similar methodology was carried out for measurement of DPPI to that employed for tryptase, chymase and carboxypeptidase. The components used are outlined in table 2.2 with those of the other in-house assays. A 96 well plate was coated with a mouse monoclonal anti-human DPPI antibody (DD1), 1:500 in coating buffer, for two hours at 22 °C. The plate was blocked with 20 % foetal bovine serum (FBS; Sigma) in PBS at 4 °C overnight. Serum samples, 1:10 in PBS, or saliva (heat-treated at 60 ° for 30 minutes), 1:4 in PBS, were added to wells in duplicate. Double dilutions (0-1000 ng/ml) of human recombinant DPPI (Unizyme) were used for standards. The plate was incubated at 22 °C for two hours. Rabbit polyclonal anti-human DPPI antibody (OXTZ), 1:1000 in 3 % FBS in PBS, was used for detection at 22 °C for two hours. Anti-rabbit HRP-conjugated antibody (Sigma) was diluted 1:8000 in 3 % FBS in PBS and incubated on a roller at 22 °C for one hour; and then added to the plate. After one hour substrate was added and colour allowed to develop. The reaction was stopped with H_2SO_4 and the optical density determined using a Thermomax microplate spectrophotometer at 450/595 nm.

Table 2.2 Components of ELISAs employed for measurement of tryptase, chymase, carboxypeptidase and DPPI. Components were produced within our laboratory unless marked with *. Human lung and skin tissue used for purification of standards was obtained from surgery following informed consent. (b): biotinylated

	ELISA			
	Tryptase	Chymase	Carboxypeptidase	DPPI
Capture antibody	E1 – rabbit polyclonal	CC1 – mouse monoclonal	CA5 – mouse monoclonal	DDI – mouse monoclonal
Standard	Purified lung tryptase	Purified skin chymase	Purified skin carboxypeptidase	Human recombinant DPPI *
Detection antibody	AA5 (b) – mouse monoclonal	CC3 (b) – mouse monoclonal	CA5 (b) – mouse monoclonal	OXTZ1 – rabbit polyclonal
Secondary	Extravidin-HRP *	Extravidin-HRP *	Extravidin-HRP *	Anti-rabbit HRP-conjugated antibody *

ELISA for angiotensin-converting enzyme

ACE measurement was performed using a commercially available ELISA Duoset kit (R&D Systems), which used very similar methodology to that described for tryptase. A 96 well plate was coated with goat anti-human ACE antibody (0.8 µg/ml in PBS), and non-specific binding sites were blocked with 3 % BSA in PBS. Serum samples, 1:200 in dilution buffer, were added to wells in duplicate. Double dilutions (0 to 8 ng/ml) of human recombinant ACE were used as standards of known concentrations. Biotinylated goat anti-human ACE antibody (200 ng/ml in dilution buffer) was used for detection, followed by streptavidin-HRP, 1:200 in dilution buffer. Substrate was added and colour allowed to develop; and the reaction was stopped with H_2SO_4. Optical density was determined 450/595 nm.

CHAPTER 3: DEVELOPMENT OF ASSAYS FOR TRYPTASE, CARBOXYPEPTIDASE AND ACE IN HUMAN BIOLOGICAL FLUIDS

3.1 Introduction

Tryptase and carboxypeptidase are stored within mast cell granules, and are released upon cell activation and degranulation (Walls *et al.*, 1990; Goldstein *et al.*, 1987). Both proteases have therefore been implicated in IgE-mediated allergic disease. Angiotensin-converting enzyme (ACE) plays a key role in degradation of bradykinin and formation of angiotensin II (Erdös *et al.,* 1976; Campbell *et al.*, 2004), and has been associated with susceptibility to severe reactions (Summers *et al.*, 2008). These mediators, and their potential involvement in allergy, have been described in detail in Chapter 1.

ELISA procedures have been developed previously for measurement of tryptase and carboxypeptidase levels; and an ELISA is commercially available for measurement of ACE. In the present studies these assays have been further developed and validated for use with human serum and saliva from patients with allergic conditions. The ELISA protocols employed have been described in Chapter 2. Validation work has been carried out separately by Dr Xiaoying Zhou for the chymase ELISA.

3.2 Methods and results

ELISAs were developed primarily in accordance with guidelines prepared by the Food and Drug Administration of the U.S. Department of Health and Human Services for validation of bioanalytical methods (2001). The key parameters specified in this guidance document include sensitivity, recovery, accuracy, precision, reproducibility and stability. These fundamental principles were used as a basis for the developmental procedures used in the current study; but recommendations were adapted where necessary and appropriate.

In brief, sensitivity of the ELISAs for tryptase and ACE was determined by assessing limits of quantification, and protease recovery studies were performed with human serum and saliva. These procedures have been carried out previously for the carboxypeptidase ELISA by Dr Xiaoying Zhou. *Ex vivo* stability of tryptase, carboxypeptidase and ACE over time at different temperatures and with increasing number of freeze thaw cycles was assessed. The variability of levels *in vivo* was investigated, and reproducibility of the assays (both inter- and intra-assay) was determined. In order to carry out these experiments for the carboxypeptidase assay, the protease was first purified from human skin. The procedure for this is described within this chapter.

Purification of carboxypeptidase from human skin

A method adapted from that of Goldstein *et al.* (1989) for extracting mast cell carboxypeptidase from human skin was employed. Macroscopically normal skin was obtained from surgical amputations following informed consent. The study was approved by Southampton and South West Hampshire Research Ethics Committee (approval number 241101). Purification involved low salt extraction (LSE) followed by high salt extraction (HSE). The high salt extract was filtered, and the enzyme purified using a potato-tuber carboxypeptidase inhibitor (PCI) column. The newly purified carboxypeptidase was assessed for use in the ELISA as a standard of known concentration.

Low and High Salt Extraction

Approximately 150 g of human skin (which had been stored at -80 °C and then thawed slowly) was chopped finely with scissors, whilst maintaining a cool temperature. The skin was blended in 600 ml of cold LSE buffer (10 mM Tris(hydroxymethyl)-aminomethane in hydrochloric acid (Tris-HCL), pH 7.4) twice for 30 seconds at low speed and twice at high speed. The solution was centrifuged at 21,000 x g for 60 minutes at 4 ⁰C using a pre-cooled rotor. The lipid layer was removed and the supernatant collected. The skin pellets were blended with 600 ml of cold LSE buffer and centrifuged twice. Each time the supernatant was collected.

Following low salt extraction the skin was blended in 600 ml of HSE buffer (10 mM Tris-HCL, pH 7.0, with 4 M sodium chloride (NaCl), 0.1 % PEG, 10 µM soybean tryptase inhibitor (SBTI)). The mixture was stirred overnight at 4 °C, and blended once for 30 seconds at low speed and twice at high speed. It was centrifuged at 21,000 x g for 30 minutes at 4 °C. As previously, the lipid layer was discarded and the supernatant collected. The skin pellets were blended with 600 ml HSE buffer, and the mixture cooled at 4 °C for 90 minutes and centrifuged as previously. The supernatant (containing extracted carboxypeptidase) was collected.

Filtration and Purification

The supernatant was passed through a 1mm mesh sieve and filtered through double layers of Whatman Number I filter papers until it passed through easily. The collection flask was kept on ice. The supernatant was then passed through Whatman 50, GF/A, GF/D, GF/F and 0.45 nm filter papers sequentially, and dialysed against salt-free HSB overnight at 4 °C.

The extracted carboxypeptidase was purified by affinity chromatography using a potato-tuber carboxypeptidase inhibitor (PCI) affinity column. The carboxypeptidase-containing supernatant was first dialyzed against PCI chromatography running buffer (10 mM Tris-HCl (pH 7.4), 1 M NaCl, 10 µM SBTI

and 0.1 % PEG) for two hours; and the column was prepared by linking 10 mg PCI (Sigma) per ml of cyanogen bromide (CNBr)-activated Sepharose 4B (Sigma); and washed twice with 50 ml acetate buffer (0.1 M sodium acetate, pH 4.0, 0.5 M NaCl) at 0.5 ml/min, followed by 70 ml Tris buffer (0.1 M Tris-HCl, Ph 8.0, 0.5 m NaCl) at 1 ml/min. Supernatant was loaded onto the column, which was then washed through with running buffer ten times, followed by running buffer without SBTI. Elution buffer (0.1 M Na_2CO_3 (pH 11.4), 2 M NaCl, 0.1 % PEG) was pumped through the column, and the eluate collected in 4.5 ml fractions. The fractions were immediately neutralized with 0.5 ml neutralizing buffer (1 M 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES; pH 6.9), 2 M NaCl, 0.1 % PEG).

Characterization

Confirmation of the identity of the purified protein was achieved by SDS PAGE, whereby the new protease preparation was run on a gel alongside carboxypeptidase that had been isolated previously. Using the carboxypeptidase specific monoclonal antibodies, CA2 and CA5, in western blotting and ELISA, it was confirmed that there was specific binding. In addition, new preparations of carboxypeptidase were systematically compared with previously purified preparations by ELISA. Protein concentration of the eluate was determined by Coomassie Blue binding assay (BioRad), with BSA as standards.

Assay development and validation

Limits of quantification

A standard curve of antigen concentration in ELISA against optical density is typically sigmoidal. A plateau occurs at the lower end due to limited sensitivity of the assay, and at the higher end due to saturation of antibody binding to epitopes of the antigen. For accurate measurement in samples, the linear part of a standard curve should be used for reference. The extremities of this are known as the upper and lower limits of quantification.

According to the guidance of the U.S. Department of Health and Human Services, at least six non-zero calibrator concentrations run in duplicate should be used, and these should cover the entire range of expected concentrations within study samples (2001). The lower limit of quantification is defined as the lowest concentration of standard that can be 'measured with acceptable accuracy and precision', and should be established by determining the coefficient of variation or appropriate confidence interval. In the current studies, we used seven non-zero calibrator concentrations, as well as a blank negative control, for each assay. The lower limit of quantification was taken to be the lowest standard concentration at which optical density readings were significantly higher ($p < 0.05$) than those measured in the negative control, as determined by calculating 95 % confidence intervals.

Tryptase

The tryptase ELISA was carried out as described in Chapter 2 using standard concentrations ranging from 0.25 to 64 ng/ml. This was repeated three times; and mean optical density readings and 95 % confidence intervals were calculated for each standard concentration. In Fig 3.1(a), optical density readings at the lower end of the standard curve are shown. The lowest concentration at which 95 % confidence intervals did not overlap was 0.5 ng/ml. This was therefore taken to be the lower limit of quantification. Fig 3.1(b) shows optical density readings for the complete standard curve. The uppermost point of the linear section of the curve was 32 ng/ml (the upper limit of quantification).

ACE

The same procedure was employed for establishing limits of detection of the ACE assay. Standard concentrations of 0.02 to16 ng/ml were used. Corresponding optical density readings for the lower end of the standard curve are shown in Fig 3.2 (a); and the complete curve is shown in Fig 3.2 (b). Upper and lower limits of quantification for the assay were determined to be 0.125 and 8 ng/ml, respectively.
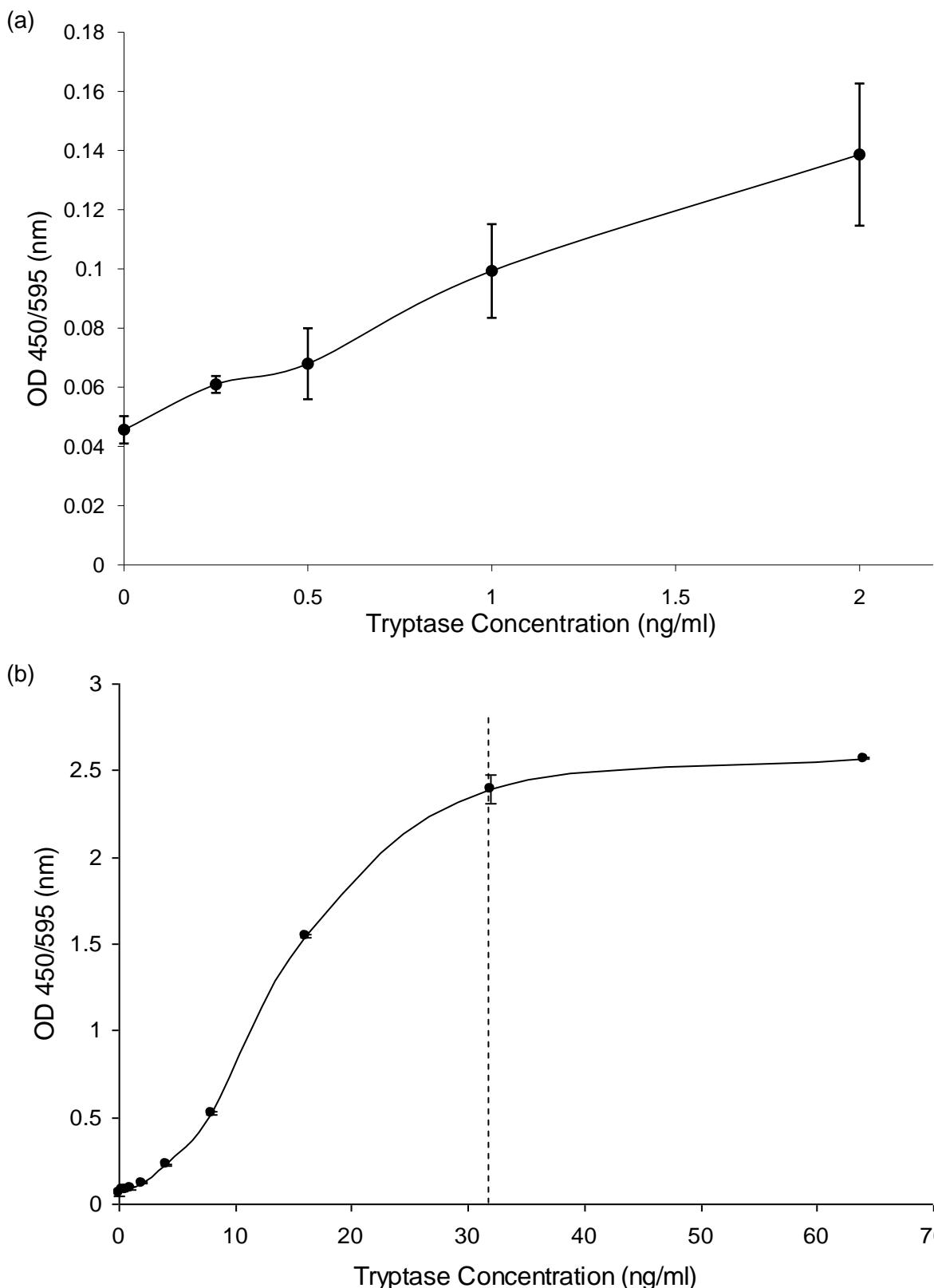
Fig 3.1 (a) Lower and (b) upper limits of detection of assay for tryptase. The lower and upper limits of detection are 0.5 and 32 ng/ml, respectively. Mean values and bars representing 95 % confidence intervals are shown (n = 3).
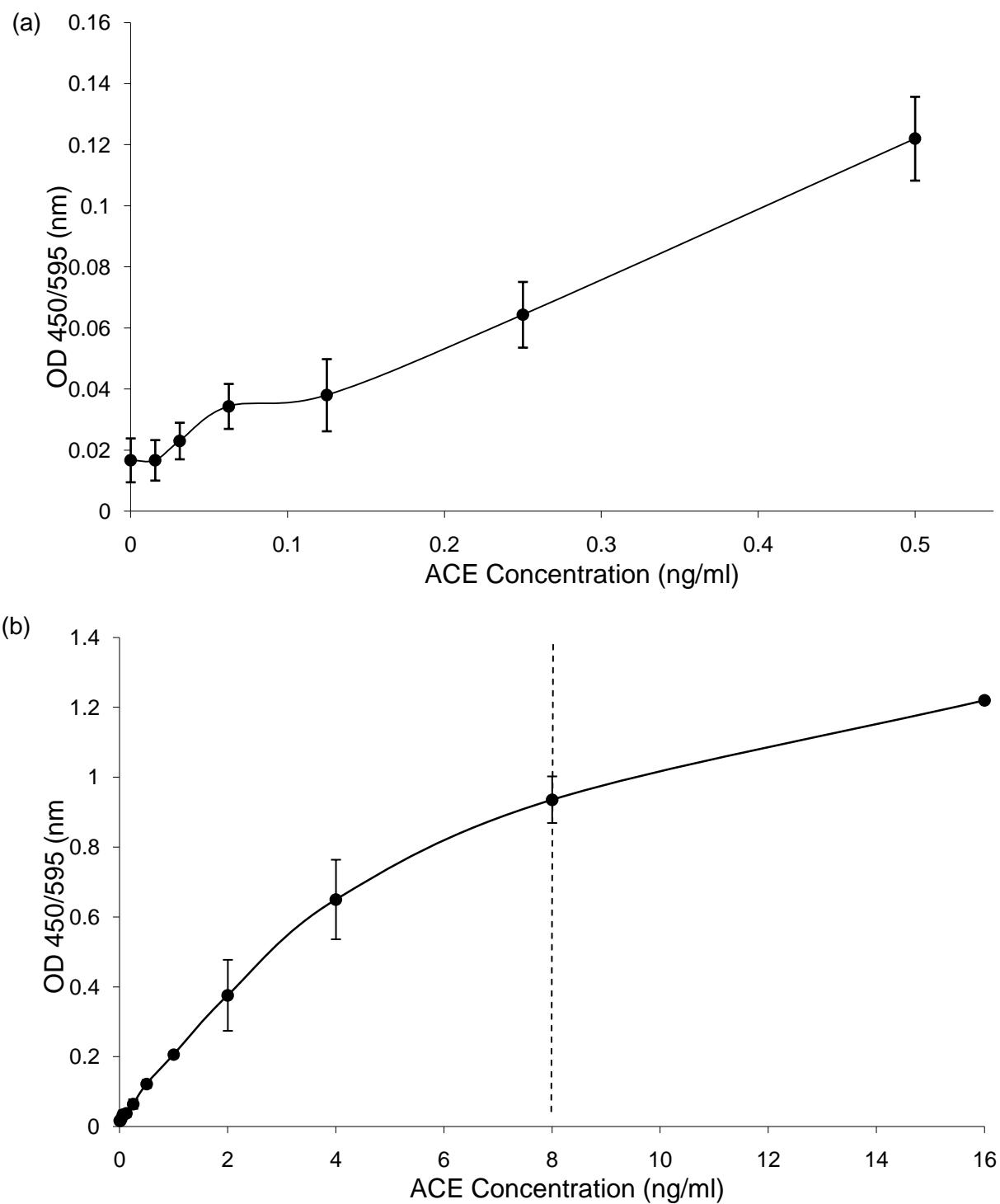
Fig 3.2 (a) Lower and (b) upper limits of detection of assay for ACE. The lower and upper limits of detection are 0.125 and 8 ng/ml, respectively. Mean values and bars representing 95 % confidence intervals are shown (n = 3).

ELISA Validation for use with human serum and saliva

Endogenous molecules in biological fluids can sometimes interfere with measurement of a protein in ELISA. This can be caused, for example, by competitive binding of other proteins, or by enzymatic degradation. The ELISA must therefore be validated for use with fluids in which the protein is to be measured. This involves assessing whether the amount of protein measured within a sample reflects the true content (recovery), and is carried out using dilution and spiking experiments. In the present studies, ELISAs have been validated for measurement of tryptase and ACE within serum and saliva.

Sample dilution

If the amount of protein measured is the true content of a sample, dilutions of the fluid should be reflected by a corresponding reduction in concentration. For example, a dilution of 1:2 should result in half the amount of the protein measured, and a dilution of 1:10 should result in one tenth being measured. Thus, determining levels of the proteases in serum and saliva samples at a range of dilutions should indicate whether there is any interference in the assay with these fluids.

Tryptase levels were measured in serum neat, or diluted by a factor of 1:2, 1:4 or 1:8 (n = 4). Results are shown in Fig 3.3, and are expressed as percentage of tryptase concentrations measured in neat serum. Dilution of samples resulted in corresponding reductions in levels of the proteases measured. When multiplied by the dilution factor, tryptase concentrations were reproducible (within 10 % of levels in undiluted serum) up to a dilution of 1:4.

A similar procedure was carried out using the ELISA for ACE. Due to the high concentration of ACE within serum, samples were diluted 1:100, 1:200, 1:400, 1:800 and 1:1600 (n = 4). Results, showing levels as a percentage of concentrations measured in serum diluted 1:100, are illustrated in Fig 3.4. Serum ACE levels were reproducible when using sample dilutions down to 1:800.
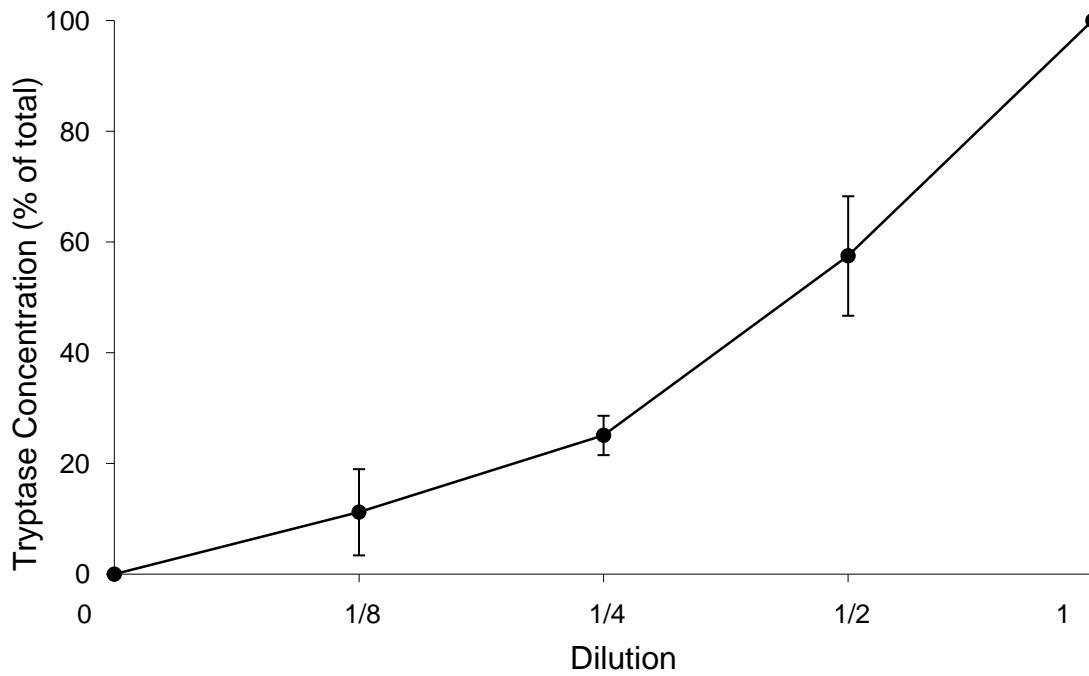
Fig 3.3 Tryptase levels with serial dilution of serum. The graph shows tryptase concentrations, as a percentage of levels measured in neat serum, against dilution factor. Average values and standard error are shown (n = 4).
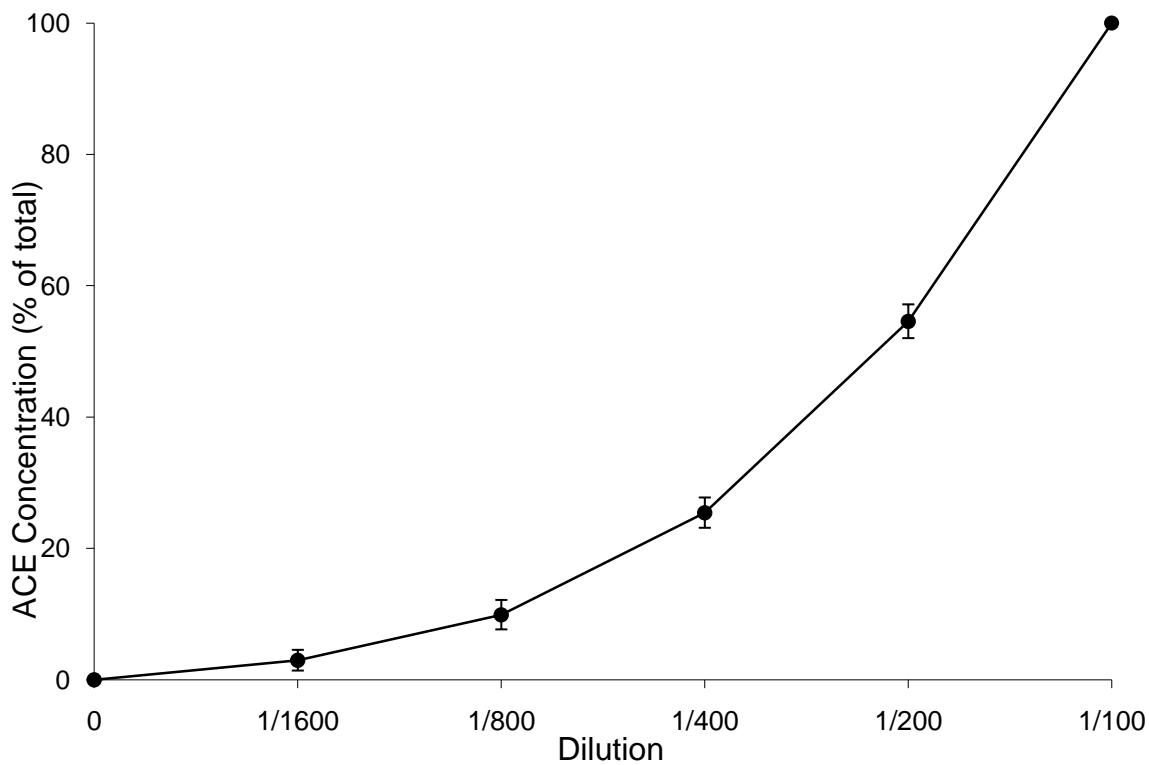
Fig 3.4 ACE levels with serial dilution of serum. The graph shows ACE concentrations, as a percentage of levels measured in serum diluted 1:100, against dilution factor. Average values and standard error are shown (n = 4).

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Serum spiking

Two methods of spiking were employed. Firstly, fixed concentrations of protease were added to a range of dilutions of serum. This method was carried out for tryptase only. It was not carried out for ACE due to the low serum concentration already used in the assay. The second method of spiking involved adding a range of concentrations of tryptase or ACE standard to neat serum. For both methods, the concentration of added standard should correspond to that measured in ELISA. If measured levels are lower, this indicates interference.

For the first spiking procedure, tryptase at a concentration of 32 ng/ml was mixed with an equal volume of serum neat, and diluted 1:2, 1:4 and 1:8 (n = 4). This gave a final protease concentration of 16 ng/ml, and final serum dilutions of 1:2, 1:4, 1:8 and 1:16. Total tryptase levels within spiked samples and un-spiked control serum were measured. Results of measured concentration minus that in un-spiked serum are shown in table 3.1. Levels were within 10 % of the spiking concentration when using serum dilutions of 1:2 or 1:4, and within 15 % at dilutions of 1:8 or 1:16. This degree of variation is acceptable according to the guidelines of the U.S. Department of Health and Human Services (2001).

For the second spiking procedure, tryptase was added to neat serum (n = 4) at 4, 16 and 64 ng/ml, giving a final serum dilution of 1:2 and standard concentrations of 2, 8 and 32 ng/ml. Total tryptase levels were measured in spiked samples, as well as un-spiked control serum. Results are shown in Fig 3.5, and represent total levels minus serum concentration. Levels measured corresponded to those added.

ACE was added to serum diluted 1:100, at concentrations of 0.5, 2 and 8 ng/ml (n = 4), giving a final serum dilution of 1:200 and standard concentrations of 0.25, 1 and 4 ng/ml. ACE levels were measured by ELISA in spiked and un-spiked serum. Results, showing measured concentration minus serum levels, are illustrated in Fig 3.6. The concentration of protease measured corresponded to that added.

Table 3.1 Serum spiking with tryptase. Serum at a range of dilutions was spiked with 16 ng/ml tryptase, and the final concentration measured by ELISA (n = 4). Results are mean values.

Serum dilution	Measured tryptase concentration (ng/ml)
1/2	16.6
1/4	16.7
1/8	17.8
1/16	18.4
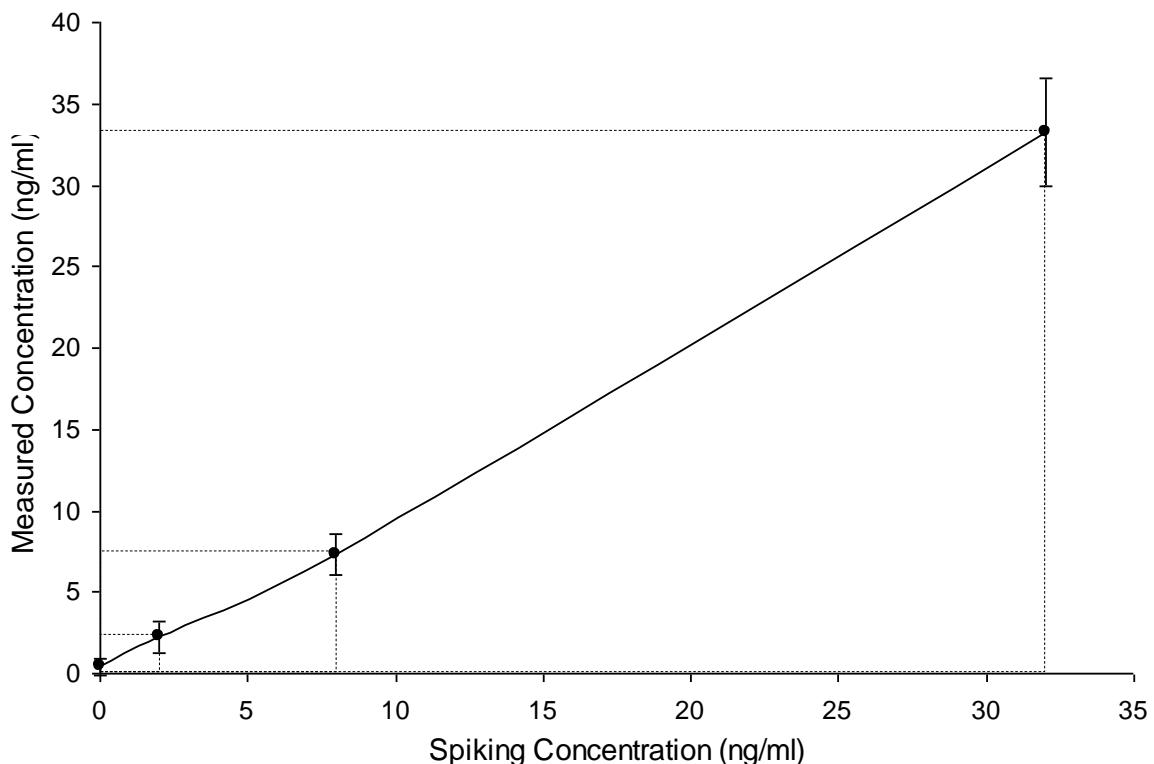
Fig 3.5 Serum spiking with tryptase. Serum, diluted one in two, was spiked with tryptase at concentrations of 2, 8 and 32 ng/ml, and total levels were measured by ELISA. The graph shows spiking concentrations plotted against corresponding values measured. Mean values and standard error are shown for all samples (n = 4).
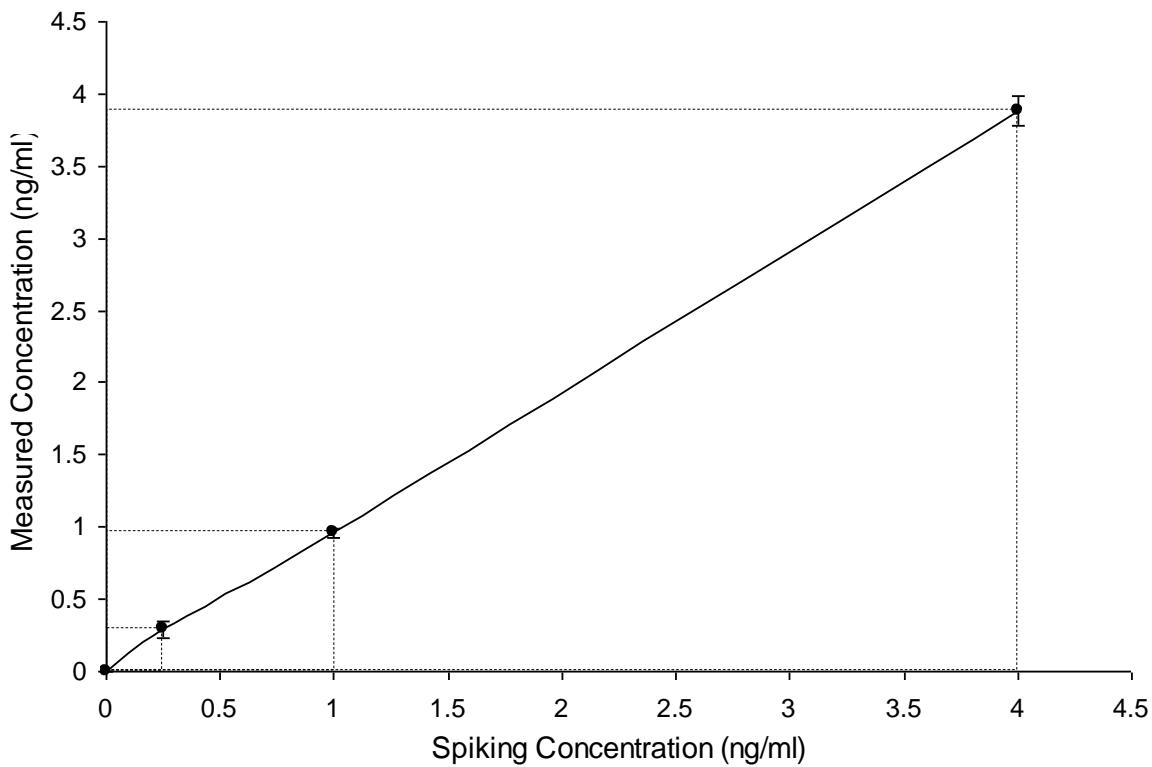
Fig 3.6 Serum spiking with ACE. Serum, diluted 1:200, was spiked with ACE at concentrations of 0.25, 1 and 4 ng/ml, and total levels were measured by ELISA. The graph shows spiking concentrations plotted against corresponding values measured. Mean values and standard error are shown (n = 4).

Saliva spiking

The same process was carried out for spiking of saliva with tryptase. ACE was not measured in saliva in this study, and validation with saliva was therefore not carried out for this assay. Neat saliva, obtained from non-smoking healthy adult control subjects, was spiked with an equal volume of tryptase standard (n = 4), giving a final saliva dilution factor of 1:2. Standard concentrations used were 1, 4 and 16 ng/ml, resulting in final concentrations of 0.5, 2 and 8 ng/ml. Total tryptase concentrations were measured in the spiked and un-spiked saliva samples. Results of total tryptase levels, minus those in un-spiked saliva, are shown in Fig 3.7. The concentration of tryptase measured in the spiked samples corresponded with concentrations of standard added. This indicates that there is little or no interference in the tryptase assay when using human saliva samples.

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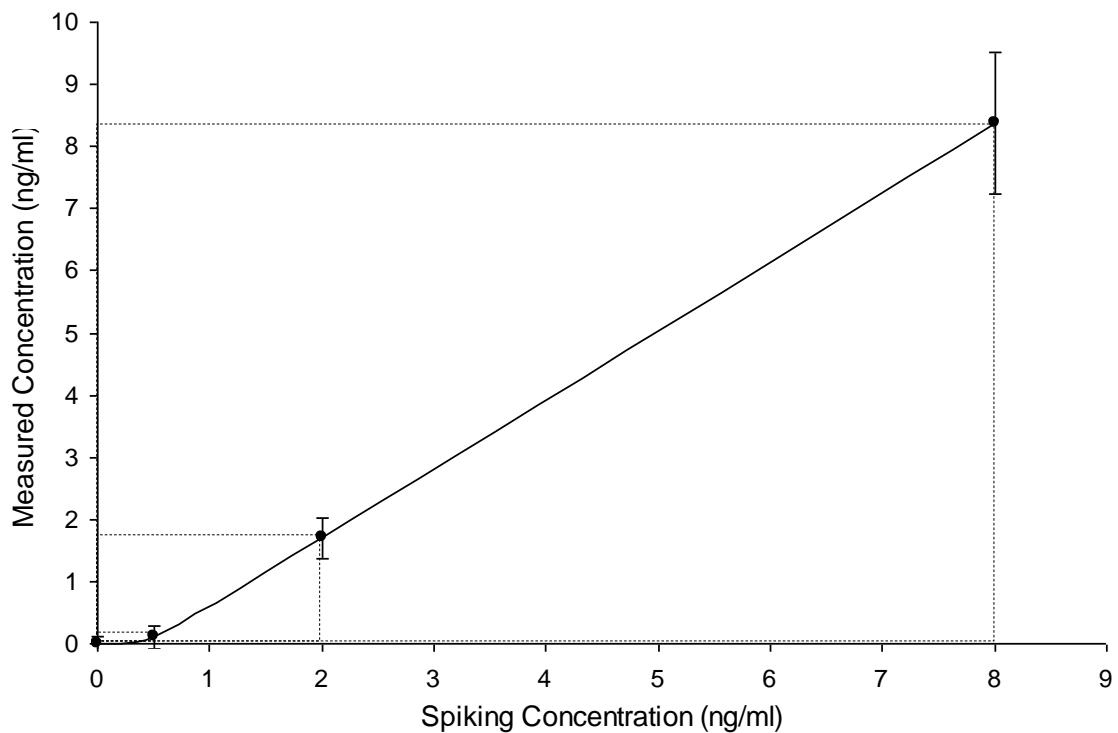
Fig 3.7 Saliva spiking with tryptase. Saliva was spiked with tryptase at concentrations of 0.5, 2 and 8 ng/ml, and total levels were measured by ELISA. The graph shows spiking concentrations plotted against corresponding values measured. Mean values and standard error are shown (n = 4).

Stability and variability of protease levels

Stability of tryptase, carboxypeptidase and ACE *ex vivo* was assessed by measuring levels in serum after different time periods at 22 °C, 4 °C and -20 °C, and following increasing numbers of freeze thaw cycles. Variation in levels of the proteases within an individual subject (*in vivo*) over time was determined by measuring levels in samples taken on different occasions. Due to the low baseline levels of tryptase and carboxypeptidase in saliva, and also the relatively low yield of saliva from an individual as compared to that required for these studies, it was not possible to repeat this work using this biological fluid.

Ex vivo stability

Serum was stored at 22 ºC, 4 ºC or -20 ºC for one, three, five, ten or 15 days before being transferred to - 80 ºC (n = 4). In addition, serum from each subject was stored at - 80 ºC immediately after collection (day 0). Levels of tryptase, carboxypeptidase and ACE were measured in the samples; and concentrations of the proteases were compared in samples that had been stored at different temperatures for different time periods.

Average concentrations of the markers in serum stored at -80 ºC from day 0 (i.e. total levels) were 5 ng/ml for tryptase, 27 ng/ml for carboxypeptidase and 250 ng/ml for ACE. At least 90 % of total tryptase levels could be measured in serum stored at 22 ºC or 4 ºC for one day; and levels were very stable (over 95 % of total) for the full 15 days at - 20 ºC. Carboxypeptidase concentrations were reduced to less than 80 % of total by one day at 22 ºC, and by three days at 4 ºC. However, levels did not decline below 98 % of total over the whole period of 15 days at - 20 ºC. ACE levels remained above 90 % of total at both 22 and 4 ºC for 15 days. Concentrations did not fall below 98 % of total by ten days at -20 ºC, or below 95 % by 15 days.

Serum was also subjected to one, two or three freeze thaw cycles (n = 4). As study samples were stored at - 80 ºC, sera undergoing freeze-thaw cycles were subjected to this temperature. For comparison, a volume of serum from each subject was not frozen, but stored at 4 ºC. Concentrations of tryptase, carboxypeptidase and ACE were measured in all samples using the specific ELISA procedures. Serum was added to plates within two hours of collection (freeze thawing was carried out within this time).

Concentrations of markers measured in serum that did not undergo any freeze thawing were taken to be total levels; and concentrations in samples subjected to freeze-thaw cycles were compared to these. Tryptase levels were reduced by over 15 % with one or two freeze thaw cycles, and by over a quarter with three cycles.

Carboxypeptidase levels were more stable, with over 90 % obtainable after one freeze thaw cycle, and over 80 % after two or three cycles. There was no reduction in ACE levels following up to three cycles.

In vivo variability

To determine the amount of variation of protease levels within individuals, serum was obtained from six subjects on two different occasions. Times between visits varied from approximately one month to one year. Tryptase, carboxypeptidase and ACE levels were measured by ELISA. Levels of all three proteases were relatively stable *in vivo*. The variation coefficient was 13% for tryptase, 18 % for carboxypeptidase, and 14 % for ACE.

Reproducibility of assays

Reproducibility of the ELISAs for tryptase, carboxypeptidase and ACE were assessed by measuring levels in serum (n = 4) at three different positions across a 96 well plate (intra-assay). In addition, concentrations were measured on different plates on two separate days (inter-assay). In the latter case, serum had been split into aliquots and frozen at - 80 °C in order to avoid multiple freeze thaw cycles. Assessment of inter- and intra-assay reproducibility provides an indication of accuracy and precision of the ELISA.

The average percentage coefficient of variation for intra-assay measurements was 14 % for tryptase, and 8 % for both carboxypeptidase and ACE. For inter-assay measurements, the percentage coefficient of variation was 8 % for tryptase, 14 % for carboxypeptidase and 8 % for ACE. These levels of variation are considered acceptable according to the guidance provided by the U.S. Department of Health and Human Sciences.

3.3 Discussion

The ELISA techniques for measurement of tryptase, carboxypeptidase and ACE have been refined and validated for use with human biological fluids. This has been carried out in accordance with guidelines presented by the U.S. Department of Health and Human Services where applicable. Studies carried out confirm that measurement of tryptase in serum and saliva, and ACE in serum, was not affected by interference. This work has previously been carried out by Xiaoying Zhou for measurement of carboxypeptidase. We have also shown that *ex vivo* stability for the tryptase, carboxypeptidase and ACE assays was excellent at -20 ⁰C, and was reasonable with up to three freeze-thaw cycles. *In vivo* variability was relatively minimal. Intra-assay variability was good (less than 15 %) for the tryptase assay, and excellent (less than 10 %) for the carboxypeptidase and ACE ELISAs. Inter-assay variability was excellent for the tryptase and ACE assays, and good for the carboxypeptidase ELISA.

Our results of *ex vivo* stability and *in vivo* variability have implications for our study in terms of methodology and interpretation of results. These have been described below.

Ex vivo stability of proteases

Our results have shown that tryptase and carboxypeptidase are very stable in serum stored at -20 °C. Both proteases are less stable at 22 or 4 °C. However, approximately 90 % of total tryptase could be measured after 24 hours at these temperatures, and about the same amount of carboxypeptidase could be measured following 24 hours at 4 °C. Serum ACE levels were very stable at 22 ⁰C, 4 ⁰C and -20 ⁰C.

From these findings it was decided that serum should be frozen at temperatures of -20 degrees or lower as quickly as possible in order to prevent degradation of certain proteases. Samples could be stored unfrozen for up to 24 hours if

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absolutely necessary, ideally at 4 ⁰C, but this should generally be avoided. In addition, all samples should be stored in identical conditions to prevent differences in protease levels due to degradation.

Although freezing of samples is often necessary to avoid degradation of components, freeze-thawing can also have this effect. In the current experiments, we found that serum tryptase levels were reduced to approximately 80 % of total after one freeze-thaw cycle, but did not reduce to the same extent with subsequent cycles. Serum carboxypeptidase levels were more stable, with over 90 % obtainable after one freeze thaw cycle. Approximately 80 % of total levels were measured after up to three cycles. ACE levels were the most stable, with no significant reduction compared to total levels following up to three freeze-thaw cycles.

Our findings suggested that, ideally, samples should not undergo freeze-thawing prior to protease measurement. However, the effect of prolonged periods at 4 ⁰C was deemed to be more detrimental (sample collection took place over two years, and assays were performed at the end of this time). Thus, it was decided that samples should be frozen, but the number of freeze-thaw cycles should be minimized. In addition, samples used within an assay should have been subjected to the same degree of freeze-thawing.

We decided upon a protocol for storage and use of samples from patients within our study based upon the concepts learnt from these experiments. Samples were stored on ice immediately upon collection; and were processed and frozen at -20 ⁰C on the same day. It was beyond the scope of the current studies to investigate stability of protease levels stored at -20 ⁰C long term. Samples were therefore transferred to -80 ⁰C within two weeks (if not used before) due to the potential for degradation. To avoid the need for multiple freeze thaw cycles, upon collection samples were split into aliquots containing enough volume for one or two assays. Once thawed, samples were stored at 4 ⁰C for a maximum of 24 hours. If sample remained following experiments, it was labelled and refrozen.

***In vivo* variability of proteases**

As part of our study, baseline levels of proteases have been investigated with respect to severity of reactions. To assess the potential usefulness of our results, the variability of proteases within individuals was examined. Our experiments revealed that levels of tryptase, carboxypeptidase and ACE were reasonably reproducible over time periods of between one and twelve months. Coefficients of variation were less than 15 per cent for tryptase and ACE, and less than 20 per cent for carboxypeptidase.

To our knowledge, there have been no previous studies looking at differences in levels of these proteases within individuals over time. Our results are promising, and are indicative that baseline levels vary minimally within individuals over time. However, all duplicate measurements were performed within one year of the previous. It would be useful to study *in vivo* levels at further time points with larger intervals, and also to study differences in matched paediatric and adult populations. In addition, the potential for protease levels to vary with season requires investigation, as concentrations may be expected to increase during months in which hay fever is most prevalent in allergic individuals. This was beyond the scope of the current study.

Summary

We have provided evidence that results obtained using our ELISAs for tryptase, carboxypeptidase and ACE are valid and accurate. We have established optimal conditions for storage and use of samples, and compliance with these conditions within the study allows for further confidence in our results. In addition, preliminary studies on levels of the proteases *in vivo* suggest that they vary only minimally over time. This is important to support our work looking at baseline levels of mediators and susceptibility to severe reactions. However, further studies are required to investigate *in vivo* variability over a longer time scale and during different seasons.

CHAPTER 4: DEVELOPMENT OF A NOVEL ELISA FOR DPPI

4.1 Introduction

Dipeptidyl peptidase I (DPPI) is a protease stored within various inflammatory cells, including mast cells. DPPI has a role in growth and differentiation of mast cells (Wolters et al., 2000), and also in activation of tryptase (McGuire *et al.*, 1993) and chymase (McEuen *et al.*, 1998). Actions are described in detail in chapter 1.

DPPI has typically been considered as an enzyme that acts intracellularly. However, there is some evidence for its release upon activation of dog mastocytomas (Wolters *et al.*, 1998), and work within our laboratory has indicated release from the mast cell line, LAD-2, and also human tonsil mast cells, as determined by measuring enzyme activity (Xiaoying Zhou, unpublished observation). The aim of the current study was to develop a specific and sensitive ELISA for measurement of DPPI in human biological fluids in order to investigate it's *in vivo* release during an allergic reaction and potential as a new biomarker.

4.2 Methods and results

A novel ELISA for measurement of DPPI in human biological fluids has been developed using specific antibodies. This was validated in accordance with guidelines prepared by the U.S. Department of Health and Human Services where applicable, as described in Chapter 3. In brief, limits of detection of the assay were determined, and its suitability for use with human biological fluids was validated. Tests for stability and reproducibility were performed. For a proof of concept study, DPPI levels were measured in nasal lavage fluid collected from patients with seasonal allergic rhinitis to grass pollen subjected to nasal allergen challenge.

Antibody production

Murine monoclonal antibodies had been prepared previously against human DPPI (Laurie Lau *et al.*, unpublished) using methods similar to those described for production of antibodies against tryptase (Chapter 7). In brief, mice were injected with DPPI, stimulating production of antibodies against the protease. Spleen cells were fused with myeloma cells, resulting in formation of hybridomas. Cloning and sub-cloning procedures were carried out to obtain monoclonal cell populations, and the hybridoma cells expanded in culture medium. Antibody-containing supernatant was collected and characterized. Six hybridoma lines secreting DPPI-specific monoclonal antibodies were produced. These were termed DD1, DD2, DD3, DD4, DD5 and DD6. Polyclonal anti-DPPI antibodies were prepared from rabbits (Laurie Lau *et al.*, unpublished). Animals were injected with DPPI (stimulating antibody production) and anti-sera collected.

Antibody binding to DPPI

Binding of antibodies to DPPI in ELISA

Binding of the new antibodies to DPPI was first established by direct ELISA. A 96 well micro-plate was coated with human recombinant DPPI (Unizyme; 1 µg/ml in

coating buffer) and refrigerated at 4 °C overnight. After blocking with 3 % BSA in PBS for two hours at 22 °C, a range of dilutions of the new antibodies in 1 % BSA in PBS were added to wells in duplicate. Monoclonal antibodies were diluted 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640; and polyclonal antibodies were diluted 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400 (antibodies are more concentrated within anti-sera than in culture supernatant).

The plate was incubated at 22 °C for two hours, and HRP-conjugated anti-mouse immunoglobulin antibody (1:1000 in 1 % BSA in PBS) or peroxidase-conjugated anti-rabbit IgG antibody (1:8000 in 1 % BSA in PBS) added as appropriate to the detection antibody. After two hours at 22 °C, TMB substrate was added and colour allowed to develop. The reaction was stopped with H_2SO_4, and the optical density measured using a spectrophotometer at 450/595 nm.

Binding of the six mouse monoclonal antibodies and rabbit anti-sera to DPPI in direct ELISA is shown in Fig 4.1. Binding occurred for all antibodies in a dose-dependent manner with increasing concentrations.

Binding of antibodies to DPPI in western blot

The molecular weight of DPPI was first established by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) using a 16 % Tris glycine gel. After assembling within an electrophoresis tank, pre-stained molecular weight markers and heat-denatured (10 min at 95 °C) human recombinant DPPI were added to lanes of the gel. Running buffer, containing 0.3 % w/v Tris (base), 1.44 % w/v glycine and 0.1 % w/v SDS, was added; and a potential difference of 95 V was applied for 20 minutes, and 195 V for approximately one hour. The gel was removed from the tank and stained with Coomassie blue. The molecular weight of DPPI was determined to be approximately 27 kDa by SDS-PAGE.
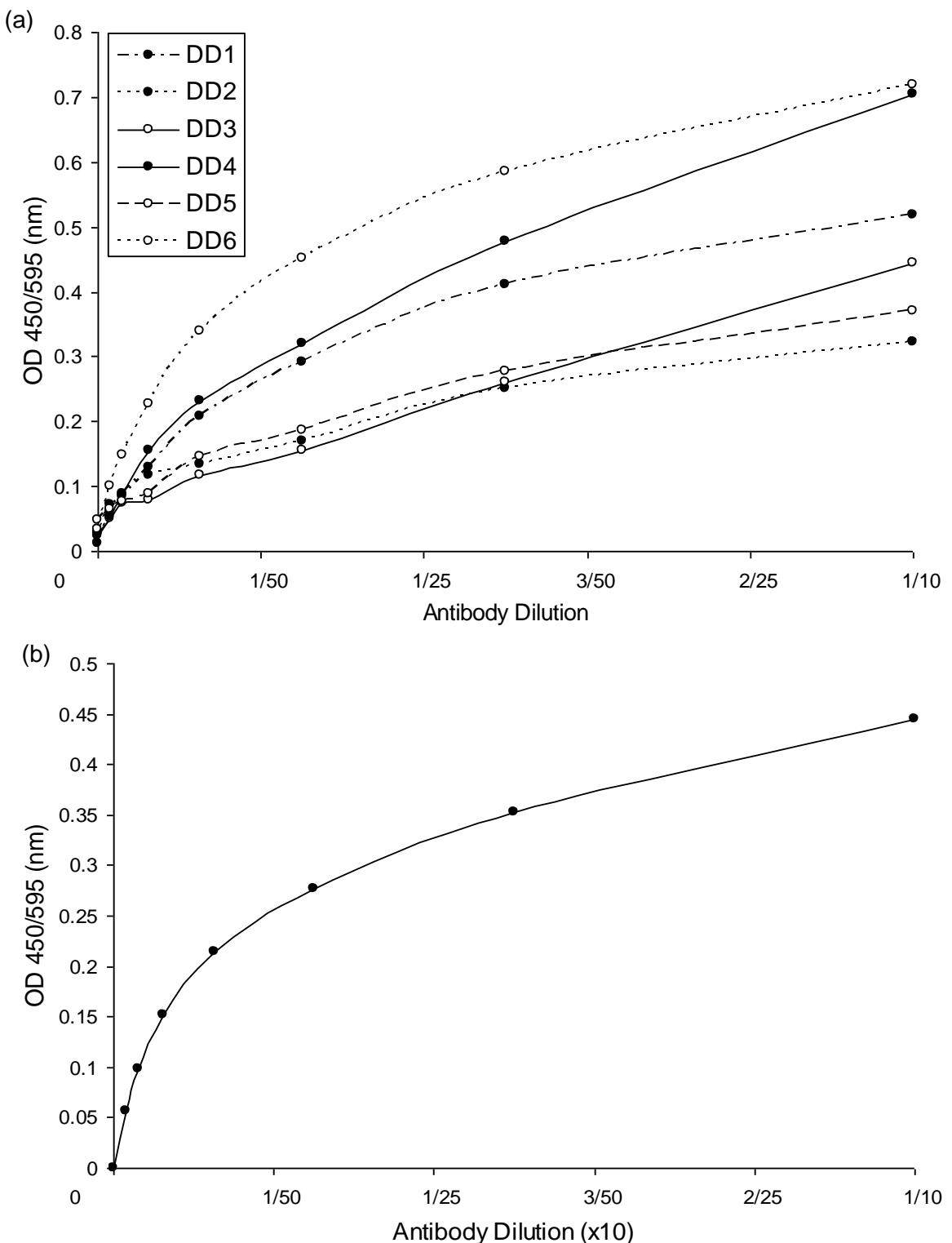
Fig 4.1 Binding of (a) DD1, DD2, DD3, DD4, DD5 and DD6 and (b) rabbit antisera to DPPI in direct ELISA. A plate was coated with human recombinant DPPI, and increasing concentrations of the antibodies were used for detection.

For western blot, molecular weight markers and DPPI were separated on a gel in SDS-PAGE as described above, and the proteins transferred to nitrocellulose membrane for immunoblotting. The gel was placed adjacent to a membrane (pre-soaked in methanol for 3 minutes), sandwiched between layers of filter paper and blotting pads, and placed within a tank containing transfer buffer (0.3% Tris (base), 1.44 % glycine, 20 % methanol). A potential difference of 100 V was applied for one hour, until the protein had transferred to the nitrocellulose membrane.

The nitrocellulose membrane was blocked in 3 % milk powder in PBS for one hour at 22 °C, and washed three times for five minutes in T-PBS. The membrane was cut into strips for each lane; and these were incubated with culture supernatant containing each of the six monoclonal antibodies (1:10 in PBS), rabbit polyclonal antibody (1:1000 in PBS), or mouse IgM isotype control (1 ug/ml in PBS). After one hour, the strips were washed as previously and incubated with HRP-conjugated anti-mouse immunoglobulin antibody (1:1000 in PBS) or peroxidase-conjugated anti-rabbit IgG antibody (1:8000 in PBS), as appropriate to the detection antibody. After washing five times for five minutes, 3,3'-diaminobenzidine (DAB) was added and colour allowed to develop,

Fig 4.2 shows binding of antibodies to DPPI in western blot. Antibodies bound to proteins at approximately 27 kDa, corresponding with the molecular mass of bands stained for DPPI on SDS-PAGE. Rabbit antisera, DD4 and DD5 also bound to proteins at other molecular weights. No staining occurred for the IgM isotype control. These studies confirmed that the antibodies bind to DPPI.

Binding of antibodies to DPPI in immunocytochemistry

Cytospins of LAD-2 cells were prepared and cells fixed with acetone for five minutes. After washing three times for five minutes with Tris buffered saline (TBS), non-specific binding sites were blocked firstly with 0.3 % H_2O_2 in 1 % sodium azide and secondly with culture medium, both for thirty minutes.
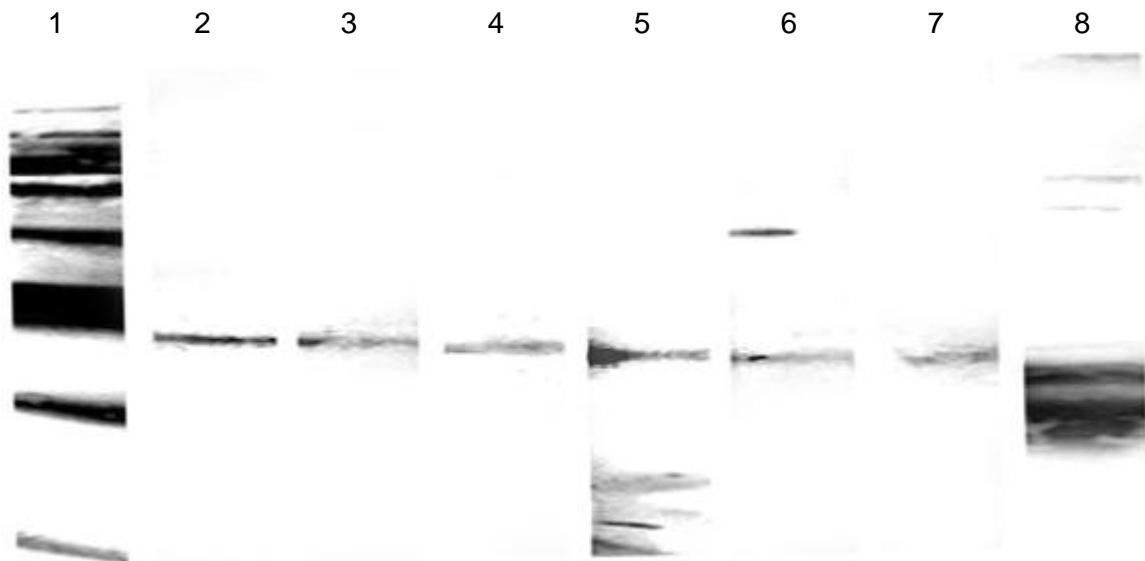
Fig 4.2 Binding of antibodies to DPPI in western blot. Lane 1: molecular weight marker, lane 2: DD1, lane 3: DD2, lane 4: DD3, lane 5: DD4, lane 6: DD5, lane 7: DD6, lane 8: rabbit DPPI antiserum. No band was seen when using IgM isotype control.

Cells were washed as previously and incubated with supernatants containing each of the six monoclonal antibodies, or rabbit antisera, at 22 °C for one hour. Mast cell-specific antibody, AA1, and TBS were used for positive and negative controls, respectively. The slides were washed, and HRP-conjugated anti-mouse immunoglobulin antibody (1:500 in TBS) or peroxidase-conjugated anti-rabbit IgG antibody (1:500 in TBS) were added as appropriate to the detection antibody. TBS was used as a negative control. After washing, cells were stained with DAB for five minutes, and then rinsed in TBS and washed under running water for five minutes. Cells were counterstained with Mayer's haemotoxylin for 90 seconds, and washed under running water for five minutes. The slides were dried and cells mounted with *p*-xylene-*bis*-pyridinium bromide (DPX) permount (Sigma); and glass cover slips were placed on top.

Staining of cells was inspected under a microscope, and could be seen in the cytoplasmic area of cells stained using the positive control and the new DPPI-specific antibodies. Negative control slides were unstained. The results of this

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technique are not shown; but confirm that the new antibodies bound to a mast cell constituent as suggested in the previous two methods of assessment.

Antibody characterization

Additive ELISAs were carried out to determine whether the six monoclonal antibodies bound to similar or to different epitopes. Direct ELISAs were performed using detection antibodies individually and in combination. When antibodies bind to different epitopes, binding saturation is likely to occur at a higher antibody concentration when combined than when used individually.

A plate was coated with DPPI (2000 ng/ml in coating buffer) at 4 °C overnight and blocked with 3 % BSA in PBS at 22 °C for two hours. Monoclonal antibody supernatants (1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640 in 1 % BSA in PBS) were added individually and paired. After two hours at 22 °C, rabbit anti-mouse HRP-conjugated antibody (1:1000 in 1 % BSA in PBS) was added. After two hours at 22 °C, TMB substrate was added and colour allowed to develop. The reaction was stopped with H_2SO_4, and optical density read at 450/595 nm.

An additive effect was seen when certain antibodies were combined, indicated by higher optical density readings with increasing antibody concentrations compared to when used individually (Fig 4.3(a)). This suggested that these antibodies may bind to different epitopes. For other pairs, the optical density curve lay between the lines produced by the antibodies separately (Fig 4.3(b)). These antibodies may bind to similar epitopes (meaning that one antibody obstructs the other). The findings suggested that DD1 and DD5 bind to different epitopes than DD2, DD3 and DD4. In addition, DD3 and DD4 may bind different epitopes. It is likely that DD1 and DD5, and also DD2 and DD4, bind similar epitopes. DD6 did not appear to bind to any epitopes different from that of any of the other antibodies. Similarities and differences in epitope binding are represented in Fig 4.4.
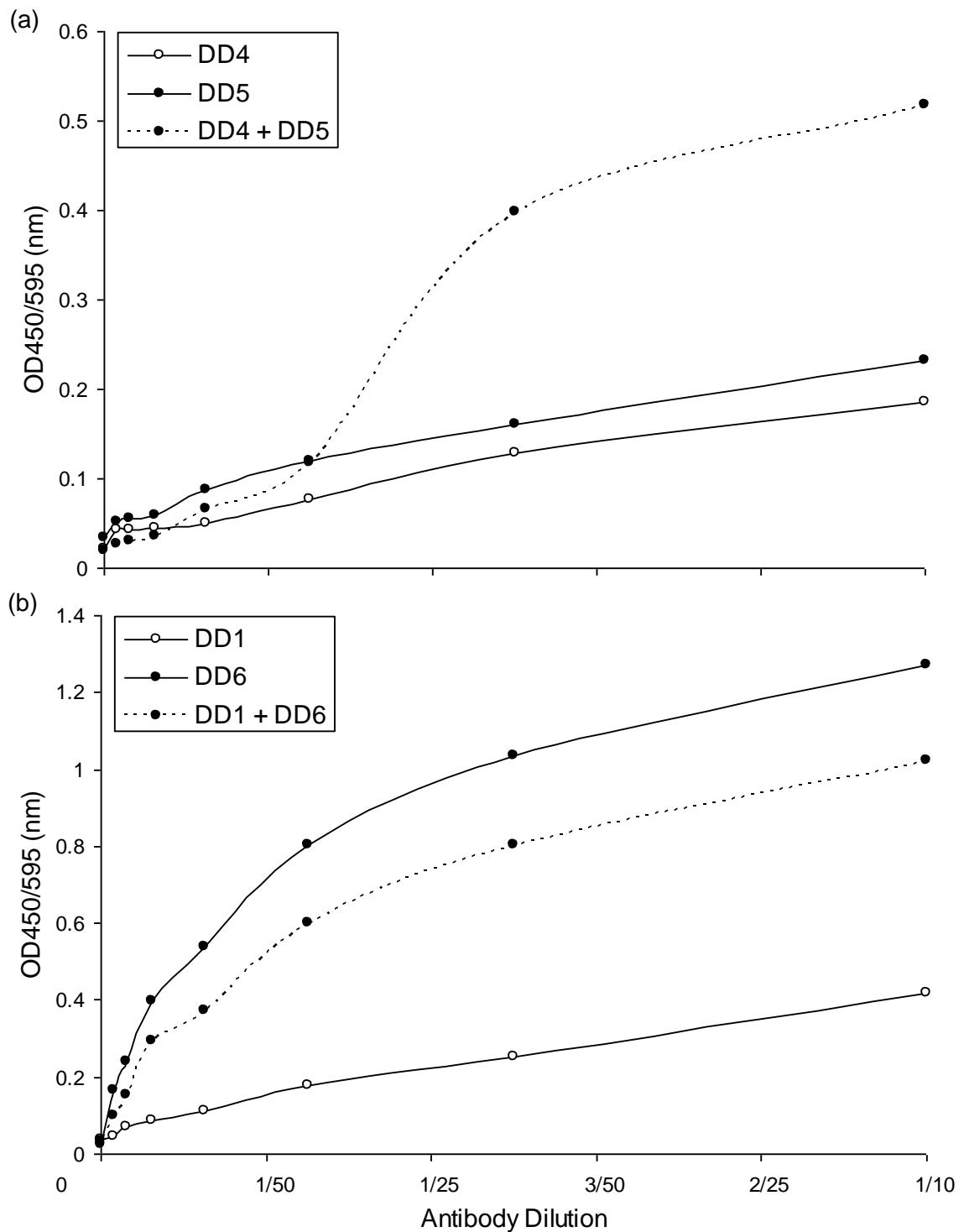
Figure 4.3 Additive ELISA with (a) DD4 and DD5, and (b) DD1 and DD6. DPPI levels were measured in ELISA using antibodies individually and paired. An additive effect indicates that antibodies bind to different epitopes.
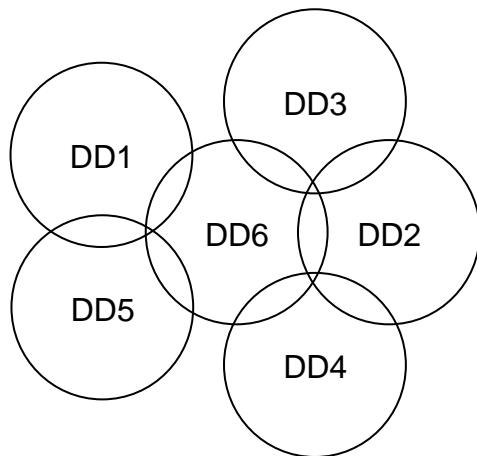
Fig 4.4 Venn diagram showing epitope binding patterns of DD1-DD6. Antibodies are represented by circles; and areas of overlap indicate binding to similar epitopes of DPPI.

Antibody Purification

Different methods of antibody purification were attempted, including affinity chromatography and antibody precipitation. Ultimately, mouse monoclonal antibodies were purified by polyethylene glycol (PEG) precipitation, and rabbit polyclonal antibody using ammonium sulphate precipitation.

Purification by affinity chromatography

The mouse monoclonal anti-human DPPI antibodies were initially purified by affinity chromatography using protein L (Thermo Scientific). Protein L, an immunoglobulin-binding protein, was immobilized on cross-linked agarose beads within a column. Antibody-containing culture supernatant (1:2 in PBS) was pumped through the column at a rate of 0.5 ml/min, allowing antibody binding to protein L. The column was washed with PBS and antibodies eluted with 0.1 M glycine (pH 2.5). The eluant was collected in glass tubes every two minutes and immediately neutralized with 100 µl 2 M Tris (pH 8.0). A coomassie blue binding assay was used to determine protein concentrations in the eluted fractions (Fig 4.5); and known concentrations of mouse γ-globulin were used for standards. Antibody fractions that contained the highest protein concentrations were pooled and dialysed against PBS overnight. The purified antibodies were stored at - 20 °C.

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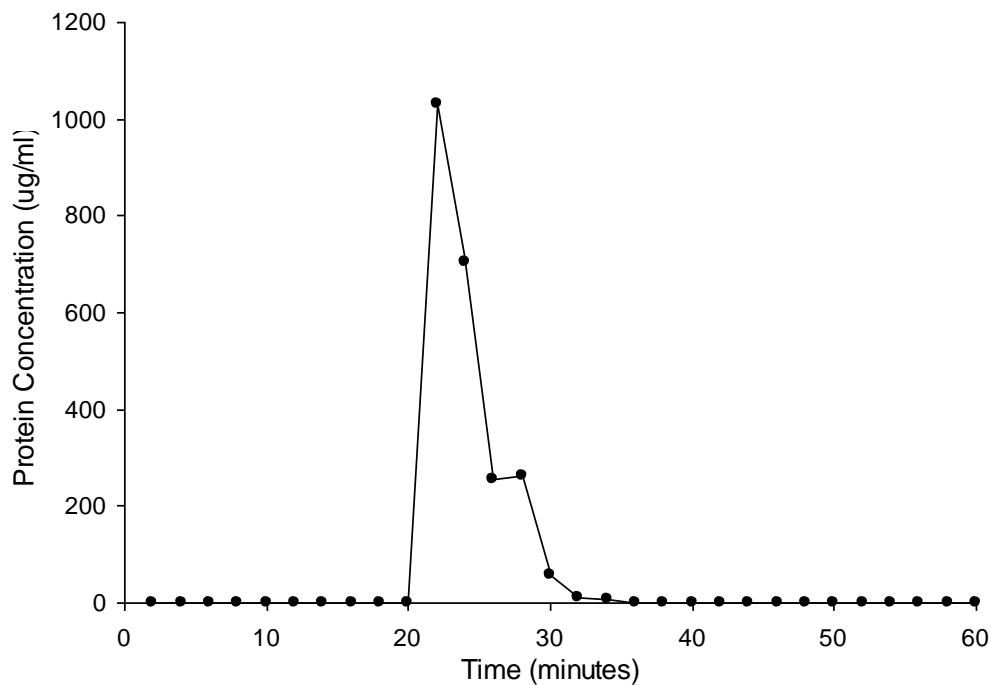
Fig 4.5 Elution curve of mouse monoclonal anti-DPPI antibody, DD1, purified by affinity chromatography. Antibodies were purified using a protein L column.

After purification by affinity chromatography, however, the ability of the antibodies to bind DPPI in a concentration-dependent manner in direct ELISA was lost, and a large degree of non-specific binding was seen. This may have been due to the harsh acidic conditions required for the elution stage, or the unstable nature of IgM. In addition, total purification of antibodies likely resulted in elimination of stabilizing proteins otherwise present in the culture supernatant. Therefore, methods of partial purification were assessed.

Partial purification by precipitation

Partial purification of monoclonal antibodies within culture supernatant was attempted by ammonium sulphate, PEG or euglobulin precipitation. Ammonium sulphate precipitation is a commonly used method for isolating crude antibodies from serum or ascitic fluids (Kent, 1999). PEG and euglobulin precipitation are additional methods that have previously been described for partial purification of IgM (Garcia-Golzaléz *et al.*, 1988; Cripps *et al.*, 1983).

For ammonium sulphate precipitation, 2.7 g $(NH_4)_2SO_4$ (ammonium sulphate, BioRad) was added to 10 ml culture supernatant. The solution was mixed on a roller for three hours at 4 °C and ultra-centrifuged at 3,000 x g for 20 minutes at 4 °C. The supernatant was discarded and the pellet re-suspended in 5 ml PBS. The partially purified antibody was dialyzed against PBS at 4 °C overnight; and again with fresh buffer for three hours. This method was also used for partial purification of the rabbit polyclonal antibodies.

For PEG precipitation, 2 ml 20 % w/v PEG 6,000 (Biochemical) in PBS, pre-cooled to 4 °C, was added to 6 ml supernatant. The solution was mixed on a roller for 30 minutes at 4 °C, and then centrifuged for 30 minutes at 2,000 x g. The pellet was drained and re-suspended in PBS. The solution was dialyzed against PBS as described for ammonium sulphate precipitation. For euglobulin precipitation, 10 ml supernatant was dialyzed against 250 ml 2 mM sodium phosphate (Sigma; pH 6.0) at 4 °C for two hours. The buffer was changed every 30 minutes. The solution was centrifuged at 2000 x g for 10 minutes at 4 °C. The supernatant was discarded and the pellet re-suspended in PBS.

A direct ELISA was carried out to assess antigen binding of antibodies purified by these techniques. The plate was coated with 0-1000 ng/ml human recombinant DPPI, and the partially purified antibodies were used for detection. Unpurified antibodies were used as positive controls. Monoclonal antibodies were diluted 1:20 in their unpurified form, and 1:100 partially purified. Rabbit antiserum was used 1:50 unpurified, and 1:200 partially purified. Buffer, containing no antibody, was used as a negative control.

PEG precipitation was the method that worked most effectively in purification of the murine antibodies. Ammonium sulphate precipitated rabbit antisera also produced a good concentration response. Standard curves for DD1 and rabbit antisera partially purified by these methods are shown in Fig 4.6.
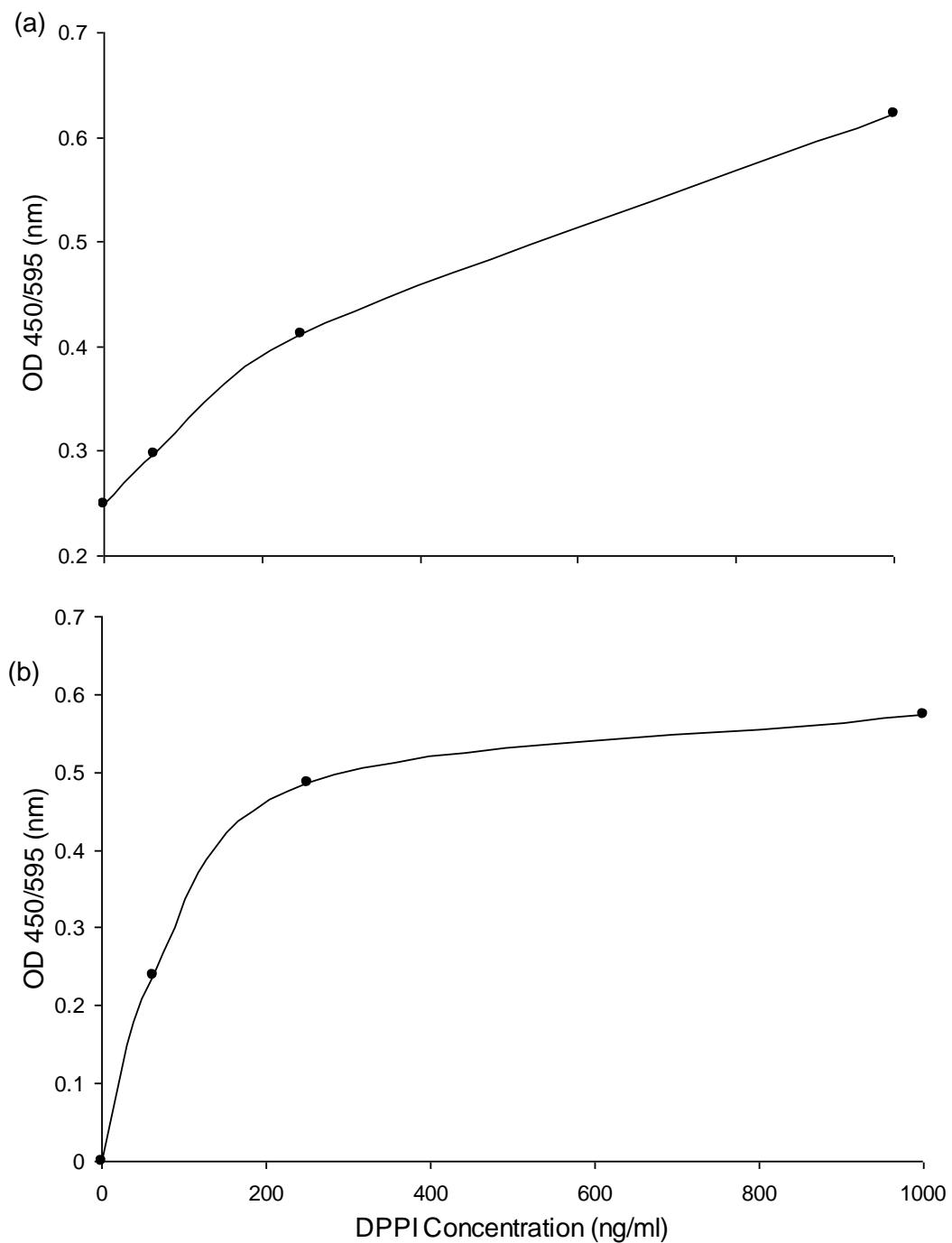
Fig 4.6 Use of (a) PEG precipitated DD1 and (b) ammonium sulphate precipitated rabbit polyclonal anti-DPPI antibody in direct ELISA. A 96 well plate was coated with DPPI (0-1000 ng/ml), and this was detected with (a) DD1 purified by PEG precipitation, or (b) rabbit antisera purified by ammonium sulphate precipitation.

Development and optimization of sandwich ELISA

Different combinations of polyclonal and monoclonal antibodies for capture and detection were assessed in sandwich ELISA. These included use of the polyclonal antibody for capture and monoclonal antibodies for detection, and vice versa; and also using monoclonal antibodies for both roles. The most sensitive and specific ELISA used PEG precipitated DD1 for capture and ammonium sulphate precipitated rabbit polyclonal antibody for detection. A peroxidase-conjugated anti-rabbit immunoglobulin secondary antibody was used in the final stage.

Conditions of the new ELISA were optimized. A range of dilutions for each antibody were assessed, and different blocking and dilution buffers were used. These included BSA, milk and foetal bovine serum (FBS). Optimal antibody dilutions were found to be 1:500 for capture, 1:1000 for detection, and 1:8000 for secondary. The most effective blocking buffer was 20 % FBS in PBS, and for dilution the FBS was diluted to 3 %.

Although a good concentration-response was seen with increasing DPPI concentrations when using these conditions, the background remained relatively high. Elimination studies, in which different components of the ELISA were excluded, revealed that the secondary antibody was binding non-specifically to proteins in the blocking buffers. The antibody was therefore incubated with dilution buffer prior to use. Finally, assessment of different blocking times revealed that the optimum duration was overnight. The final protocol for the assay is described in Chapter 2; and the standard curve for the assay using the optimized conditions is shown in Fig 4.7.
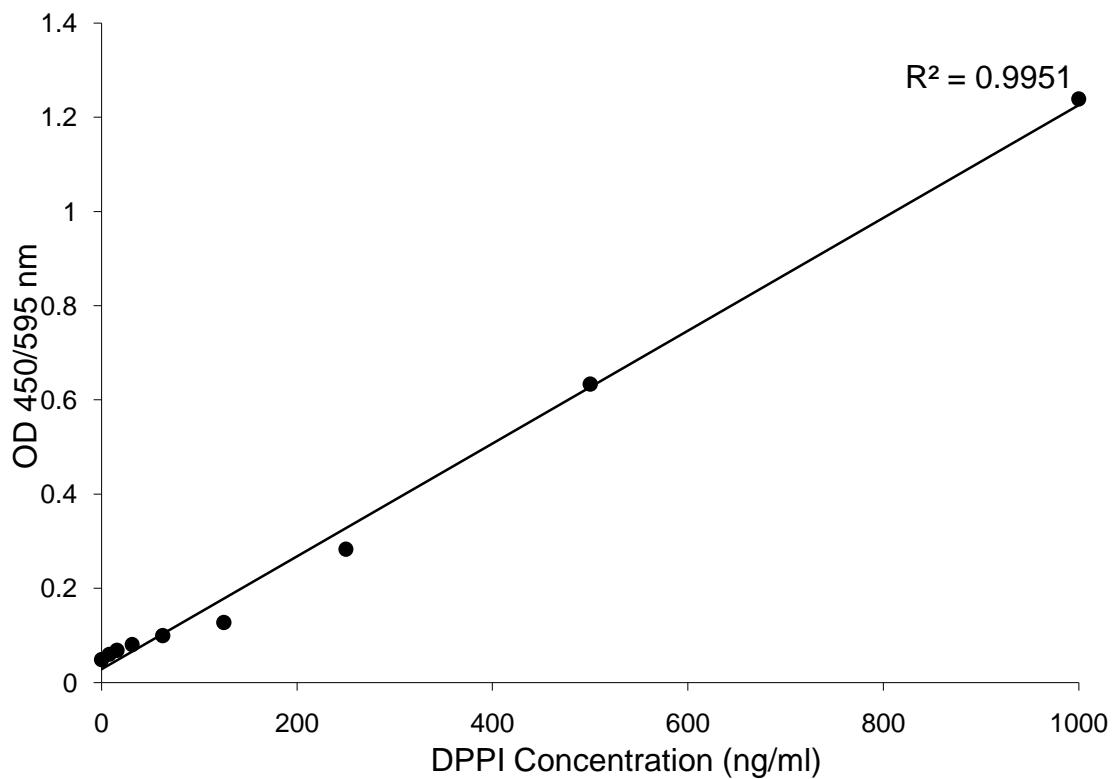
Figure 4.7 DPPI standard curve in optimized ELISA. A sandwich ELISA using PEG precipitated DD1 for capture and ammonium sulphate precipitated rabbit antisera for detection. Foetal bovine serum was used for blocking and dilution.

Limits of detection

The protocol for the DPPI assay was carried out as described in Chapter 2; and a wide range of standard concentrations were used (0-2000 ng/ml, double diluted). This was carried out five times, and the mean optical density readings and 95 % confidence intervals were calculated for each standard concentration.

Fig 4.8(a) shows the lower region of the standard curve. The lowest standard concentration at which 95 % confidence intervals did not overlap with those of the standard below was 7.81 ng/ml. This is therefore the lower limit of the assay. Fig 4.8(b) shows the full standard curve. Optical density plateaus after a DPPI concentration of 1000 ng/ml, but is linear up to this point. This is therefore the upper limit of detection.
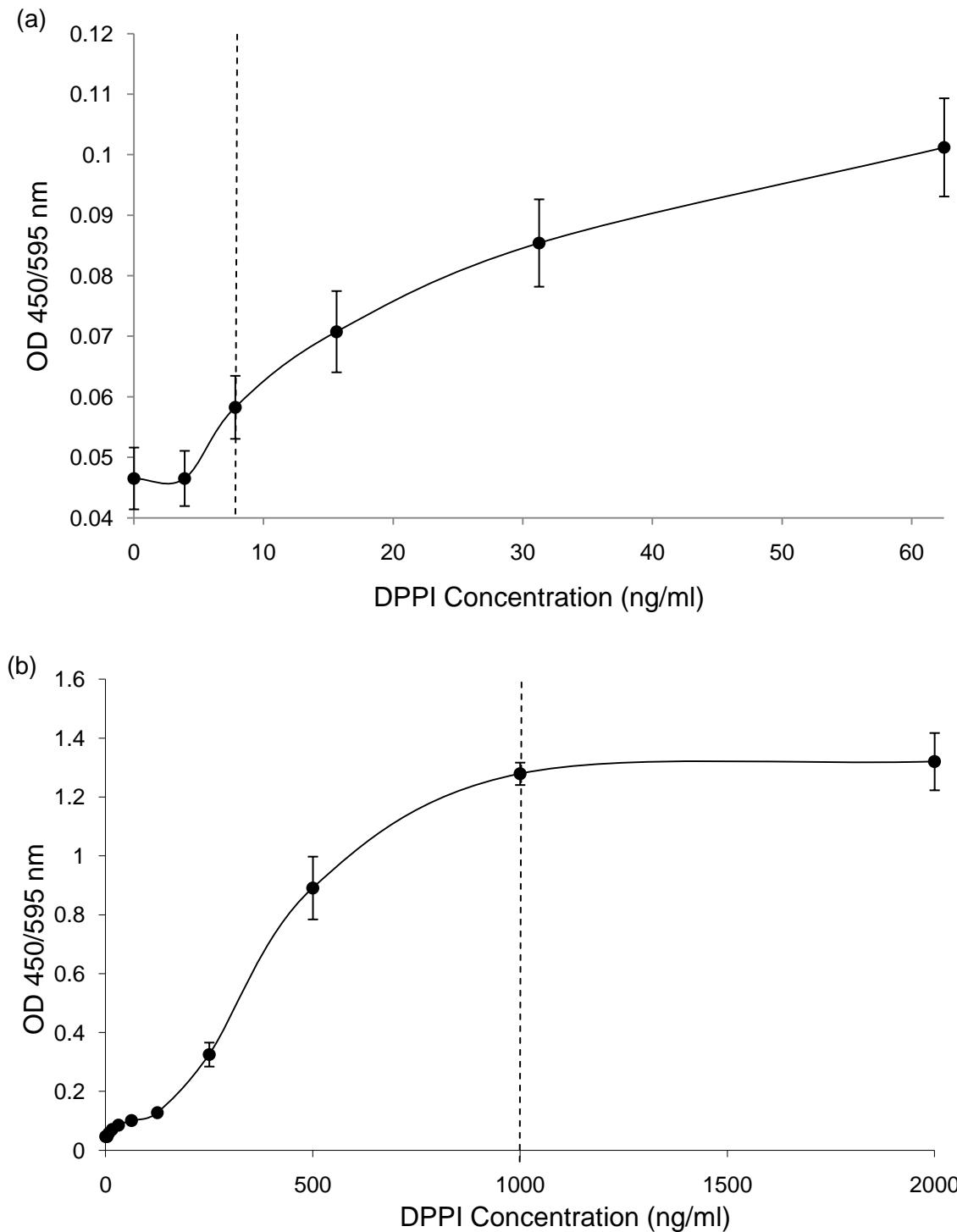
Fig 4.8 (a) Lower and (b) upper limits of detection of ELISA for DPPI. ELISAs were carried out using a wide range of standards (0-2000 ng/ml; n = 5). Graphs show mean optical density readings, and the bars represent 95 % confidence intervals. The dotted lines indicate the detection limits.

ELISA validation for use with human serum and saliva

Serum dilutions

DPPI concentrations were determined in serum neat and diluted 1:2, 1:4, 1:8, 1:16 and 1:32 (n = 4) using the optimized ELISA protocol (Chapter 2). This could not be carried out for saliva due to low baseline levels of DPPI. Results are shown in Fig 4.9 as percentages of levels measured in undiluted serum. Measured and projected results are illustrated.

Serial dilutions of serum resulted in a stepwise reduction in measured DPPI concentration, as illustrated by a linear curve, down to a dilution of 1:16 (levels in serum diluted 1:32 were mostly below the limit of detection of the assay). However, multiplication of the concentrations by the dilution factors revealed a bell shaped curve. This indicates that at the higher dilutions the assay is less sensitive, and at the lower dilutions there is interference. Concentrations plateaud between serum dilutions of 1:8 and 1:16. Thus, for this assay, serum must be diluted within this range.

Serum and saliva spiking

Serum

Human recombinant DPPI at concentrations of 125, 500 and 2000 ng/ml was added to equal volumes of serum (n = 4) diluted 1:5. This gave final DPPI concentrations of 62.5, 250 and 1000 ng/ml, and a final serum dilution of 1:10. DPPI concentrations were measured within the spiked samples and in un-spiked control serum (diluted 1:10). Results of total DPPI concentration in spiked serum minus levels in un-spiked serum are shown in Fig 4.10. The results show that, at a serum dilution of 1:10, the DPPI levels measured in ELISA reflect those added by spiking. This indicates that there is no interference in the assay at this dilution, and serum should therefore be diluted 1:10 for use in the assay.
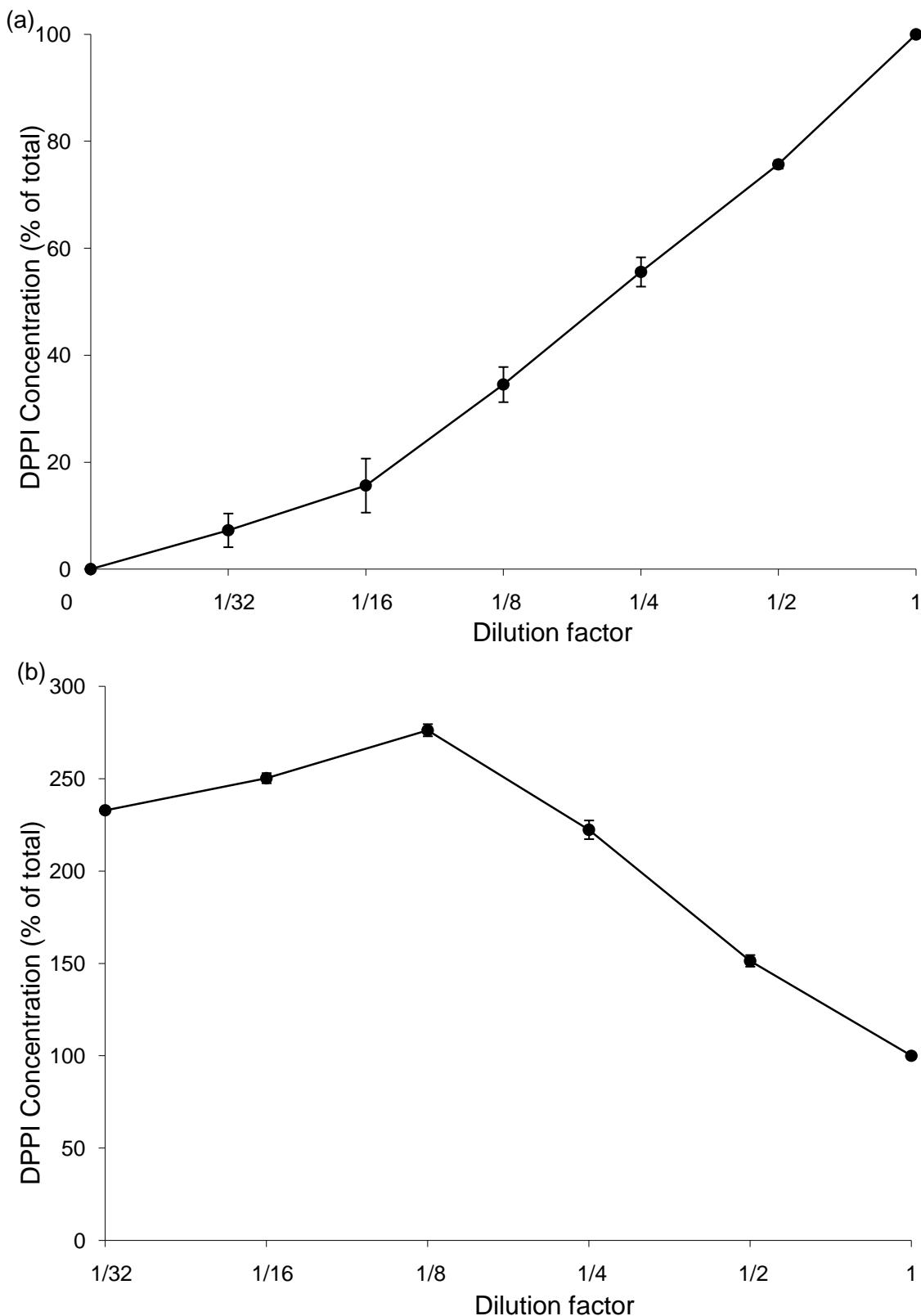
Fig 4.9 Concentrations of DPPI measured by direct ELISA in (a) dilutions of serum, and (b) multiplied by the dilution factor. Graphs illustrate measured concentrations (a) and projected concentrations (b) as a percentage of levels in undiluted serum. Average values and standard error are shown.
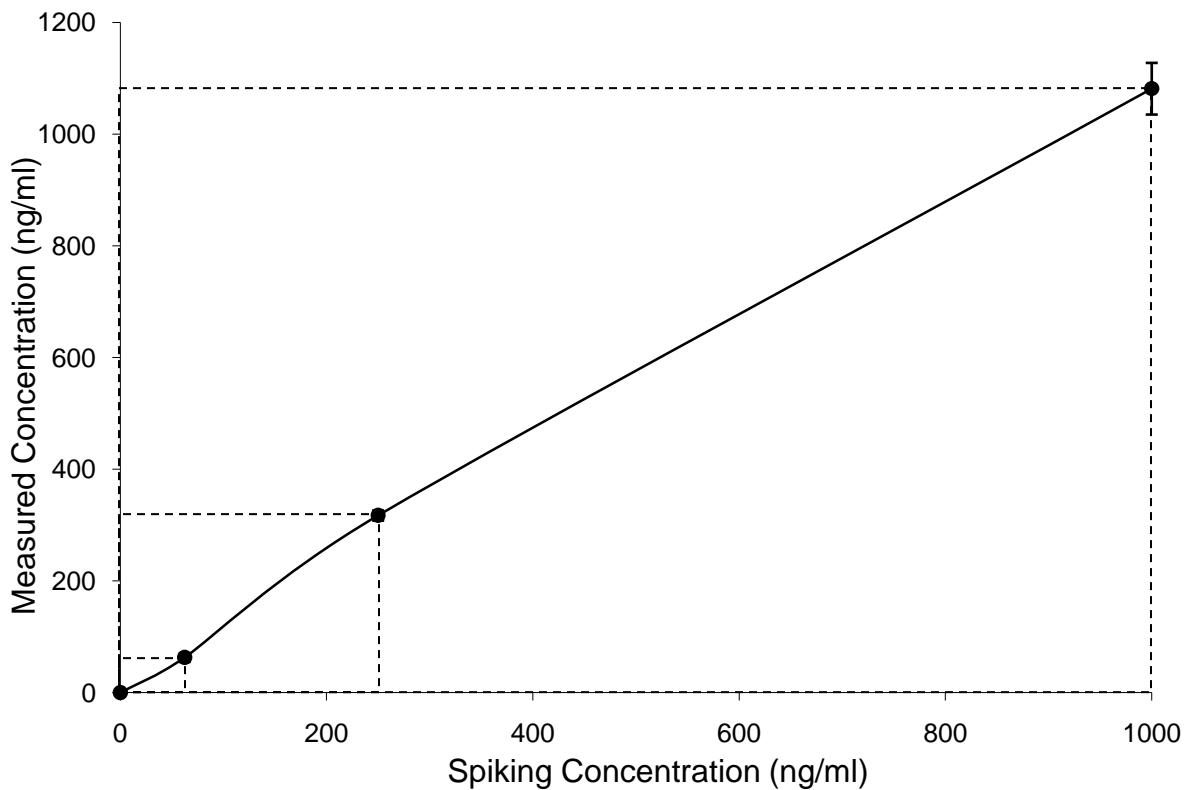
Fig 4.10 Serum spiking with DPPI. Serum (n = 4), diluted 1:10, was spiked with DPPI at concentrations of 62.5, 250 and 1000 ng/ml, and total levels were measured by ELISA. The graph shows spiking concentrations plotted against corresponding measured concentrations. Mean values and standard error are shown.

Saliva

Saliva spiking was carried out using a procedure similar to that for serum spiking. Saliva (n = 5) was diluted 1:4, and standard concentrations of 31.25, 125 and 500 ng/ml were used. DPPI levels were measured by ELISA. However, the concentration of DPPI measured was lower than that added to the samples. This indicated that factors in saliva (e.g. enzyme activity) cause interference with the assay. Saliva could not be diluted any further as endogenous DPPI levels were likely to be below the lower limit of detection of the assay.

Methods for reducing interference within the assay when using human saliva were assessed. Spiking experiments were performed as described above using saliva from five subjects that had been heat-treated for 10 minutes at 95 ºC or for 30

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minutes at 60 ºC, or diluted in a buffer containing a high salt content (0.5 mM NaCl in PBS) or T-PBS. DPPI levels were measured by ELISA in spiked and un-spiked saliva.

Total DPPI levels in spiked saliva minus those in un-spiked control saliva are shown in Fig 4.11. Interference was observed upon heat treating the saliva at 95 ºC and using buffers containing a high salt content or detergent. However, it was considerably reduced when heat treating the samples at 60 ºC for 30 minutes: the levels of DPPI measured reflected those added to the saliva. Thus, the optimal dilution factor for saliva was 1:4 following heat treatment for 30 minutes at 60 ºC.

Variability and stability of DPPI

Ex vivo stability and *in vivo* variability of DPPI in human serum were studied. Due to the low baseline levels of DPPI in saliva, and also the relatively low yield of saliva from an individual as compared to that required for these studies, it was not possible to repeat this work using this biological fluid.

Ex vivo stability

To study stability of DPPI *ex vivo*, serum from four healthy control subjects was stored at 22 °C, 4 °C or -20 °C for one, three, five, ten or 15 days, and then transferred to -80 °C. For comparison, some serum from each subject was stored at -80 °C on the day of collection (day 0). In addition, serum was subjected to one, two or three freeze-thaw cycles (n = 4). Some serum did not undergo freeze-thawing and was stored at 4 ºC. Levels of DPPI were measured in all samples by ELISA. This was carried out on the day of collection in samples subjected to freeze-thawing to prevent degradation of DPPI within the control serum at 4 ºC.
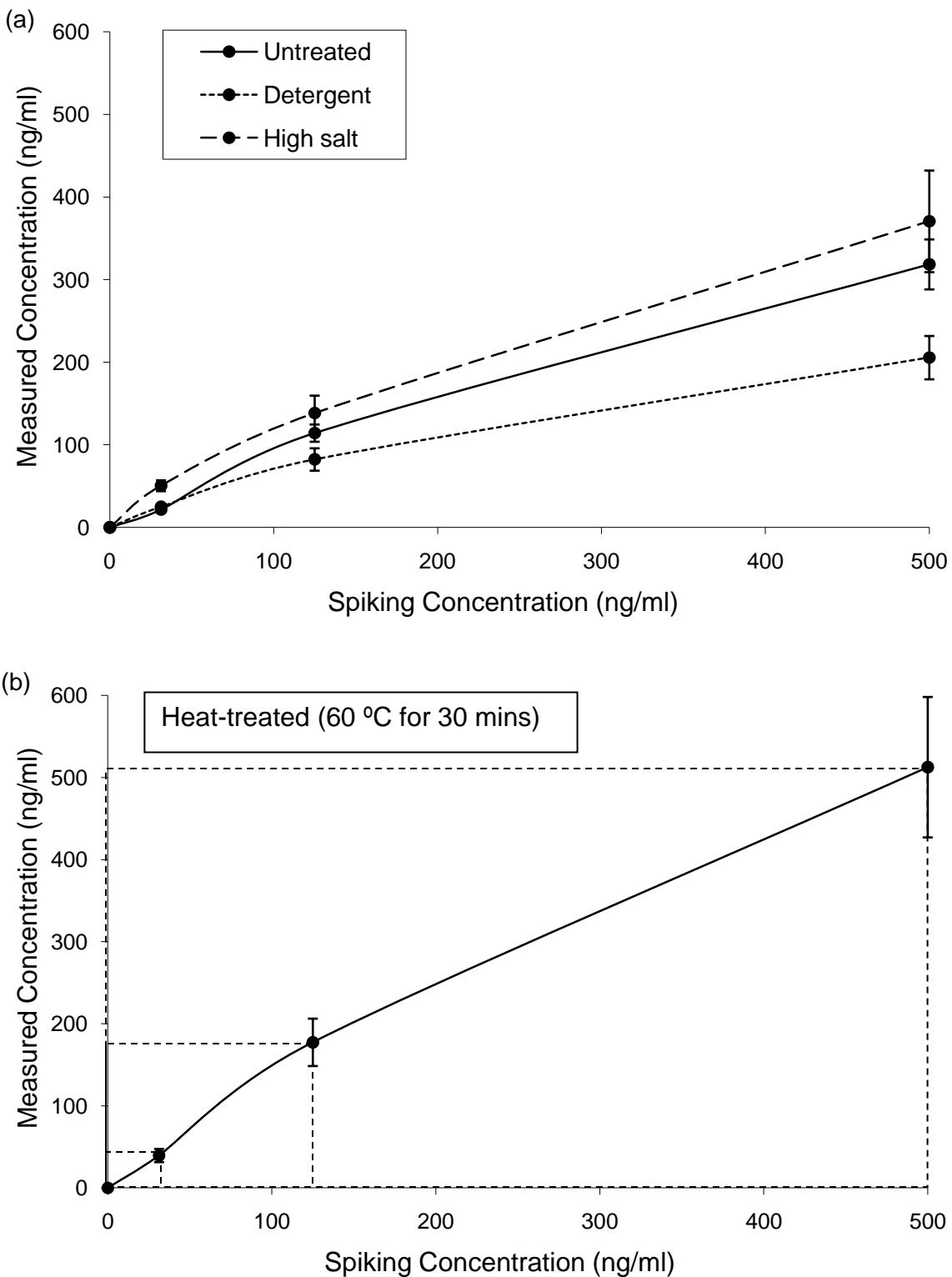
Fig 4.11 Saliva spiking with DPPI following (a) no treatment or treatment with detergent or high salt, and (b) heat treatment. DPPI (31.25, 125 and 500 ng/ml) was added to saliva (n = 5; diluted 1:4) that was either untreated or pre-treated under various conditions. Total DPPI levels were measured by ELISA. Mean levels and standard error are shown.

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Results for the time course studies are shown in Fig 4.12. Concentrations of DPPI at each time point are expressed as a percentage of levels in serum that were frozen at -80 °C on day 0 (total levels). The average total level measured was 710 ng/ml. The results show for the first time that DPPI concentrations were relatively stable at 22 °C and 4 °C, falling below 90 % of total only on days 5 and 10, respectively. At -20 °C, levels varied by no more than 2 % over the full 15 days.

The results of freeze-thawing are shown in Fig 4.13. The graph shows DPPI concentrations as a percentage of levels measured in serum that was not subjected to any freeze-thaw cycles (total levels). Again for the first time, results show that DPPI concentrations were reduced by less than 5 % of total with up to two freeze thaw cycles; and over 80 % was still obtainable following three cycles.

In vivo variability

To determine how variable levels of DPPI are *in vivo*, blood was taken from six subjects on two separate visits. Times between visits varied from approximately one to twelve months. Serum levels of the protease were determined and compared for the different time points. The results for each subject are shown in Fig 4.14. Concentrations *in vivo* appear to be relatively stable. The percentage coefficient of variation was calculated to be 15 %.

Reproducibility of assay

DPPI levels were measured in serum from four subjects at three different positions across a 96 well ELISA plate in order to assess intra-assay reproducibility. To measure inter-assay variation, concentrations of the protease were measured in serum samples on different days (n = 4). The serum used was obtained on the same day, and divided into aliquots to be frozen at -80 °C. Different aliquots were used when performing the ELISAs to prevent degradation due to freeze-thawing.
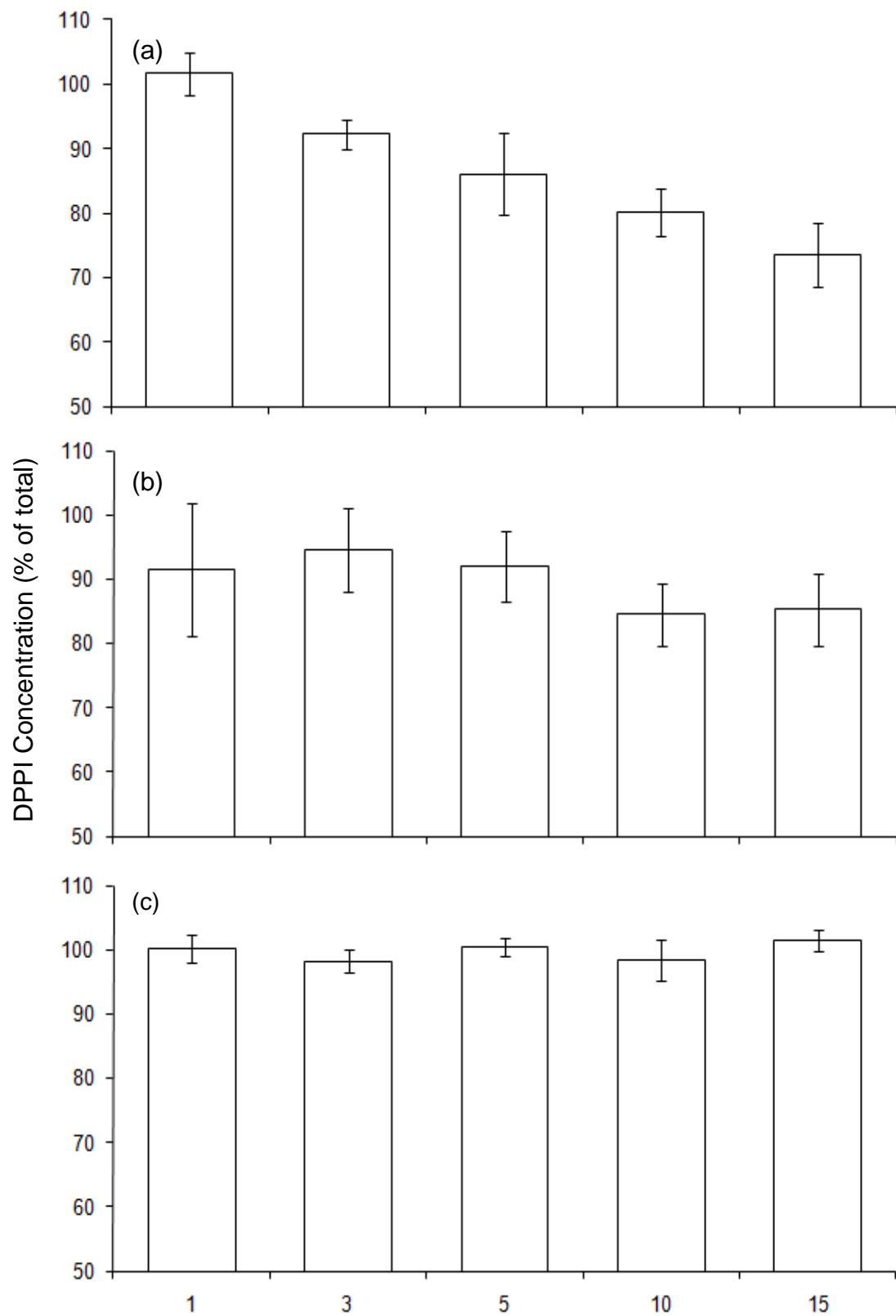
F**ig 4.12 Stability of DPPI in serum at (a) 22 °C, (b) 4 ºC and (c) -20 ºC.** Serum was stored at the different temperatures for 1, 3, 5, 10 or 15 days, and DPPI levels were measured by ELISA. Results are shown as a percentage of total levels. Mean values and standard error are shown (n = 4).
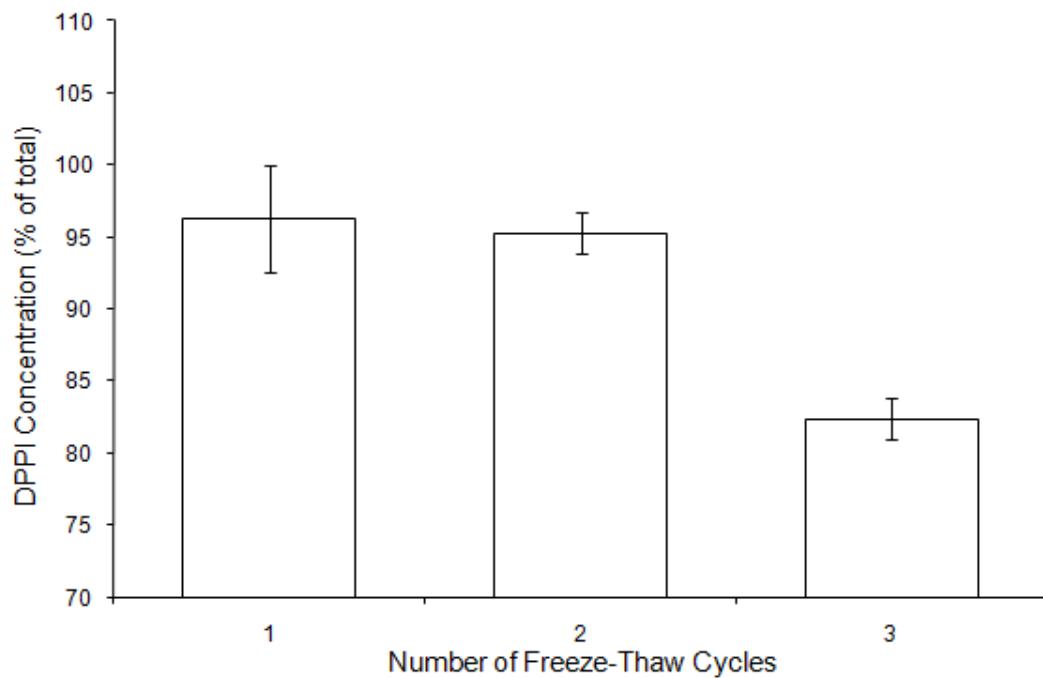
Fig 4.13 Stability of DPPI in serum following freeze-thaw cycling. Serum was subjected to 1, 2 or 3 freeze thaw cycles and DPPI levels measured by ELISA. Results are presented as percentage of total levels. Mean values and standard error are shown (n = 4).
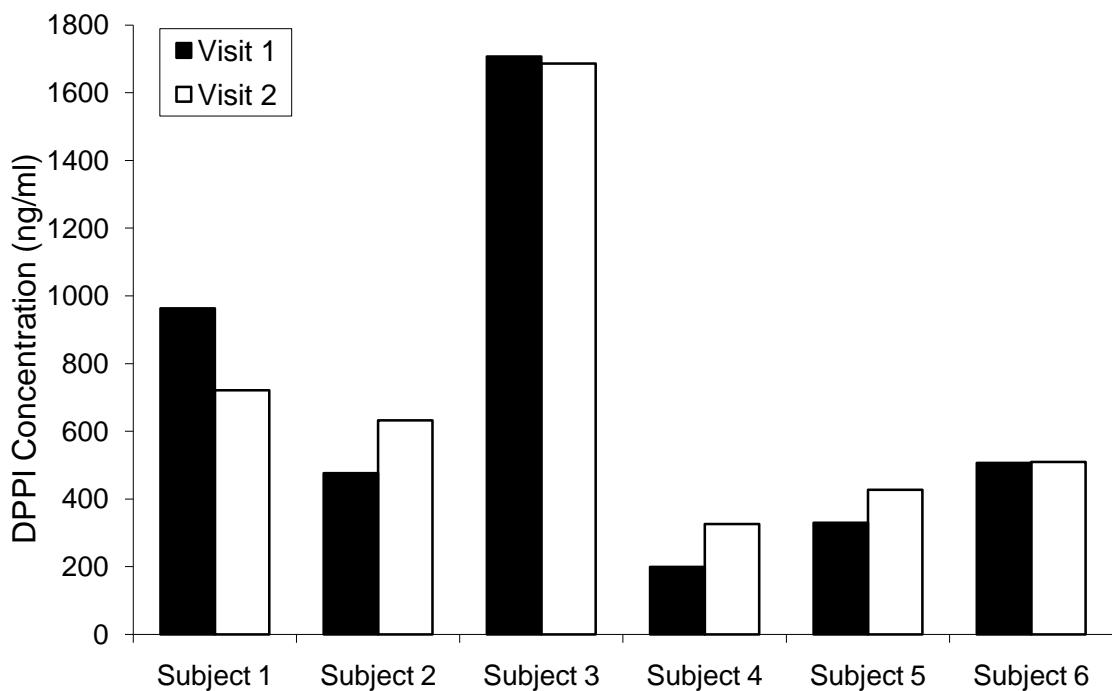
Fig 4.14 Variability of DPPI in serum *in vivo*. Serum was collected from six subjects on two different visits, and DPPI was measured by ELISA. The graph shows levels for each subject on each of the visits.

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Figure 4.15 (a) shows results for intra-assay reproducibility. Levels of DPPI measured in serum at different positions across the plate varied minimally. Results for inter-assay variation are shown in Fig 4.15 (b), and indicate that measurements are reproducible when carried out on different days. The percentage coefficients of variation for intra- and inter-assay reproducibility were 6 and 8 %, respectively.
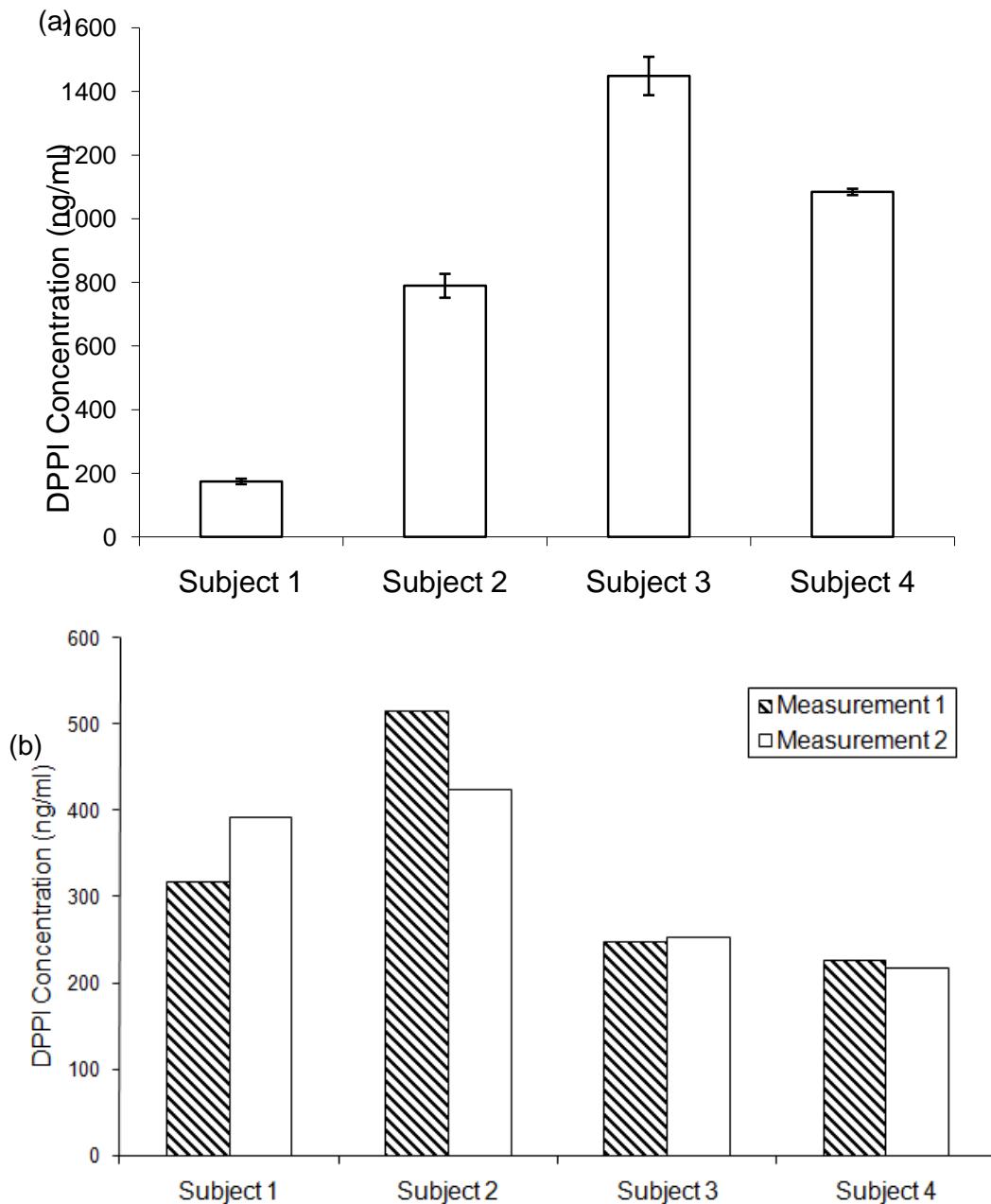
Fig 4.15 Intra- and inter-assay reproducibility. DPPI levels were measured in serum from four subjects at three positions across a plate (a). Mean levels and standard error are shown. Levels were also measured in serum from four subjects on two different days (b). The graph shows the two measurements for each subject.

4.3 Release of DPPI in seasonal allergic rhinitis

The main objective of the current research was to investigate potential markers of allergic reactions to food. This is described in detail in the following chapters. However, for a proof of concept study utilizing the new ELISA technique, levels of DPPI were measured in nasal lavage fluid of patients with seasonal allergic rhinitis to grass pollen.

Rhinitis refers to inflammation of the nasal mucosa, and can be allergic, non-allergic or infective. Allergic rhinitis affects over one fifth of the UK population, and can be caused by a range of allergens including moulds, animal dander or pollens. In the latter case, the disease is typically seasonal (reviewed in Angier *et al.*, 2010). Although systemic reactions can occur, symptoms of allergic rhinitis are generally localised to the upper respiratory tract and oral/nasal mucosa. These include sneezing, itchy nose/palate, rhinorrhea, or nasal congestion. More severe symptoms include wheezing or shortness of breath. Itching and swelling of the eyes can also occur.

Due to the mostly localised nature of allergen exposure and presenting symptoms in allergic rhinitis, we hypothesized that an increase in levels of allergic mediators would be observed in nasal lavage fluid upon exposure to allergen. We therefore investigated changes in nasal lavage concentrations of DPPI, alongside tryptase and histamine, following direct nasal challenge with grass pollen in allergic volunteers. Albumin levels were studied as an indicator of extra-vascular leakage.

Methods

Sample collection

Nasal lavage fluid was collected from ten volunteers (aged 17-51) with seasonal allergic rhinitis upon challenge with grass pollen. Patients were recruited and samples collected by Professor Peter Howarth and colleagues as part of a larger

study carried out previously (approved by the Southampton Joint University and Hospitals Ethics Committee). All subjects had experienced symptoms of seasonal allergic rhinitis over the preceding two years, and tested positive to mixed grass pollen in SPT (>5 mm wheal diameter).

Following informed consent, subjects attended for allergen challenge. Baseline measurement of nasal cavity volume and minimal nasal cross-sectional area (a-min) was performed using an acoustic rhinometer (GM Instruments, Kilwinning, Scotland), and nasal lavage fluid was collected. Each nostril was flushed for ten seconds with 2.5 ml sterile saline (pre-warmed to 37 ºC) using a syringe and nasal adaptor; and fluid was withdrawn into the same syringe. This procedure was repeated five times, and the fifth sample was used as the pre-challenge baseline sample.

Subjects were challenged first with intra-nasal 0.9 % saline, and 30 minutes later with mixed grass pollen allergen (HAL Allergen Laboratory BV, Brussels). Allergen was administered using a Valois nasal pump spray, and was applied half hourly at incremental concentrations (starting at 125 allergen units). Measures of a-min were performed at 5, 10, 15 and 20 minutes after each dose; and the challenge was ceased when a 50 % drop in a-min was observed.

Nasal volume and a-min were measured, and nasal lavage fluid collected, at 0.5, 1, 2, 4 and 6 hours after challenge cesation. Peak nasal inspiratory flow was also measured using an inspiratory flow meter with nasal adaptor (Clement Clerk International, Harlow, England). In addition subjects completed a visual analogue scale before challenge and at the times listed to quantifiably indicate self-perceived severity of symptoms, including nasal itch, congestion, sneezing and rhinorrhea. The procedure for sample collection and recording of signs and symptoms is illustrated in Fig 4.16.

Nasal lavage samples were stored on ice upon collection, and centrifuged at 400 x g for 8 minutes at 4 ºC. Supernatant was frozen at -80 ºC.

Measurement of mediators

Histamine, tryptase and albumin concentrations had previously been determined by immunoassay. Histamine levels were measured by commercially available ELISA kit according to the manufacturer's instructions (Beckman Coulter, England). 'In house' ELISAs were used for measurement of tryptase (described in Chapter 2) and albumin concentrations (described in Salib *et al*., 2005).

DPPI concentrations were measured in the nasal lavage samples using the ELISA procedure developed as part of the present studies. Validation studies were performed using dilution and spiking experiments (as described above) for use of the assay with this biological fluid. These confirmed that there was no interference in the ELISA, and that DPPI levels could be measured in nasal lavage fluid accurately (data not shown).

Nasal lavage fluid collected
Clinical signs and symptoms recorded

| Baseline | | 0.5 hr | 1 hr | 2 hr | 4 hr | 6 hr |

Nasal allergen
challenge
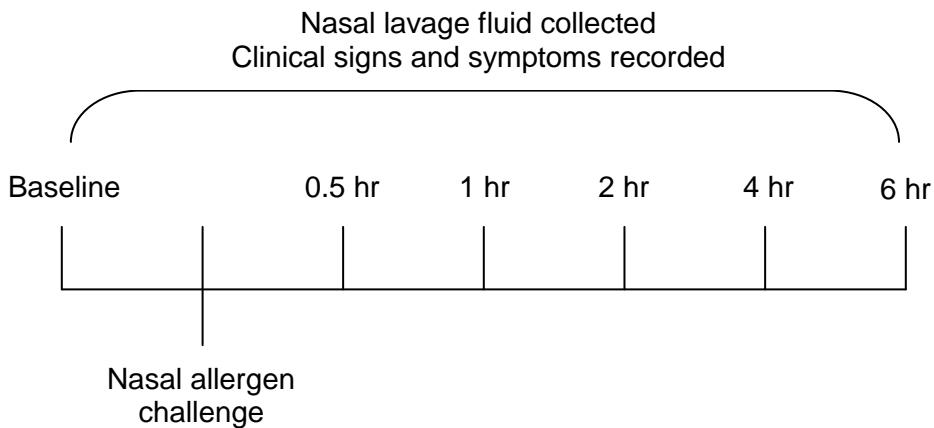
Fig 4.16 Nasal allergen challenge. Nasal lavage fluid was collected and clinical parameters recorded before and at 0.5, 1, 2, 4 and 6 hours after challenge with grass pollen extract.

Analysis

Analysis was carried out using SPSS Statistics 17.0. Data for mediator measurements had a skewed distribution, and thus non-parametric tests were

used for analysis. Friedman's two-way analysis of variance by ranks and related-samples Wilcoxon signed ranks test were used to examine changes in mediator concentrations over the time course. Spearman's rank correlation coefficient was used to investigate associations between continuous variables.

Results

DPPI was detected in all samples of nasal lavage fluid. Levels were significantly increased compared to those at baseline by one hour following challenge with grass pollen extract ($p = 0.015$), reaching peak levels by 2 hours ($p = 0.005$; Fig 4.17). Tryptase was not measurable in nasal lavage fluid from any subjects at baseline, but was detected in half of subjects by 30 minutes ($p = 0.043$; Fig 4.18(a)). Levels declined thereafter. Histamine and albumin were detected in all samples, and were raised compared to baseline by 30 minutes after allergen challenge ($p = 0.047$ and 0.005, respectively; Fig 4.18(b) and (c)). Concentrations of both markers reduced after this time. However, histamine levels appeared to reach a second peak by four hours (non-significant). Concentrations of DPPI were closely associated with those of histamine (but not albumin or tryptase) at two hours ($p = 0.02$; Fig 4.19). DPPI levels did not correlate with any of the markers at any other time point.

Nasal area and volume, and also peak nasal inspiratory flow, were reduced by 30 minutes after allergen challenge. Measurements increased gradually after this time. In addition, visual analogue scale scores for nasal itch, congestion, sneezing and rhinorrhea were raised compared to at baseline by 30 minutes, and declined thereafter. At the same time point, DPPI levels were inversely correlated with peak nasal inspiratory flow ($p = 0.01$), and positively associated with visual analogue scores for nasal congestion ($p = 0.04$) and rhinorrhea ($p = 0.03$; Fig 4.20). This was not seen for minimal nasal cross-sectional area or volume measurements, or for visual analogue scale scores for nasal itch or sneezing.
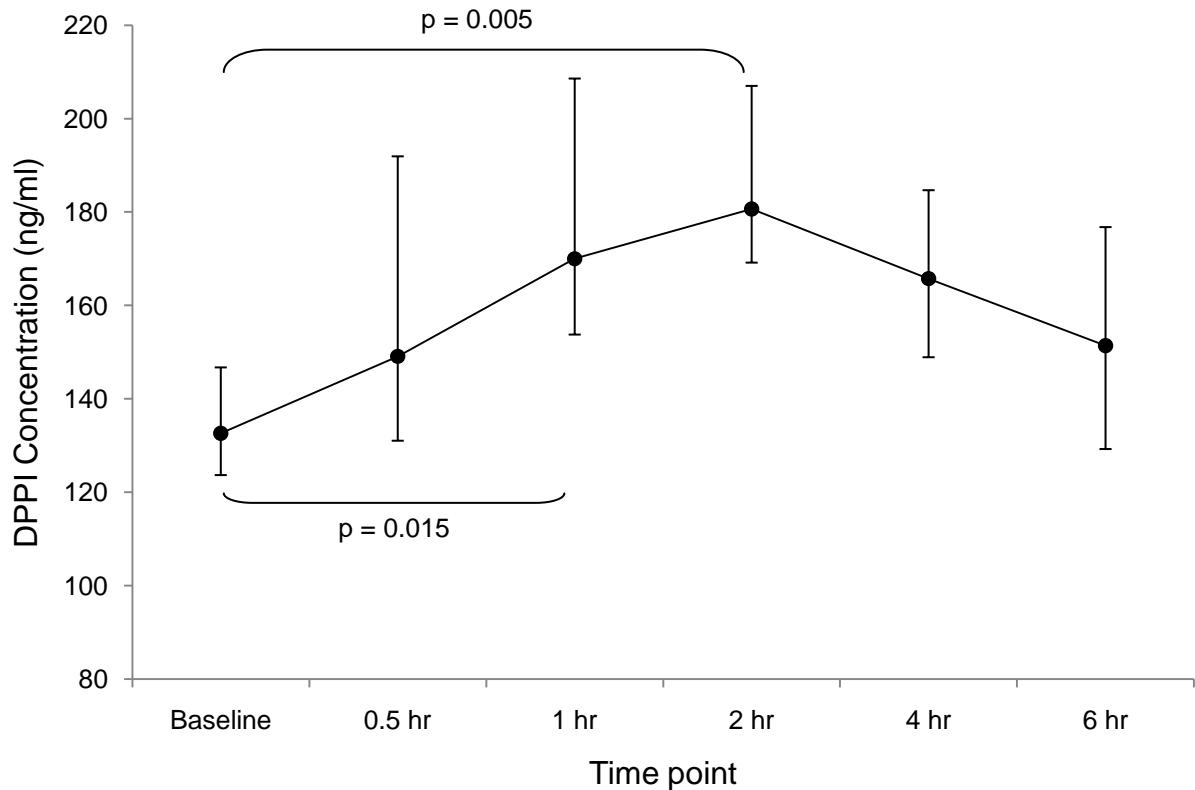
Fig 4.17 DPPI levels in nasal lavage fluid before and after grass pollen allergen challenge. Concentrations were measured by ELISA. Results were analyzed by related-samples Friedman's two-way analysis of variance by ranks, and related-samples Wilcoxon signed ranks test. Median values and inter-quartile range are shown (n = 10).
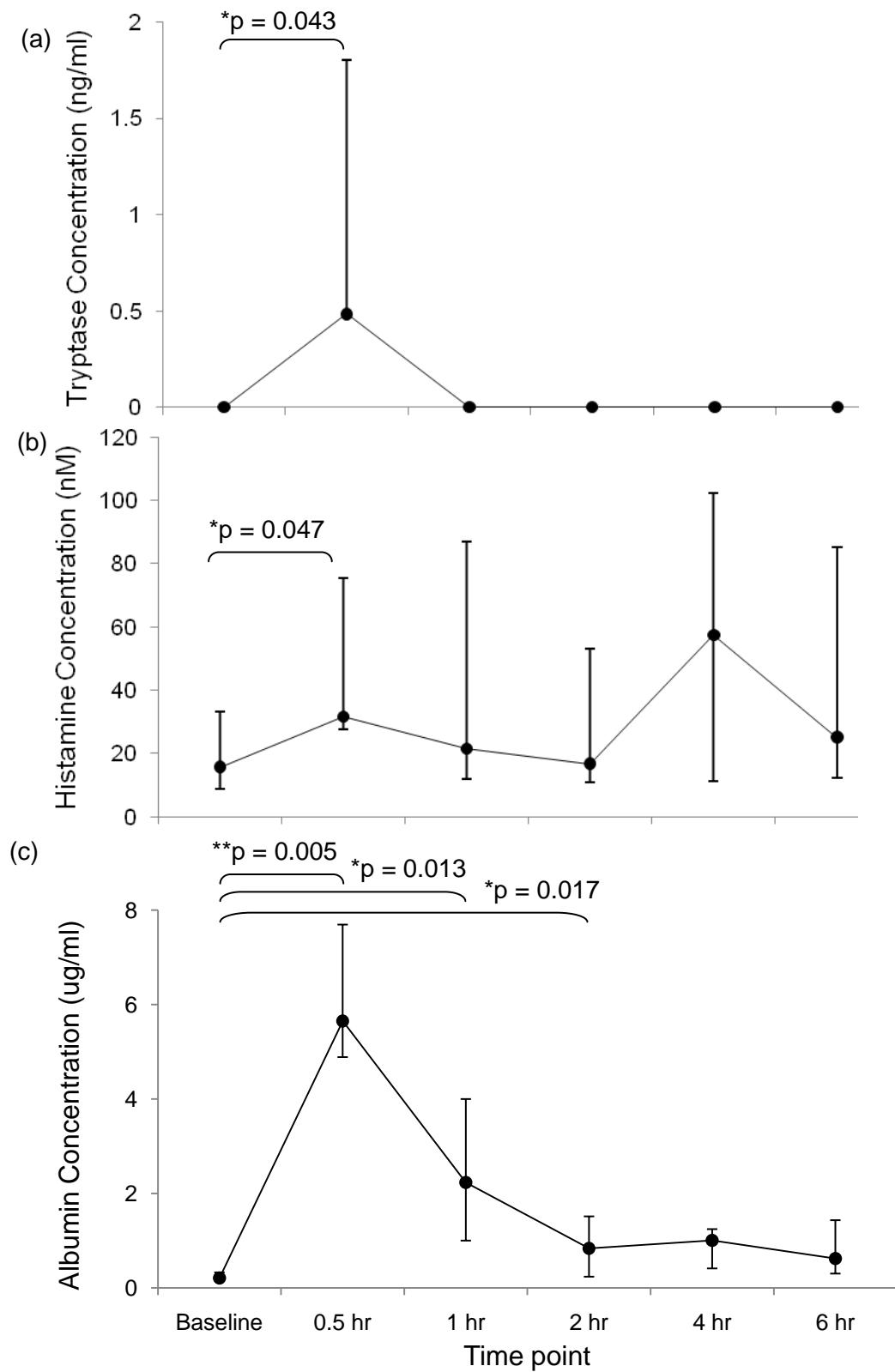
Fig 4.18 Levels of (a) tryptase, (b) histamine and (c) albumin in nasal lavage fluid before and following allergen challenge. Results were analyzed by related-samples Friedman's two-way analysis of variance by ranks, and related-samples Wilcoxon signed ranks test. Median values and inter-quartile range are shown (n = 10).
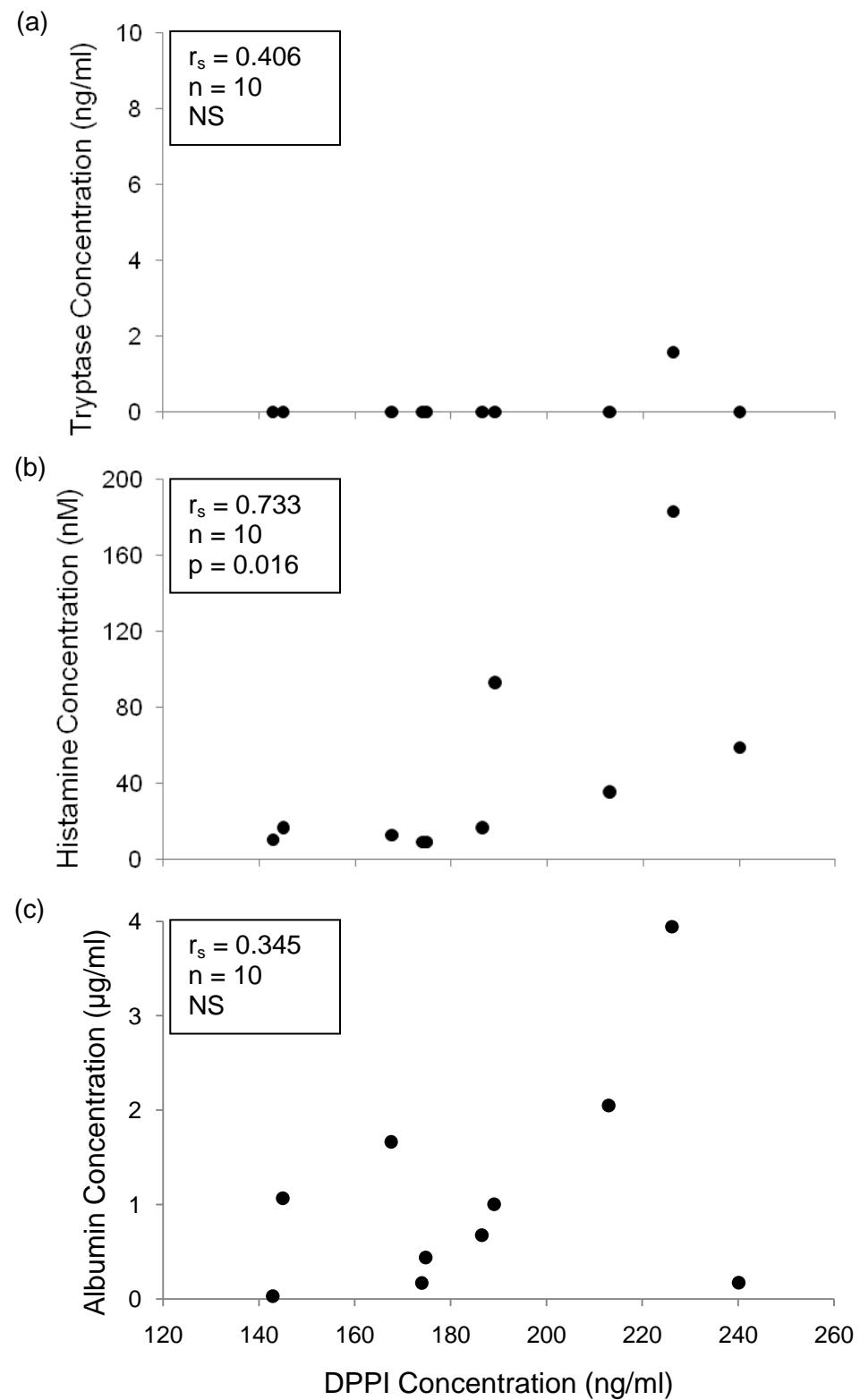
Fig 4.19 Association between DPPI and (a) tryptase, (b) histamine and (c) albumin levels in nasal lavage two hours after challenge. Spearman coefficients of rank correlation are shown. NS: non-significant.
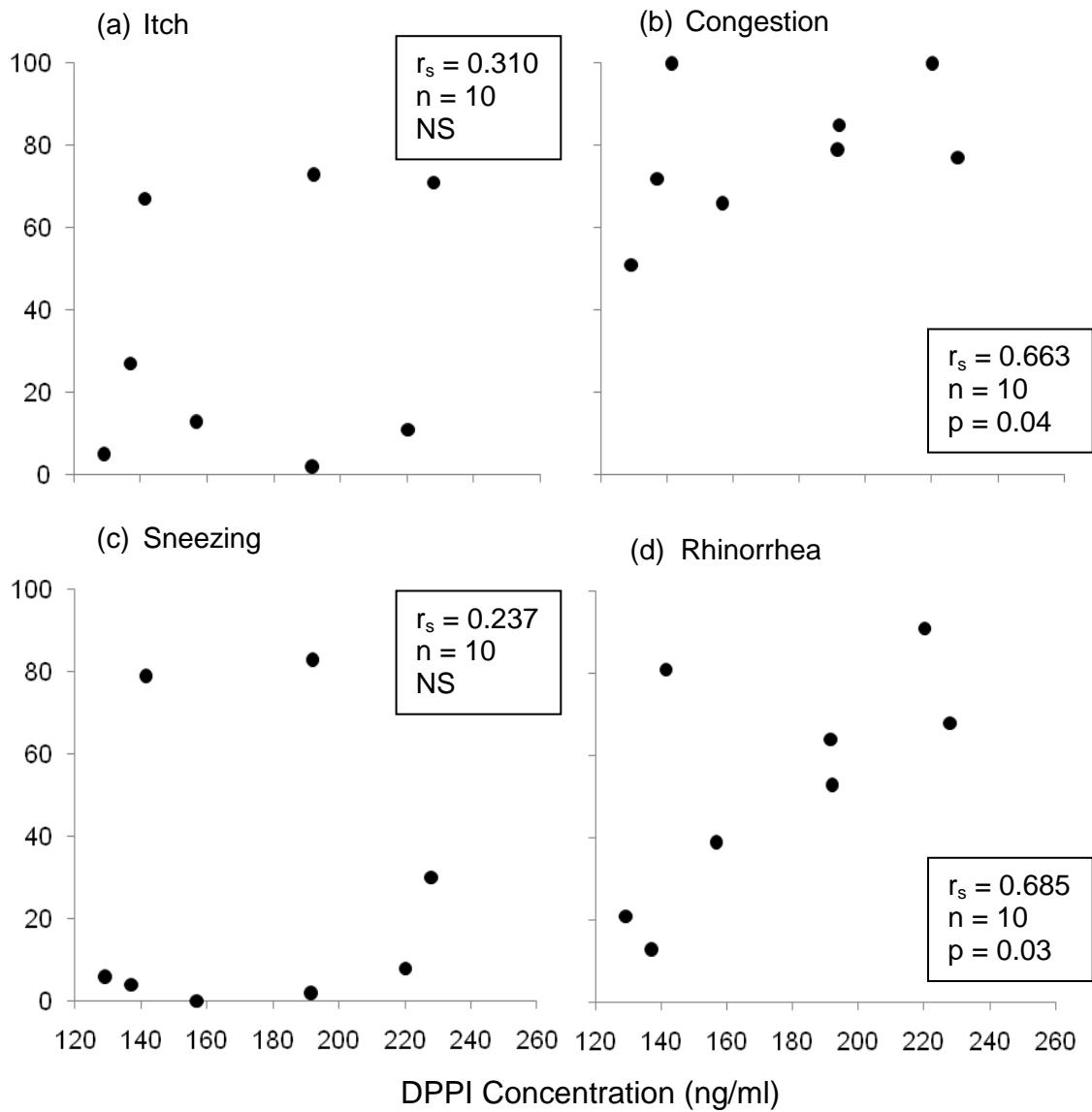
Fig 4.20 Association between DPPI levels and VAS scores for (a) nasal itch, (b) congestion, (c) sneezing and (d) rhinorrhea 30 minutes after challenge. DPPI levels were measured by ELISA. Y-axes show visual analogue scale (VAS) scores. NS: non-significant.

4.4 Discussion

Novel assay for DPPI

Using specific antibodies prepared in our laboratory, we have developed for the first time an ELISA for DPPI. The assay was shown to be suitable for use with human serum and saliva, as well as other body fluids such as nasal lavage, and has been validated for such studies. Specific protocols were successfully developed for measurement of DPPI levels in the various biological fluids.

For individual subjects there was little variation in baseline levels of DPPI in serum collected in visits up to one year apart. After collection this protease was relatively stable in an immune reactive form in serum. At least 80 % of total levels could be measured in samples stored at 22 ºC for ten days, or at 4 ºC for 15 days. Levels did not reduce significantly during this time in samples stored at -20 ºC. The ELISA technique was found to have excellent intra- and inter-assay variability.

DPPI is stored within mast cells, and there is *in vitro* evidence that it is released upon cell activation (Wolters *et al.*, 1998). Our ELISA may prove to be a valuable tool in investigating *in vivo* release of the protease in allergic conditions, and therefore perhaps in confirming an IgE-mediated hypersensitivity response.

DPPI release in seasonal allergic rhinitis

In preliminary studies we have shown that DPPI is released into nasal lavage fluid during an allergic response to grass pollen. Furthermore, levels were associated with those of histamine, and with clinical signs and symptoms.

An association of DPPI release in the upper airways with an early increase in histamine levels supports the idea that mast cells are a major cellular source of DPPI in allergic reactions. However, levels did not correlate with those of tryptase. Peak tryptase levels occurred at 30 minutes after allergen exposure and declined

rapidly thereafter; whereas DPPI concentrations reached a maximum by 2 hours. This could reflect a difference in sizes of the two proteases (Walls *et al.*, 1990; Wolters *et al.*, 1998), which may result in different rates of accumulation within nasal lavage fluid. Alternatively, tryptase and DPPI may be degraded at different rates. *In vivo* mechanisms of tryptase degradation are currently unknown. Basophils also contain and release histamine, and numbers of this cell type have been shown to be increased in the nasal mucosa of subjects with allergic rhinitis following allergen challenge with grass pollen (KleinJan *et al.*, 2000). The potential for DPPI storage in, and release from, basophils requires investigation.

In the current study, release of DPPI into the nasal cavity was associated with clinical signs and symptoms of allergic reactions to grass pollen. Levels were higher in subjects who scored symptoms worse on visual analogue scales. DPPI has previously been implicated in activation of tryptase and chymase from their pro forms (McGuire *et al.*, 1993; McEuen *et al.*, 1998). Thus perhaps severity of reactions is worse with higher levels of DPPI release due to increased activation of other mast cell proteases. In our study total tryptase levels were measured within nasal lavage fluid, and these showed no association with DPPI concentrations. However, levels of mature tryptase may be expected to increase upon release of DPPI.

Summary

We have developed a sensitive and specific ELISA for measurement of DPPI in a range of human biological fluids. Using this new technique we have shown that the protease is released into the nasal cavity alongside histamine during grass pollen-induced allergic rhinitis. Furthermore, the extent of release was associated with reaction severity. These preliminary results suggest that our new ELISA may have the potential to be of value in clinical diagnosis of allergic conditions. The findings of this proof of concept study show promise for further investigations of the use of DPPI as a marker of allergic reactions to foods.

CHAPTER 5: CONFIRMATORY MARKERS OF ALLERGIC REACTIONS

5.1 Introduction

Diagnosis of allergic reactions can be difficult. A broad range of symptoms can present and there is a lack of useful diagnostic tests (Simons, 2005). National and international guidelines recommend measurement of serum tryptase levels for confirming a reaction (Project Team of The Resuscitation Council (UK), 1999; Lieberman *et al.*, 2005). However, evidence supporting the use of this test is limited, particularly in cases of food allergy (Treudler *et al.*, 2008; Lin *et al.*, 2000; Vila *et al.*, 2001). There is a need for tests that have greater reliability in diagnosis of the condition. In this study we therefore aimed to investigate changes in levels of various potential markers in human biological fluids during allergic reactions.

Markers of interest included the mast cell proteases, tryptase, chymase, carboxypeptidase and DPPI, which are released upon cell activation (Walls *et al.*, 1990; Wintroub *et al.*, 1986; Goldstein *et al.*, 1987; Wolters *et al.*, 1998). In addition, we have studied angiotensin-converting enzyme (ACE), as low activity levels have been linked with severe reactions to peanuts and tree nuts (Summers *et al.*, 2008). The rationale for the study, and the reasons for selecting the markers of interest, is provided in detail in Chapter 1. The potential for these markers to be used in predicting susceptibility to severe reactions is addressed in Chapter 6.

5.2 Methods

The methodology for this study is described in detail in Chapter 2. In brief, levels of tryptase, chymase, carboxypeptidase, DPPI and ACE were investigated in serum and saliva samples from paediatric and adult patients with allergic reactions. These included children undergoing diagnostic food challenge, adults undergoing skin testing to drugs, and patients presenting at the accident and emergency department with anaphylaxis or allergic asthma exacerbation. Serum was also taken from healthy atopic and non-atopic volunteers.

Food challenges

Diagnostic allergen challenges were performed on children under investigation for food allergy. Serum and/or saliva levels of the markers listed above were measured before, during and after the challenge. Changes in levels of the proteases were investigated with respect to challenge outcome and severity or types of symptoms. Reactions in food challenge were scored according to the degree of allergen exposure and severity of symptoms. In addition, reactions were divided into those that could be considered subjective or objective; and also those involving isolated oral allergy or systemic allergy. Symptoms were recorded as 'subjective' if described by the patient but could not be measured or seen visually (e.g. pruritus, nausea or abdominal pain). Other reactions were divided into those with 'mild objective' symptoms such as localised urticaria or rhinitis, or 'moderate/severe objective symptoms' such as oedema, vomiting or wheeze. The term 'isolated oral allergy' encompassed reactions in which symptoms were confined to the oral mucosa. 'Systemic allergy' was used to describe reactions involving the skin or gastrointestinal, respiratory or cardiovascular systems.

Drug challenges

Changes in levels of the markers described were investigated in adults undergoing investigation for drug allergy. Drug challenges involved skin prick and intra-dermal

testing with therapeutic agents; and serum and saliva levels of the proteases were determined before and after drug testing. A positive result in skin testing was taken to indicate allergy. In some patients, symptoms were also experienced.

Anaphylaxis and allergic asthma exacerbation

Concentrations of the markers indicated were also measured in serum from adults diagnosed clinically with anaphylaxis or allergic asthma exacerbation to foods, drugs, insect venom or other allergens at the Accident and Emergency Department. The results were analysed together with those obtained from patients with anaphylaxis in allergen challenge; and were compared with those from patients who experienced no reaction or less severe reaction. Adults with either no allergic history, or a history of mild allergy to allergens excluding foods, drugs or insect venom, were used as additional controls (non-atopic and atopic, respectively).

Analysis

Statistical analysis was performed using methods appropriate to the data. The majority of data was of skewed distribution, and thus non-parametric tests were used. Comparison of continuous variables across two unpaired groups, for example serum levels of a marker in children who reacted compared to in those who did not, was carried out using Mann Whitney U Test. Analysis of continuous variables across two paired groups, such as serum levels of a marker post challenge compared to pre challenge, was carried out using Related-Samples Wilcoxon Signed Ranks Test. Where more than two groups were present, tests used for unpaired and paired data were the Kruskal-Wallis test and Friedman's Two-Way Analysis of Variance, respectively. Spearman's Rank Correlation Coefficient was employed for examining associations between two continuous variables. A p-value of less than 0.05 was considered significant.

5.3 Results

Paediatric food challenges

Ninety two children (aged 7-18 years) presenting for 124 diagnostic food challenges were recruited. The majority of challenges were to peanut, tree nuts, egg or milk, and approximately one third of children reacted. All children recruited on to the study provided saliva samples; but not all gave blood. In some cases only one blood sample was taken. The details of recruited patients and food challenges are presented in table 5.1.

Table 5.1 Patients and food challenges. Ninety two children attending for 124 diagnostic food challenges were recruited.

Age	Range: 7 – 18 years; Mean: 12 years
Gender	Male: 62; Female: 30
Number of challenges	One: 71; Two: 16; Three: 2; Four: 3
Foods challenged	Peanut: 21; Tree nuts: 50; Egg: 30; Milk: 10; Seafood: 3; Fruit/veg: 2; Seeds: 1; Other: 7
Outcome	Positive: 43; Negative: 81

Serum Markers

Serum levels of tryptase, chymase, carboxypeptidase, DPPI and ACE were measured before and after food challenge. We examined firstly differences in levels of the individual markers either at baseline or post food challenge in children with positive and negative outcomes, and secondly changes in levels over the course of the challenge. This was also investigated in children who reacted with differing severity or types of symptoms.

Tryptase

Neither baseline nor post challenge levels of tryptase were significantly different in children who reacted in food challenge compared to those who did not (Fig 5.1(a)). Furthermore, levels did not reach concentrations above normal (>12 ng/ml) in any of the patients. There was a trend for an increase in tryptase levels over the course of the food challenge in subjects who reacted with systemic symptoms; but this did not reach significance (p = 0.07). No such trend was seen for subjects with no reaction or isolated oral reactions. No differences were seen between children with objective or subjective symptoms, and levels were not associated with specific symptoms or reaction severity (Fig 5.2(a)).

Chymase

Serum chymase levels were similar in children with positive and negative challenges at all time points; and concentrations did not change over the course of the challenge in either group (Fig 5.1(b)). There were no differences between children with reactions of differing severity or types of symptoms (Fig 5.2(b)).

Carboxypeptidase

Serum levels of carboxypeptidase were significantly higher both at baseline (p = 0.019) and post challenge (p = 0.049) in patients who reacted than in those who did not (Fig 5.3(a)). Baseline (but not post challenge) carboxypeptidase concentrations were associated with severity of reactions in children who had systemic symptoms in food challenge (p = 0.003). This is discussed further in Chapter 6. Levels of serum carboxypeptidase did not change over the course of the challenge in children with either positive or negative challenges. No differences in post challenge carboxypeptidase concentrations were seen between those who reacted with differing severity or types of symptoms (Fig 5.4(a)).
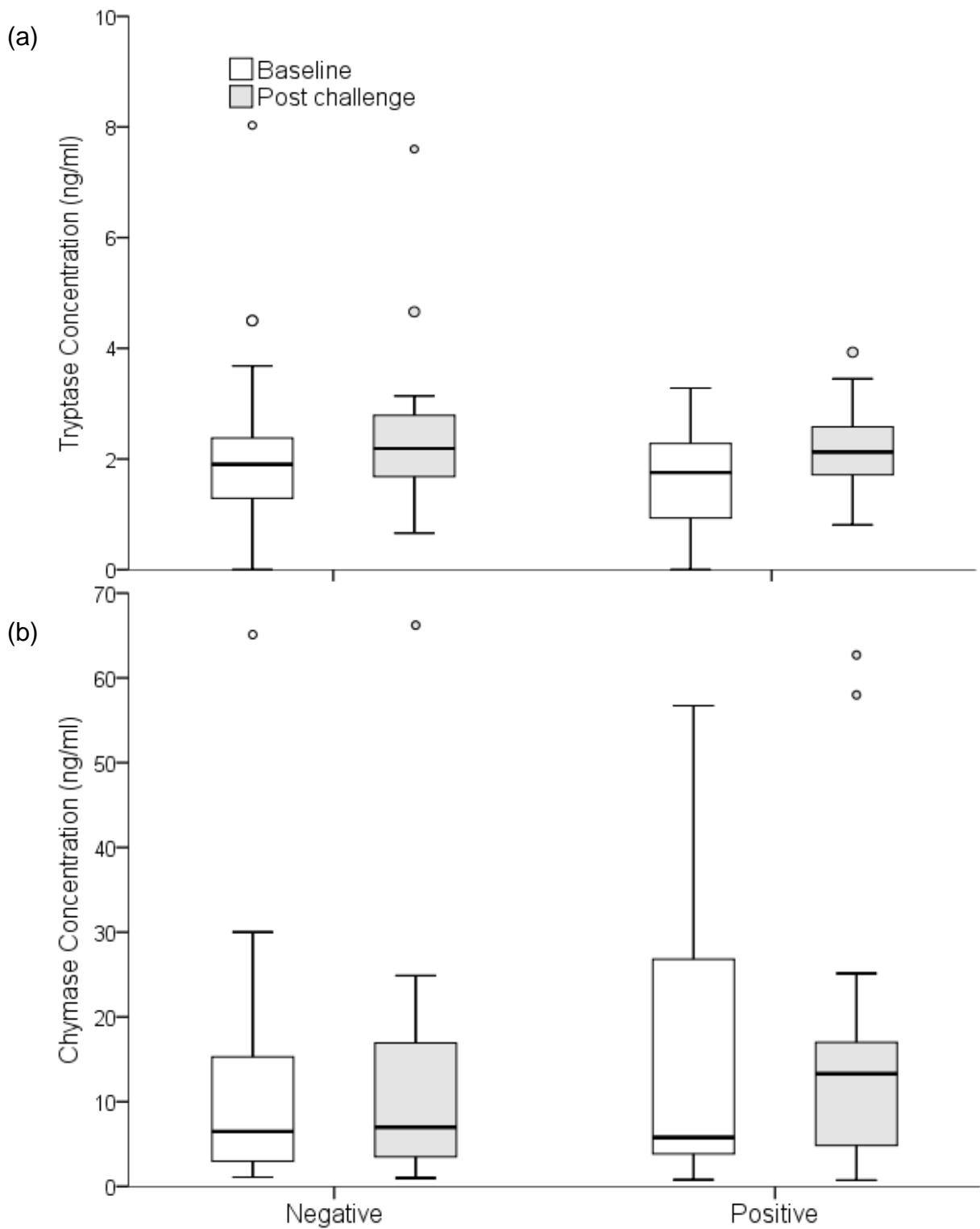
Fig 5.1 Baseline and post challenge levels of serum (a) tryptase and (b) chymase in positive and negative challenges. Tryptase and chymase levels were measured by ELISA in serum collected pre and post food challenge. Results are shown for positive (n = 22) and negative (n = 40) outcomes. The middle bar indicates the median, and the box and whiskers show the inter-quartile range and extremities of the data, respectively. Circles outside of this range represent outliers. Statistical analysis was performed using Mann-Whitney U Test.

(a)

$r_s = -0.294$
$n = 20$
$p = 0.209$

Tryptase Concentration (ng/ml)

(b)

$r_s = 0.118$
$n = 17$
$p = 0.652$

Chymase Concentration (ng/ml)

Reaction Severity Score
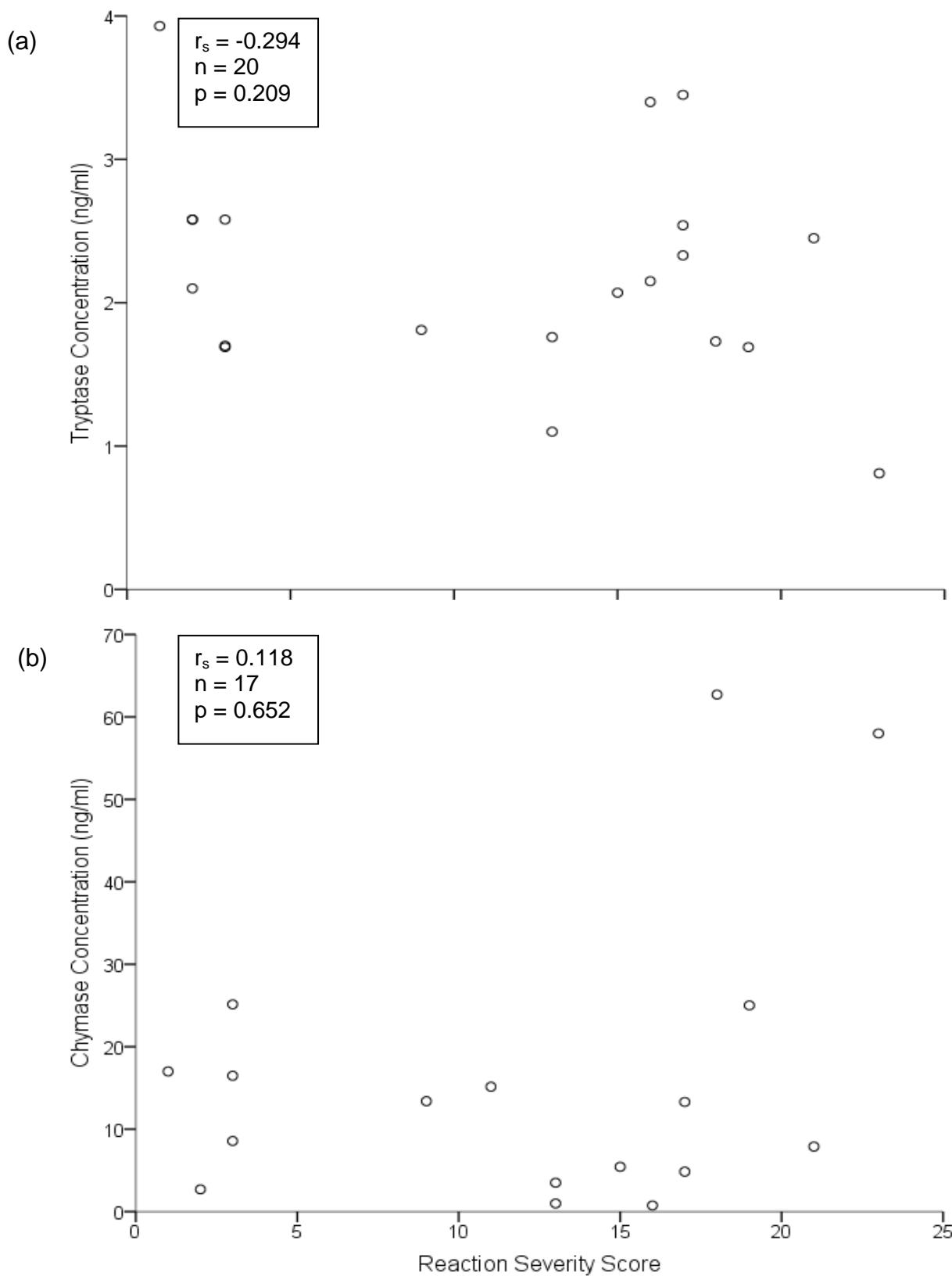
Fig 5.2 Post food challenge levels of serum (a) tryptase and (b) chymase and reaction severity score. Concentrations of tryptase and chymase after challenge were investigated with respect to reaction severity. Results are shown for subjects who reacted only. Statistical analysis was performed using Spearman's Rank Correlation Coefficient.
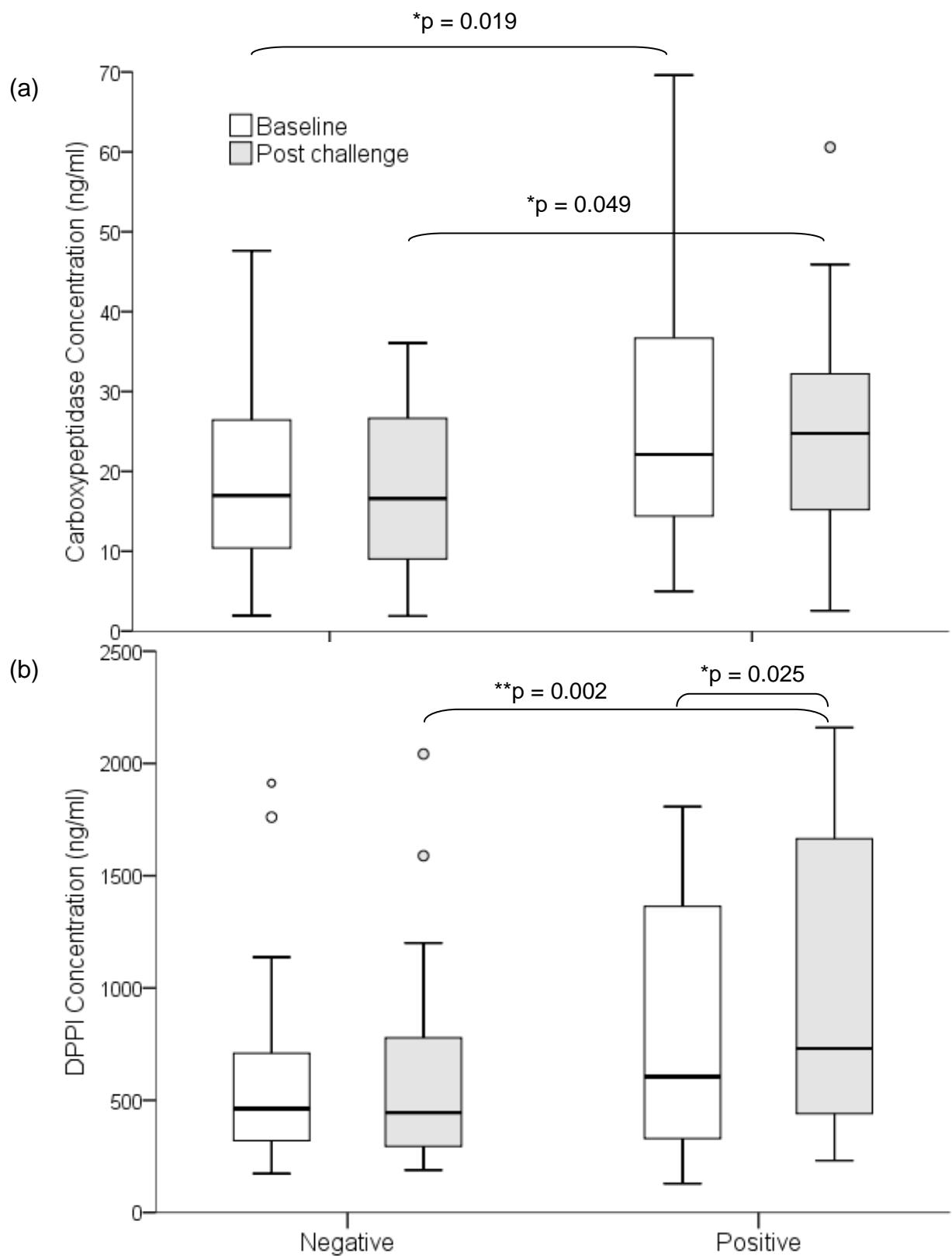
Fig 5.3 Baseline and post challenge levels of serum (a) carboxypeptidase and (b) DPPI in positive and negative challenges. Carboxypeptidase and DPPI levels were measured by ELISA in serum collected pre and post food challenge. Results are shown for positive (n = 22) and negative (n = 40) outcomes. Statistical analysis was performed using Mann-Whitney U Test.
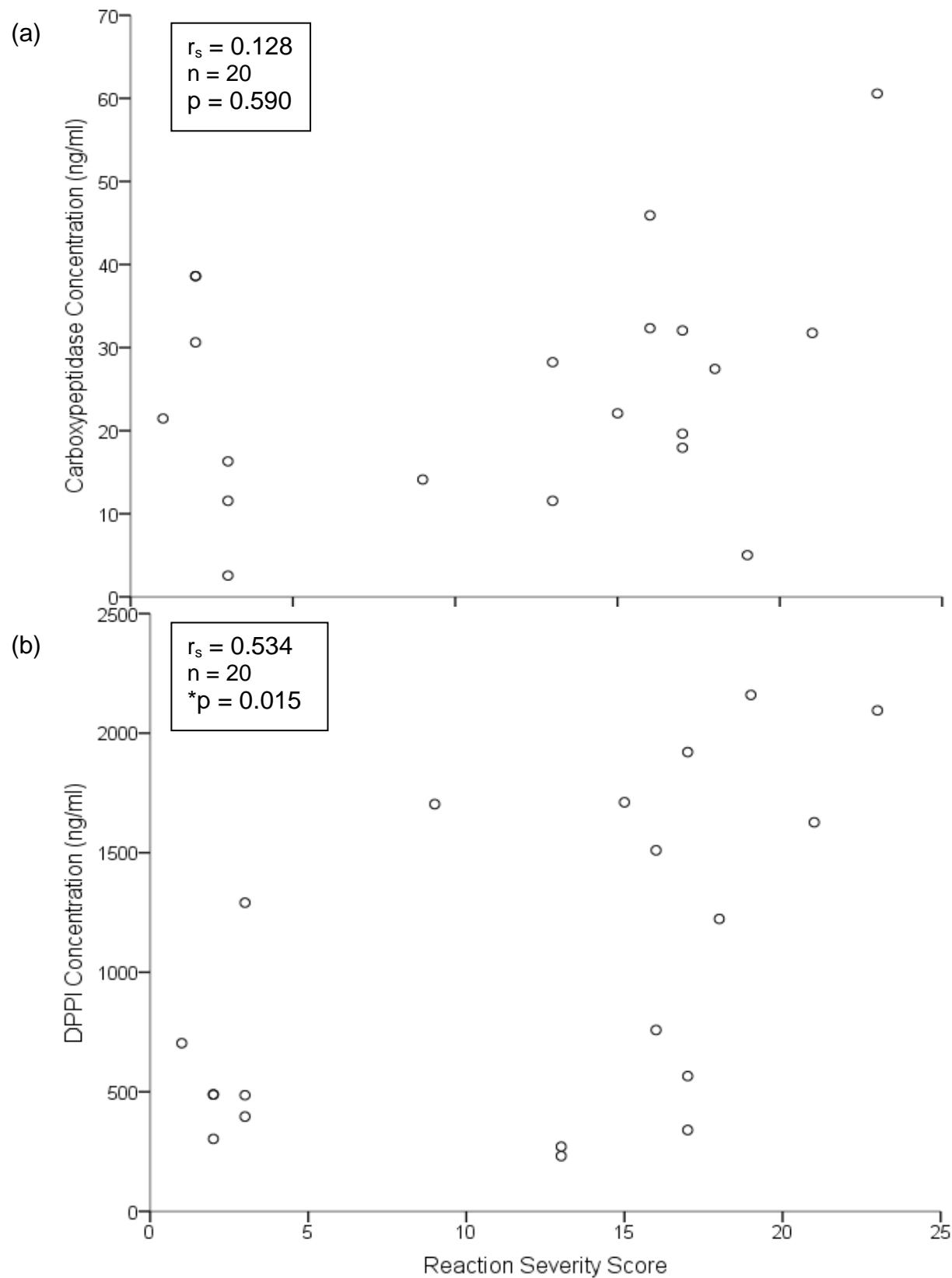
Fig 5.4 Post food challenge levels of serum (a) carboxypeptidase and (b) DPPI and reaction severity score. Concentrations of tryptase and chymase after challenge were investigated with respect to reaction severity. Statistical analysis was performed using Spearman's Rank Correlation Coefficient.

DPPI

There was a trend for higher baseline serum DPPI levels in children with positive than negative challenges (non-significant), and post challenge concentrations were significantly higher in children who reacted (p = 0.025; Fig 5.3(b)). More specifically, post challenge levels were higher in patients with moderate/severe systemic symptoms than in those with no reaction (p = 0.017), oral allergy (p = 0.013) or mild systemic allergy (p = 0.05; Fig 5.5). Post challenge DPPI concentrations correlated with severity scores in children with positive food challenges (0.015, r_s = 0.534, n = 20; Fig 5.4(b)). The same effect was seen for baseline levels (p = 0.022 r_s = 0.487, n = 22). Serum levels of DPPI were higher pre (p = 0.01) and post challenge (p = 0.037; Fig 5.6(d)) in children with throat tightness than in those without. Baseline levels are discussed further in Chapter 6.

There was a significant increase in DPPI levels over the course of the challenge in children who reacted (p = 0.022), but not in those who did not. The increase occurred specifically in patients with objective symptoms (p = 0.007), and in particular in those with moderate/severe objective symptoms (p = 0.004; Fig 5.7). Levels did not increase in subjects with subjective symptoms. Similarly, DPPI concentrations increased over the course of the challenge in patients with systemic symptoms (p = 0.017) but not in those with isolated oral allergy.

The cut-off value of DPPI concentration post challenge for confirming a reaction to food with the greatest sensitivity and specificity was determined using receiver operator characteristic (ROC) curve analysis. Using a cut-off of 1210 ng/ml, the specificity was 94%, but the sensitivity only 45 % (Fig 5.8(a)). However, this gave a higher positive likelihood ratio (7.6), and the negative likelihood ratio remained low (0.58). The same cut-off value gave a specificity and sensitivity of 93 % and 62 %, respectively, for confirming a moderate or severe objective reaction (Fig 5.8(b)). The resulting positive likelihood ratio was 8.4, and the negative likelihood ratio only 0.4.
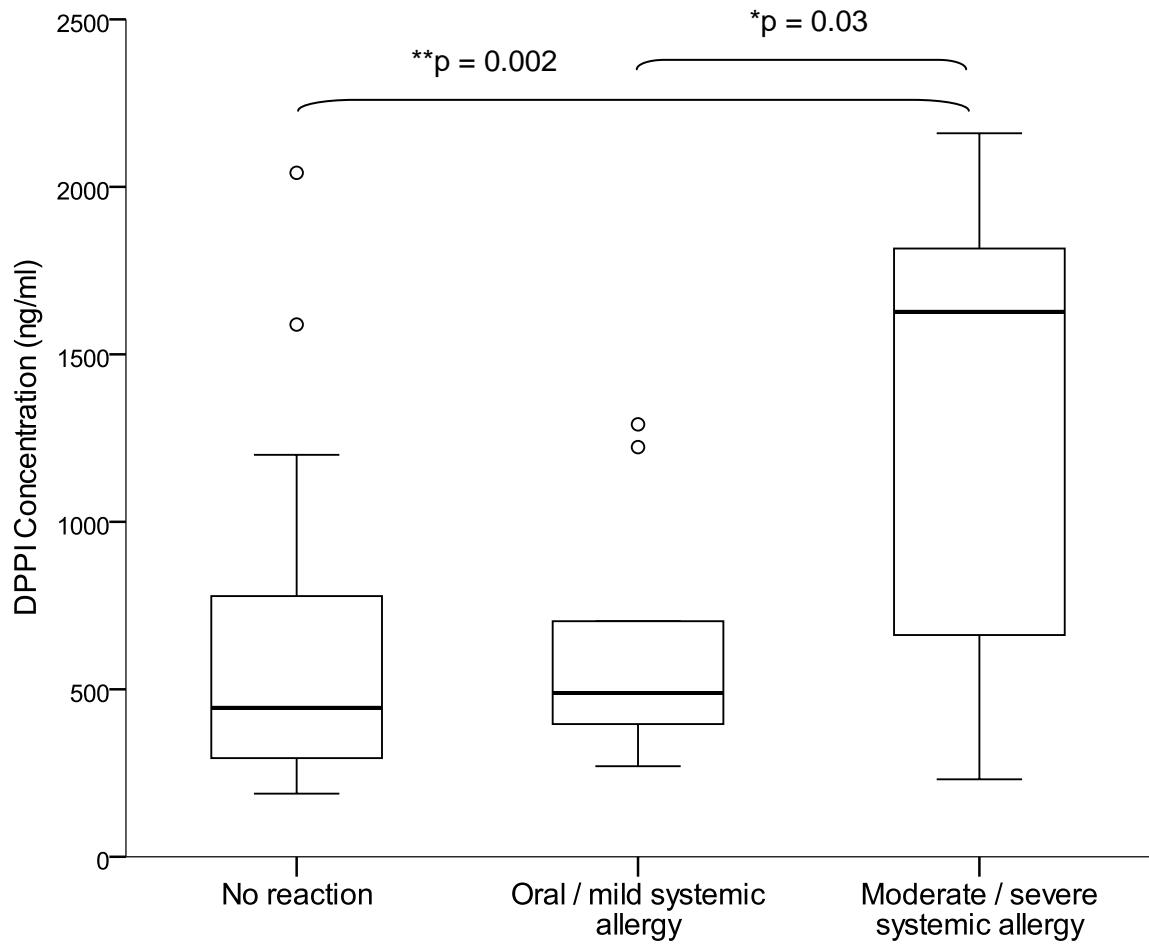
Fig 5.5 Serum DPPI levels post challenge and type of reaction. DPPI levels were compared in serum collected post challenge in children who had no reaction (n = 34), oral/ mild systemic reactions (n = 9), or moderate/severe systemic reactions n = 11). Statistical analysis was performed using Independent-Samples Kruskal-Wallis Test and Mann-Whitney U Test.
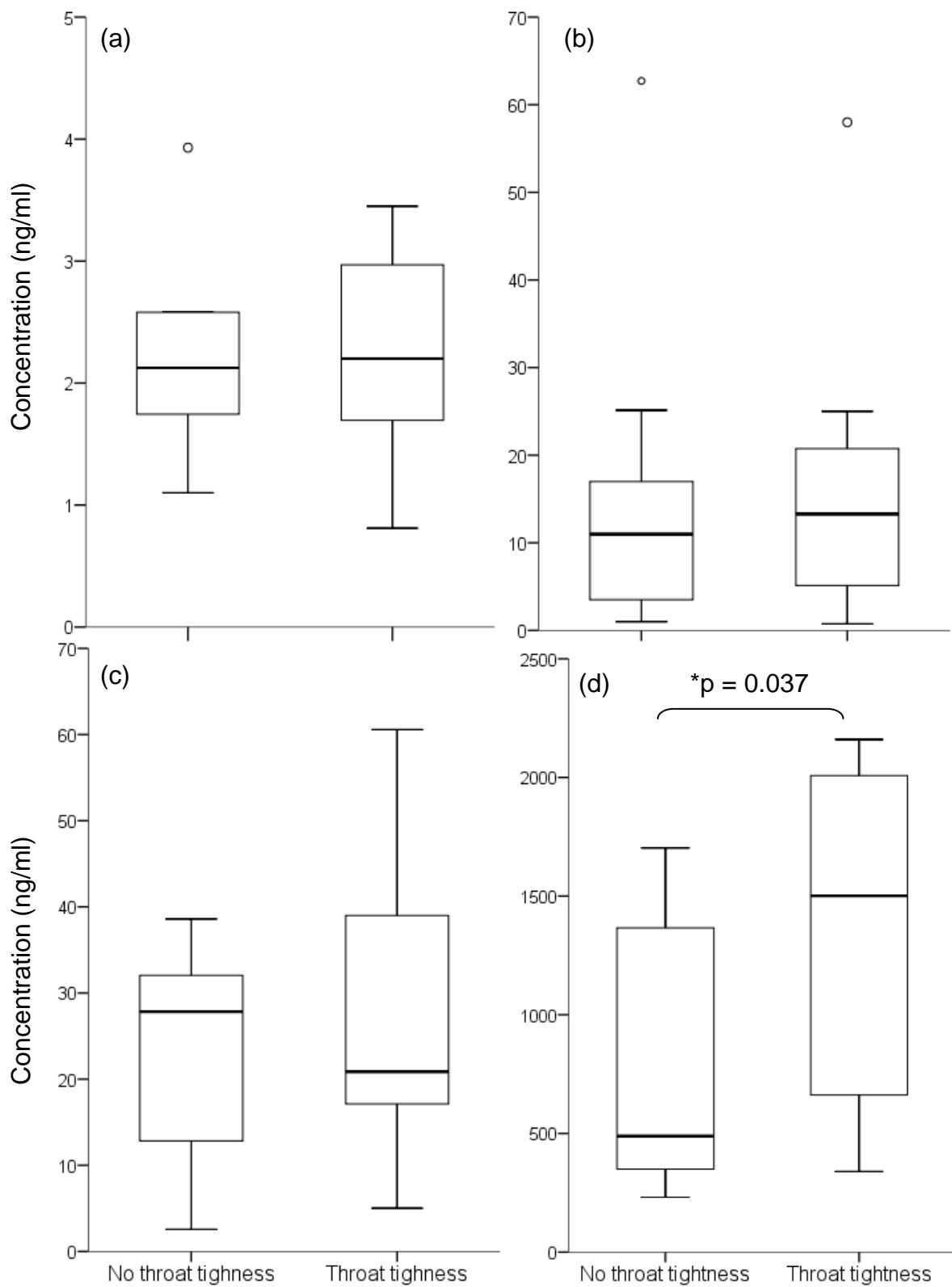
Fig 5.6 Post challenge serum levels of (a) tryptase, (b) chymase, (c) carboxypeptidase and (d) DPPI in children with and without throat tightness. Concentrations of the markers were measured by ELISA in serum taken after challenge in subjects who did (n = 8) and did not (n = 12) experience the symptom in food challenge. Statistical analysis was performed using Mann Whitney U Test.
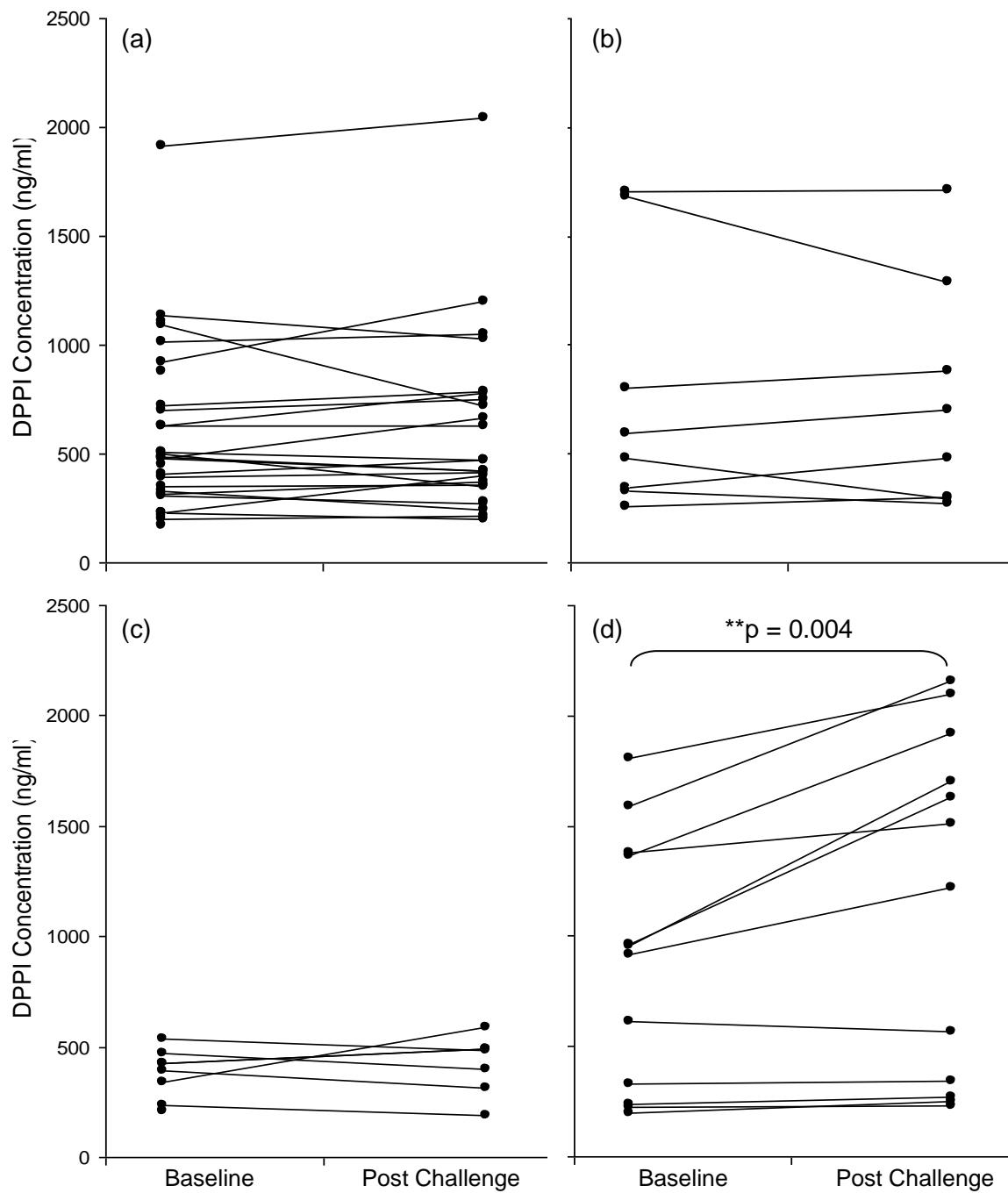
Fig 5.7 Change in serum DPPI levels during food challenge in patients with (a) no reaction, and (b) subjective, (c) mild objective or (d) moderate/severe objective symptoms. DPPI levels were compared pre and post challenge. Statistical analysis was performed using Related-Samples Wilcoxon Signed Ranks Test.
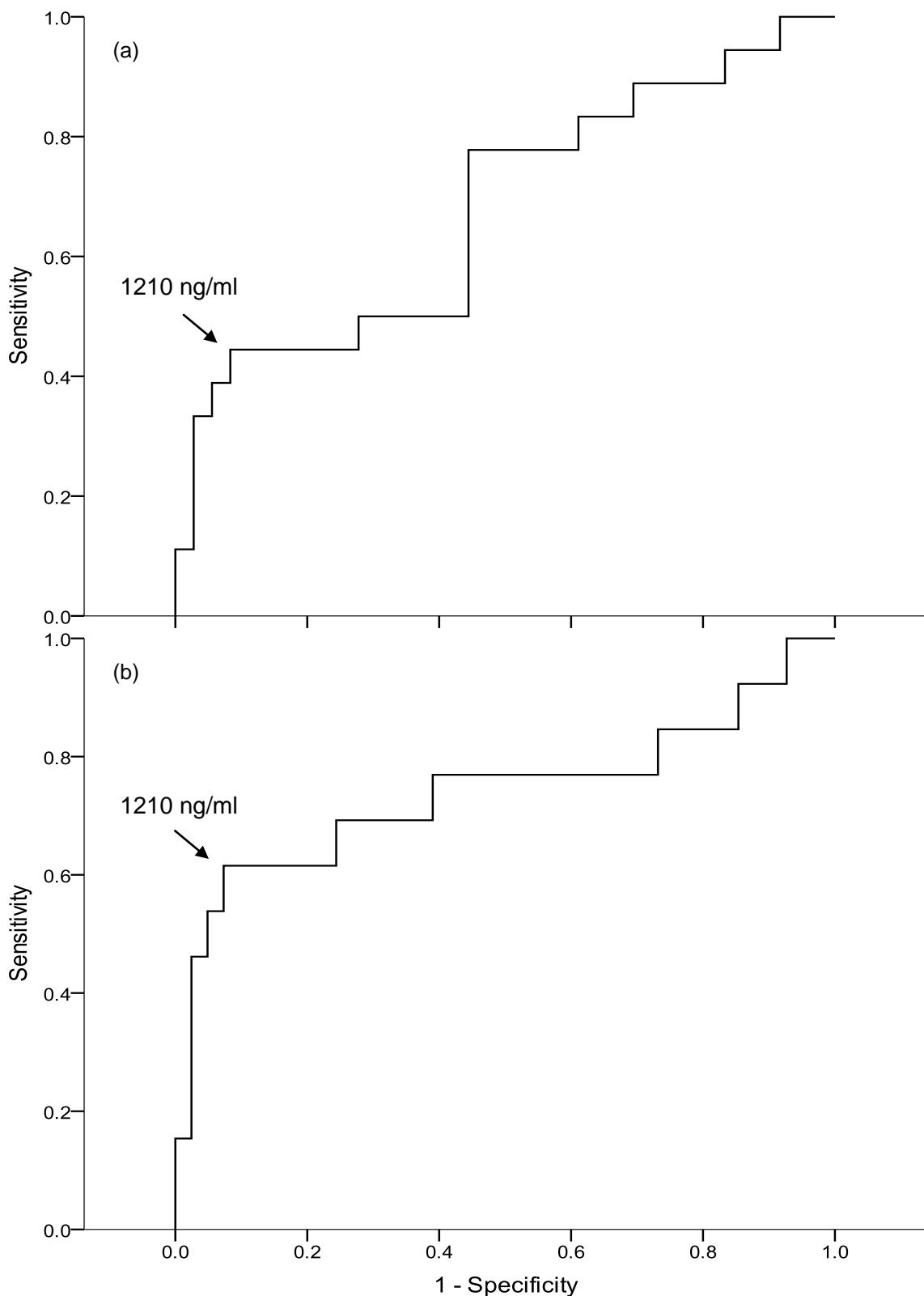
Fig 5.8 ROC curve analysis of DPPI assay for confirming (a) all reactions to food and (b) moderate/severe objective reactions to food. The concentration yielding the greatest sensitivity and specificity was determined.

ACE

Serum levels of ACE were not significantly different in children with positive or negative challenges at either time point; and concentrations did not change over the course of the challenge (Fig 5.9). Concentrations did not differ in children who had reactions of differing severity (Fig 5.10). However, baseline levels of serum ACE were significantly higher in patients who experienced oral oedema in food challenge than those who did not (p = 0.024). Associations between baseline levels of markers and specific symptoms are discussed further in Chapter 6.

Saliva markers

Saliva levels of tryptase, carboxypeptidase and DPPI were measured before challenge (baseline), fifteen and thirty minutes after initial allergen exposure, and immediately and two hours after challenge. Levels of the markers were compared in children with positive and negative challenges at each time point. In addition, changes in concentrations over the course of the challenge were investigated. Children with reactions of differing severity or symptom type were compared.

Tryptase

Tryptase concentrations in saliva were mostly below the lower limit of detection of the assay. Levels did not differ between children with positive or negative challenges at any time point. Levels were also not significantly different in subjects with reactions of differing severity or types of symptoms. Saliva tryptase levels did not significantly change over the course of the challenge in children with positive or negative challenges. However, concentrations were increased 15 minutes after initial allergen exposure compared to at baseline in children who specifically experienced isolated oral allergy (p = 0.05). There was also a trend for increased levels immediately after challenge compared to at baseline. However, this did not reach statistical significance (p = 0.071). These differences were not seen in children who experienced no symptoms or systemic symptoms in food challenge.
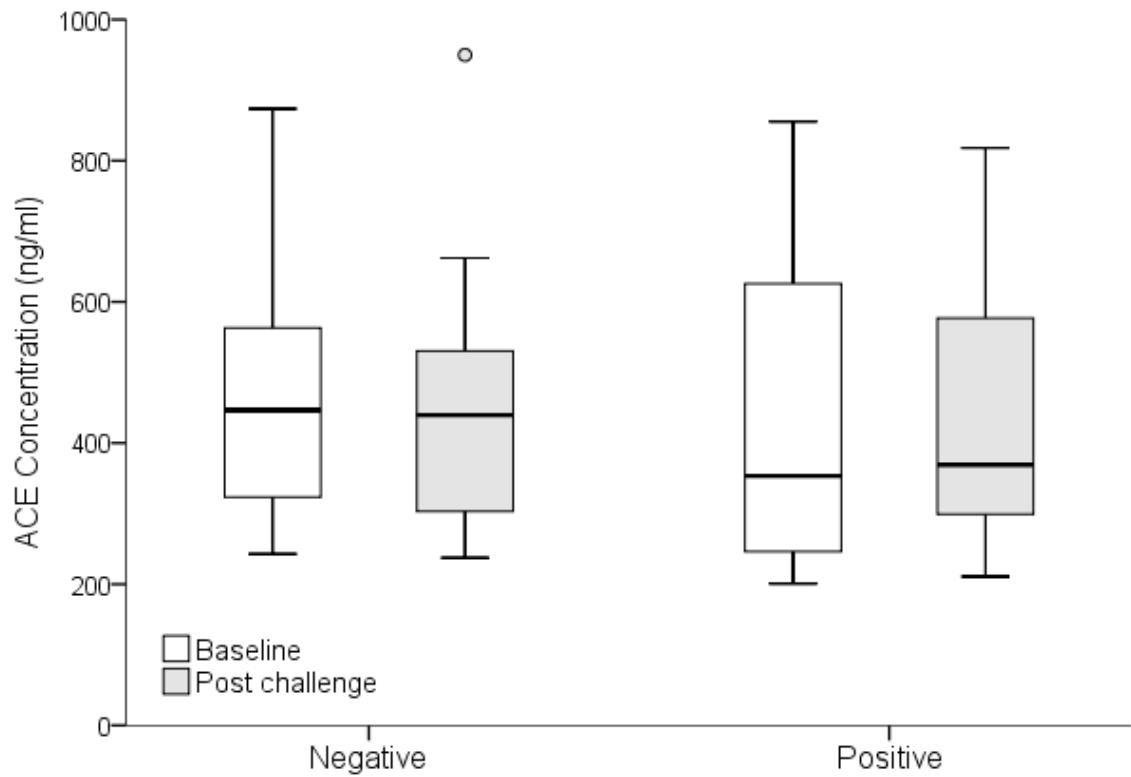
**Fig 5.9 Baseline and post challenge levels of serum ACE in positive and negative
challenges.** ACE levels were measured by ELISA in serum collected pre and post food
challenge. Results are shown for positive (n = 22) and negative (n = 40) outcomes.
Statistical analysis was performed using Mann-Whitney U Test.
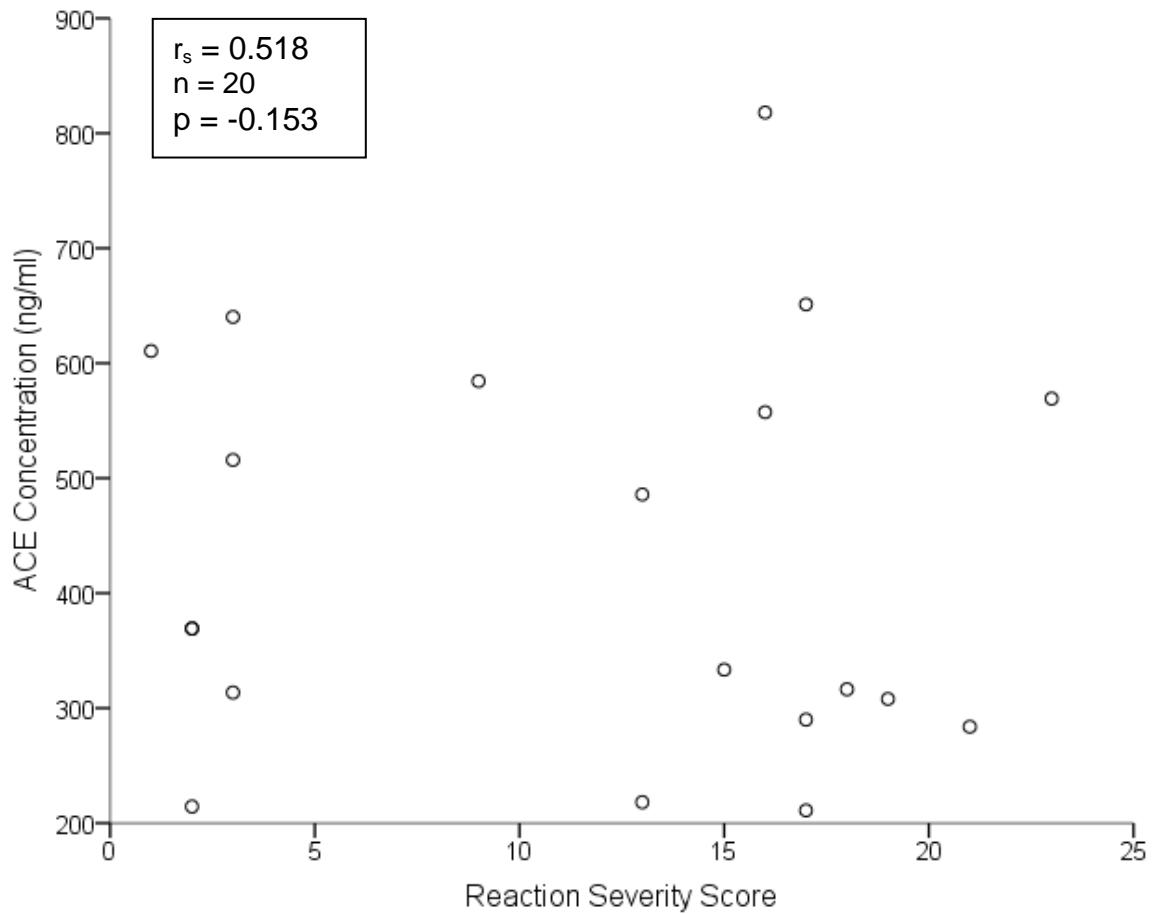
**Fig 5.10 Post food challenge levels of serum ACE and reaction severity score.**
Concentrations of tryptase and chymase after challenge were investigated with respect to reaction severity. Statistical analysis was performed using Spearman's Rank Correlation Coefficient.

Levels of salivary tryptase at baseline were significantly higher in children who reported oral pruritus in than in those who did not (p = 0.007); and levels two hours after challenge were higher in those who experienced throat tightness (p = 0.04; Fig 5.11). Most subjects without these symptoms had saliva tryptase levels below the lower detection limit of the assay. Associations between baseline levels of saliva markers and specific symptoms are discussed further in Chapter 6.

Carboxypeptidase

As for tryptase, salivary carboxypeptidase concentrations were frequently below the lower limit of detection of the assay. No differences in levels were observed between children who did and did not react to food challenge at any of the time points. Furthermore, carboxypeptidase levels did not change over the course of the challenge in either group. No differences were seen between subjects with reactions of differing severity or types of symptoms. Unlike saliva tryptase and DPPI, carboxypeptidase concentrations were not associated with throat tightness (Fig 5.12)

DPPI

DPPI was detectable in most saliva samples, but levels did not significantly differ in saliva from children with positive or negative food challenges at any of the time points. In addition, no significant change in concentrations was observed over the course of the challenge in those who did or did not react. No differences were seen between subjects who reacted with differing severity. However, saliva levels were higher by 15 minutes after initial allergen exposure in children who experienced throat tightness than those who did not (p = 0.014; Fig 5.13(a)). Concentrations were also higher at 30 minutes after initial allergen exposure (p = 0.027; Fig 5.13(b)), and there was a trend for a similar pattern at two hours after challenge (p = 0.062; Fig 5.13(d)).
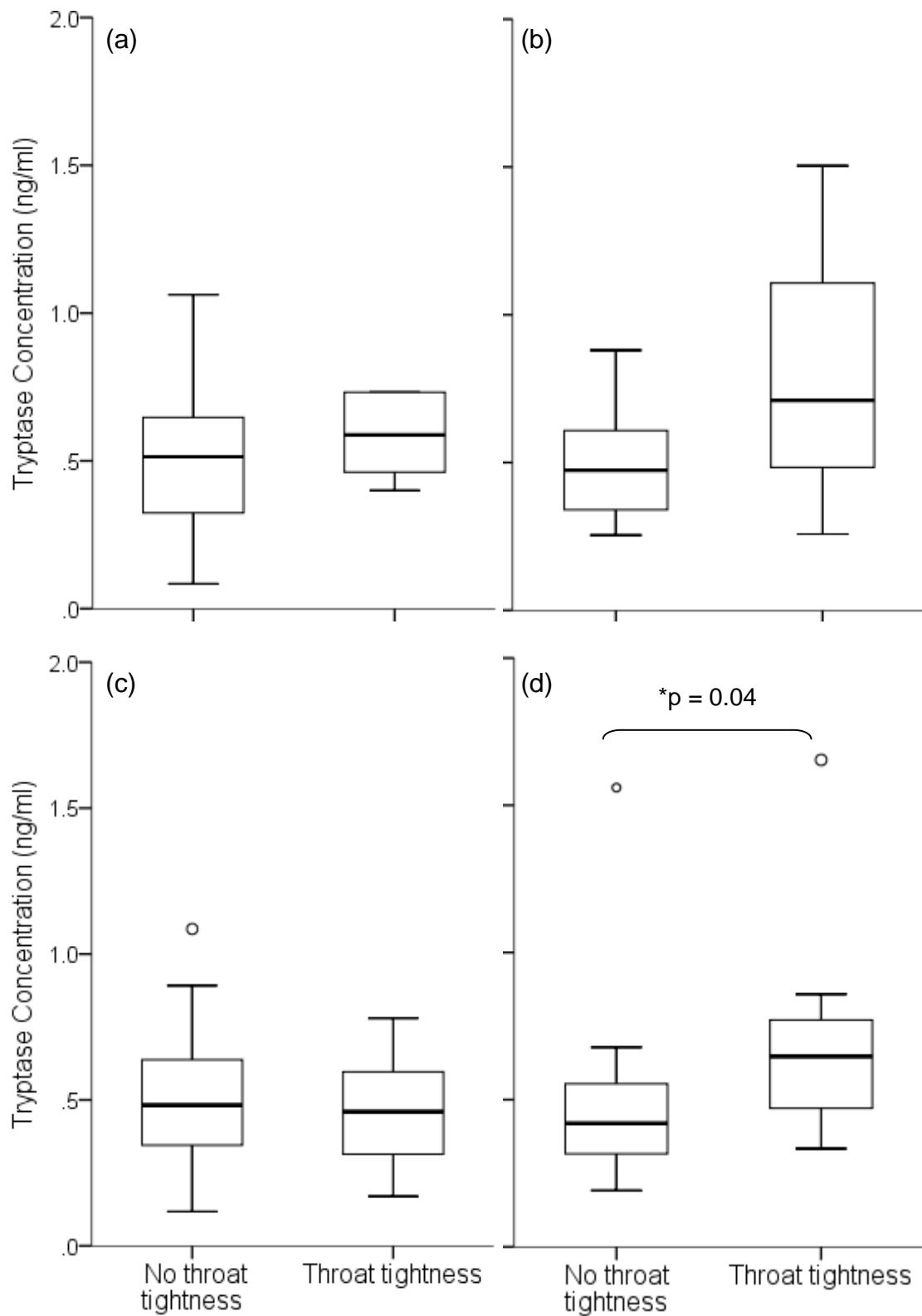
Fig 5.11 Saliva concentrations of tryptase at (a) 15 and (b) 30 minutes after initial allergen exposure, and (c) immediately and (d) 2 hours after food challenge in children with and without throat tightness. Levels were measured by ELISA. Eleven children experienced the symptoms and 32 did not. Analysis was performed using Mann Whitney U Test.
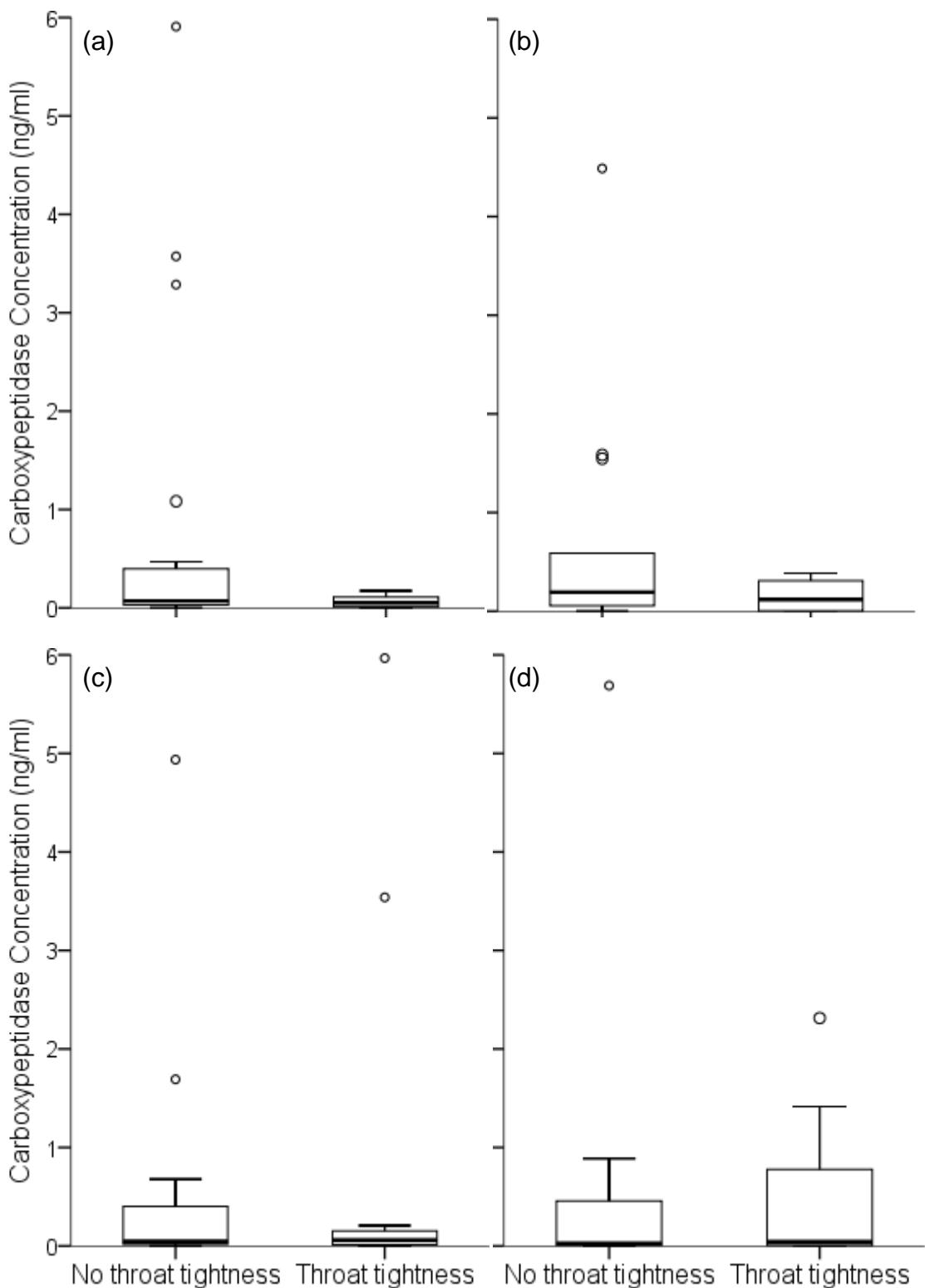
Fig 5.12 Saliva concentrations of carboxypeptidase at (a) 15 and (b) 30 minutes after initial allergen exposure, and (c) immediately and (d) 2 hours after food challenge in children with and without throat tightness. Levels were measured by ELISA. Eleven children experienced the symptoms and 32 did not. Analysis was performed using Mann Whitney U Test.
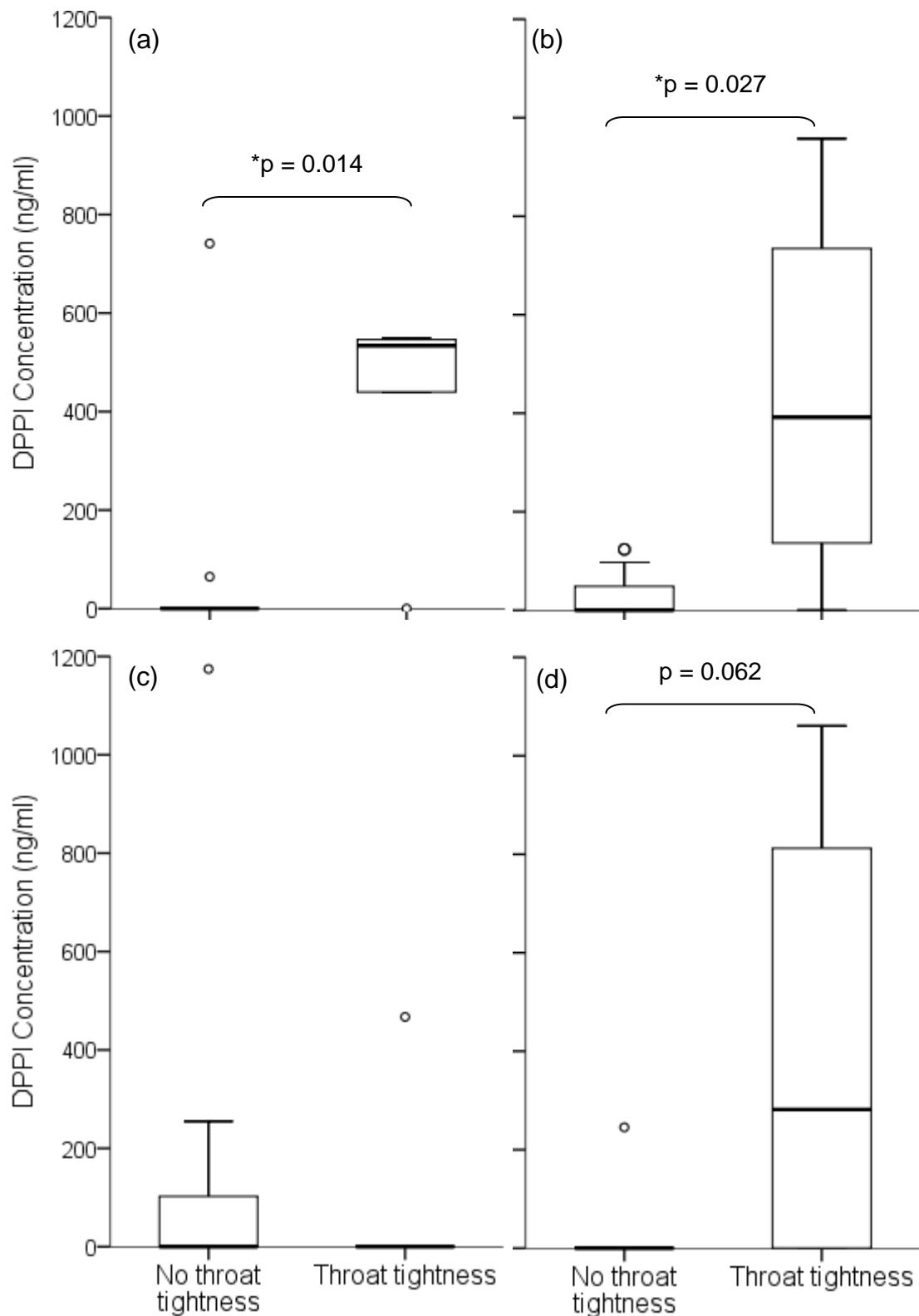
Fig 5.13 Saliva concentrations of DPPI at (a) 15 and (b) 30 minutes after initial allergen exposure, and (c) immediately and (d) 2 hours after food challenge in children with and without throat tightness. Levels were measured by ELISA. Eleven children experienced the symptoms and 32 did not. Analysis was performed using Mann Whitney U Test.

Correlations between markers

Serum tryptase or ACE levels did not correlate with those of any other marker at any time point. Serum chymase and DPPI levels were associated both at baseline ($p < 0.001$, $r_s = 0.566$, $n = 42$) and post challenge ($p = 0.012$, $r_s = 0.362$, $n = 47$). Similarly carboxypeptidase and DPPI concentrations within serum were correlated before ($p = 0.006$, $r_s = 0.345$, $n = 64$) and after challenge ($p = 0.004$, $r_s = 0.39$, $n = 54$). The level of significance was greatest between baseline serum levels of carboxypeptidase and post challenge levels of DPPI ($p < 0.001$, $r_s = 0.49$, $n = 51$).

Saliva tryptase levels were correlated with those of DPPI, but not any other marker, by 30 minutes after initial allergen exposure ($p = 0.004$, $r_s = 0.513$, $n = 30$). This association was strongest when looking only at subjects who reacted in food challenge ($p < 0.001$, $r_s = 0.838$, $n = 13$). Levels of tryptase and DPPI in saliva were also correlated two hours after challenge ($p = 0.037$, $r_s = 0.664$, $n = 10$). Saliva concentrations of carboxypeptidase were not associated with any other marker at any time point.

Adult drug challenges

Thirty two patients undergoing allergen testing to drugs were recruited. Subjects were challenged to between one and five different therapeutic agents in 36 challenges. Drugs most commonly tested were antibiotics and non-opiate analgesics. As multiple drugs were tested at once, challenges in which responses were seen to at least one agent were considered positive. In addition to a positive result in skin testing, some patients also experienced symptoms such as pruritus or angioedema. Details of patients and drug challenges are provided in table 5.2.

Table 5.2 Patients and drug challenges. Thirty two adults attended for diagnostic drug challenge were recruited. NMBA: neuromuscular blocking agent.

Age	Range: 17 – 82 years; Mean: 50 years
Gender	Male: 6; Female: 26
Number of visits	One: 29; Two: 4
Number of drugs tested	One: 5; Two: 11; Three: 8; Four: 5; Five: 3
Drugs challenged	NMBA: 8; Hypnotic: 9; Opiate: 9; Antibiotic: 23; Non-opiate analgesic: 18; Antiseptic: 9; Other: 10
Outcome of skin testing	Positive: 15; Negative: 21
Additional symptoms	Present: 9; Not present: 6

Serum and saliva markers

Serum levels of tryptase, chymase, carboxypeptidase, DPPI and ACE, and salivary tryptase, carboxypeptidase and DPPI concentrations, were measured pre and post drug challenge. Levels at each of the time points were compared in patients with positive and negative outcomes. In addition, changes in levels over the course of the procedure were studied. Results were compared in patients with positive skin tests who did and did not experience symptoms. As not all allergic patients experienced symptoms in challenge, if was not possible to study in depth the relationship between markers and specific symptoms in this setting.

Tryptase

Neither serum nor salivary levels of tryptase differed in patients who reacted or did not react in drug testing at baseline or after challenge (Fig 5.14). No changes in serum or saliva levels were observed in either group. In addition, no differences were seen between patients who did and did not experience symptoms in addition to testing positive in skin test (Fig 15(a) and Fig 16(a), respectively).

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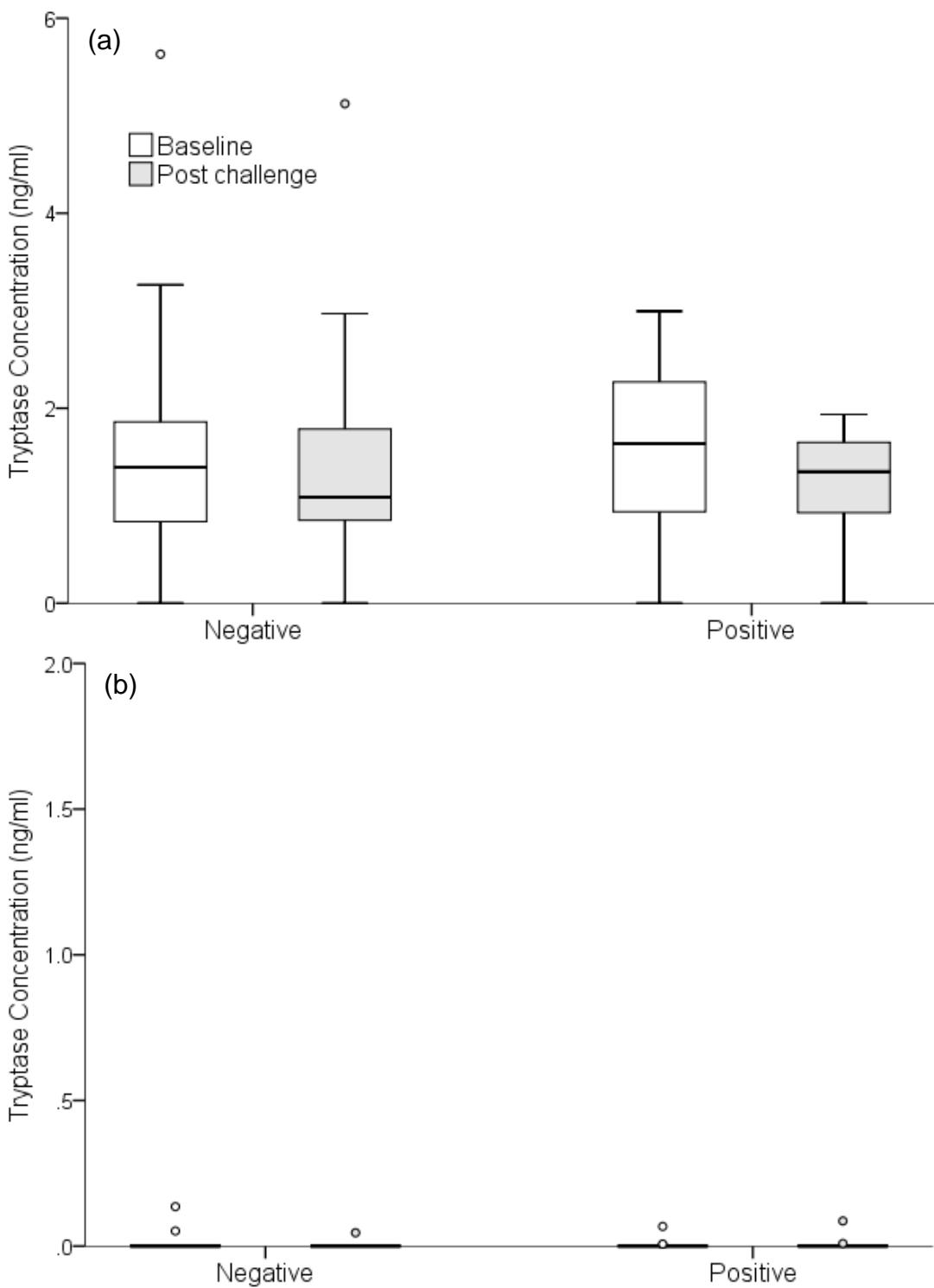
Fig 5.14 Baseline and post challenge levels of (a) serum and (b) saliva tryptase in positive and negative challenges. Levels were measured by ELISA in serum collected pre and post food challenge. Results are shown for positive (n = 15) and negative (n = 21) outcomes. Statistical analysis was performed using Mann-Whitney U Test.
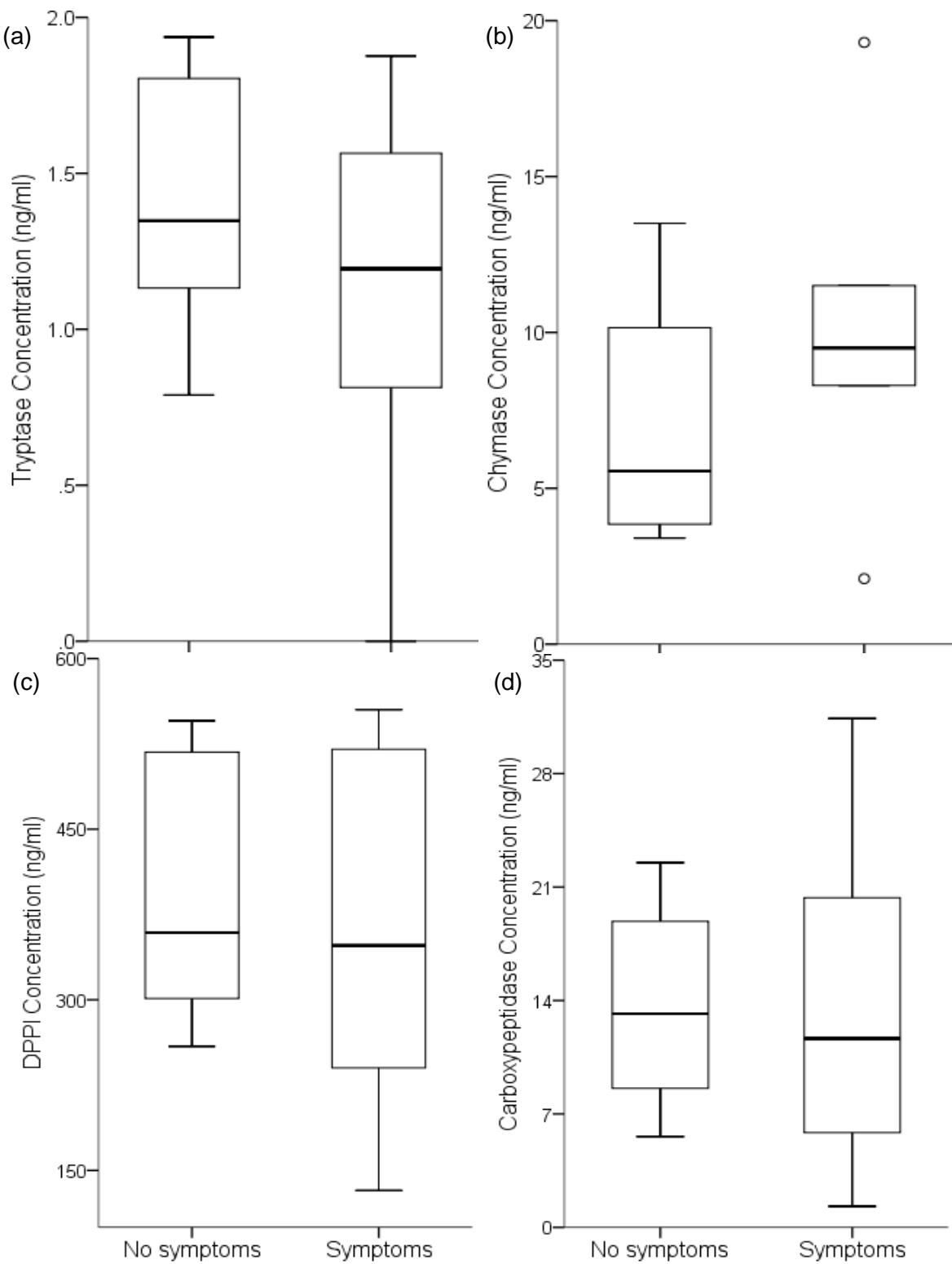
Fig 5.15 Post challenge serum levels of (a) tryptase, (b) chymase, (c) carboxypeptidase and (d) DPPI in patients who did and did not experience symptoms in drug challenge. Levels were measured by ELISA, and examined in patients who presented with symptoms not localised to the site of skin testing (n = 9) compared to those who did not (n = 6). Statistical analysis was performed using Mann Whitney U Test.
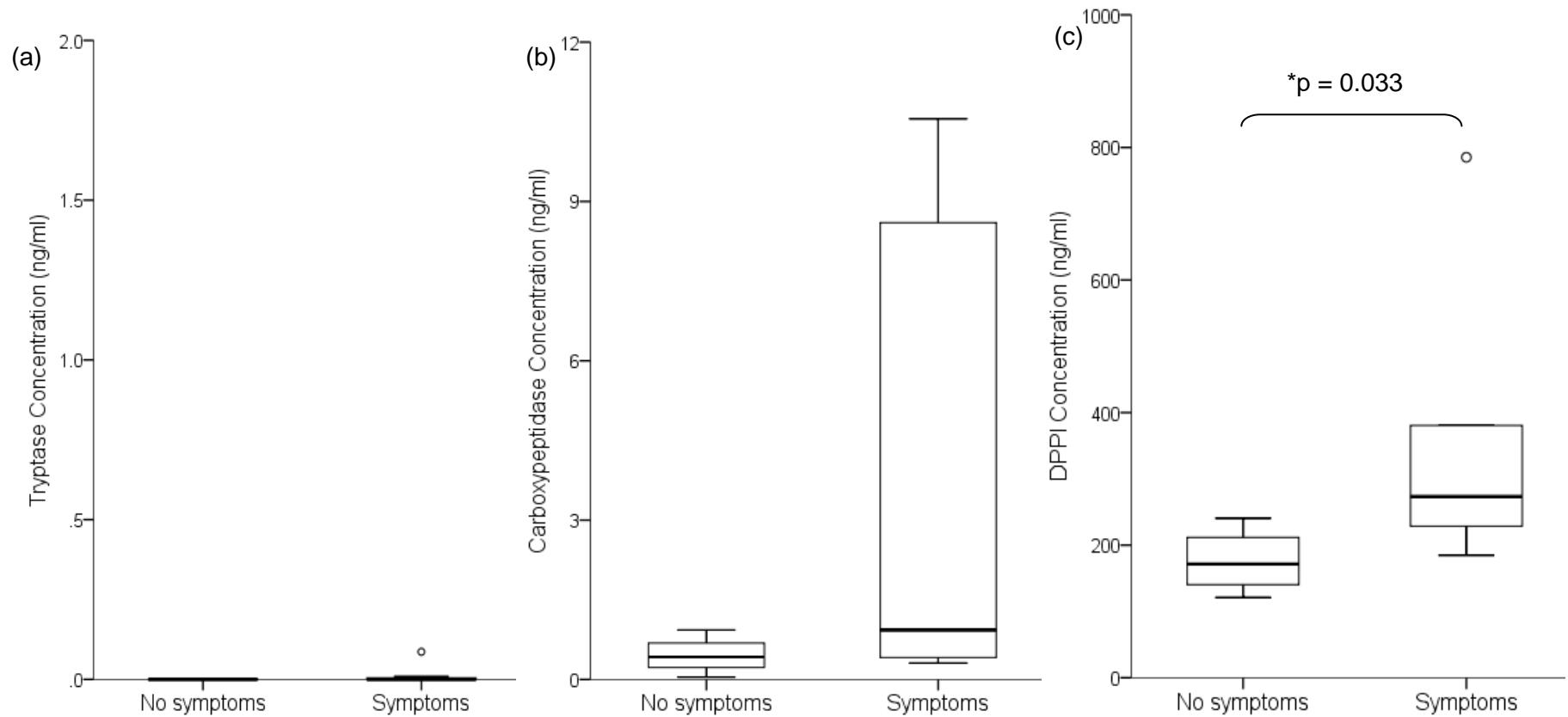
Fig 5.16 Post challenge saliva levels of (a) tryptase, (b) carboxypeptidase and (c) DPPI in patients who did and did not experience symptoms in drug challenge. Nine patients experienced symptoms not localised to the site of skin testing, and six did not. Statistical analysis was performed using Mann Whitney U Test.

Chymase

Serum chymase levels were higher post challenge in patients who reacted in drug challenge than in those who did not (p = 0.018; Fig 5.17). This difference was not seen in baseline concentrations of the protease, but no increase in concentrations post compared to pre challenge was seen in either group. ROC curve analysis was employed to determine the optimum cut off value of chymase concentration post challenge in confirming a positive outcome. A cut off of 6.4 ng/ml gave a specificity of 92 % and sensitivity of 70 % (Fig 5.18), giving positive and negative likelihood ratios of 8.4 and 0.3, respectively. Levels of chymase were no different in serum of patients who experienced symptoms compared to those who did not (Fig 5.15(b)).

Carboxypeptidase

Serum levels of carboxypeptidase did not significantly differ in patients with positive and negative challenges either at baseline or after challenge, and levels did not change over the course of the challenge. However, saliva levels were increased over the course of the challenge in patients who tested positive in SPT or intra-dermal testing (p = 0.021), but not in those who did not (Fig 5.19). Baseline levels of salivary carboxypeptidase were significantly higher in patients who tested positive in skin testing and had additional symptoms than in other patients (p = 0.022; discussed further in Chapter 6). This effect was not seen for post challenge levels of serum (Fig 5.15(c)) or saliva (Fig 5.16(b)).

DPPI

Concentrations of DPPI in serum and saliva were not significantly different, either before or after challenge, in patients who did or did not react (Fig 5.20). No increase in levels was observed over the course of the challenge. However, post challenge salivary DPPI concentrations were higher in patients tested positive in skin testing and also experienced symptoms compared to those in other patients (p = 0.021; Fig 5.16(d)). This effect was not seen for serum levels (Fig 5.15(d)).
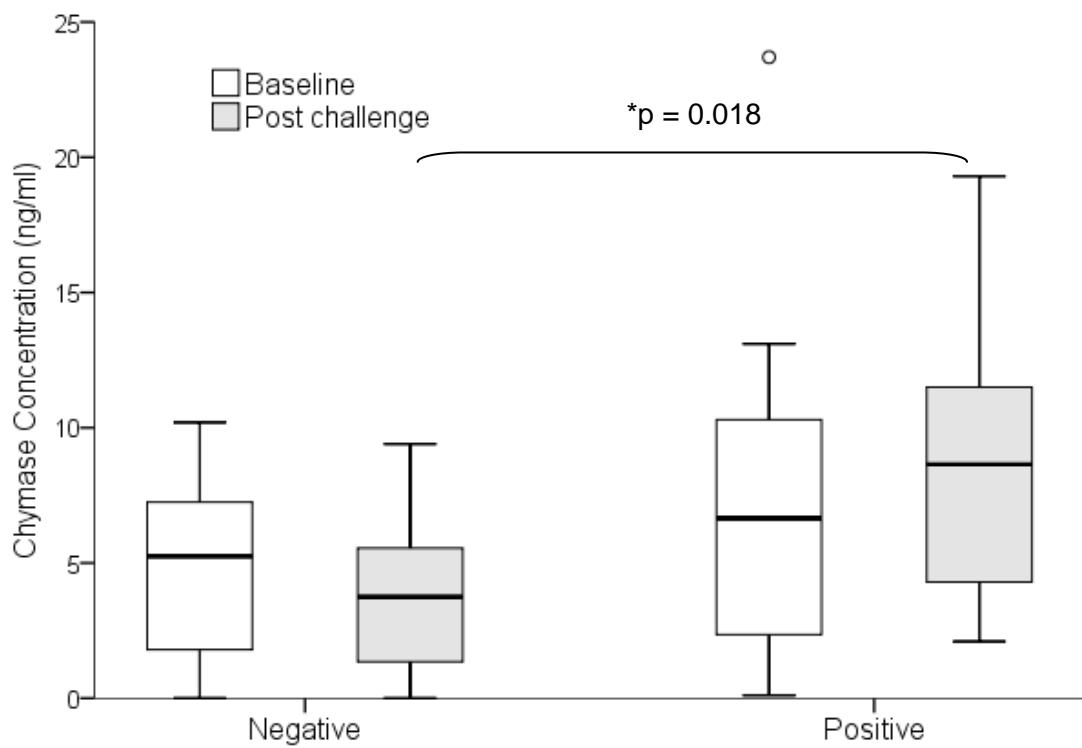
Fig 5.17 Baseline and post challenge levels of serum chymase in positive and negative challenges. Levels were measured by ELISA in serum collected pre and post food challenge. Results are shown for positive (n = 15) and negative (n = 21) outcomes. Statistical analysis was performed using Mann-Whitney U Test.
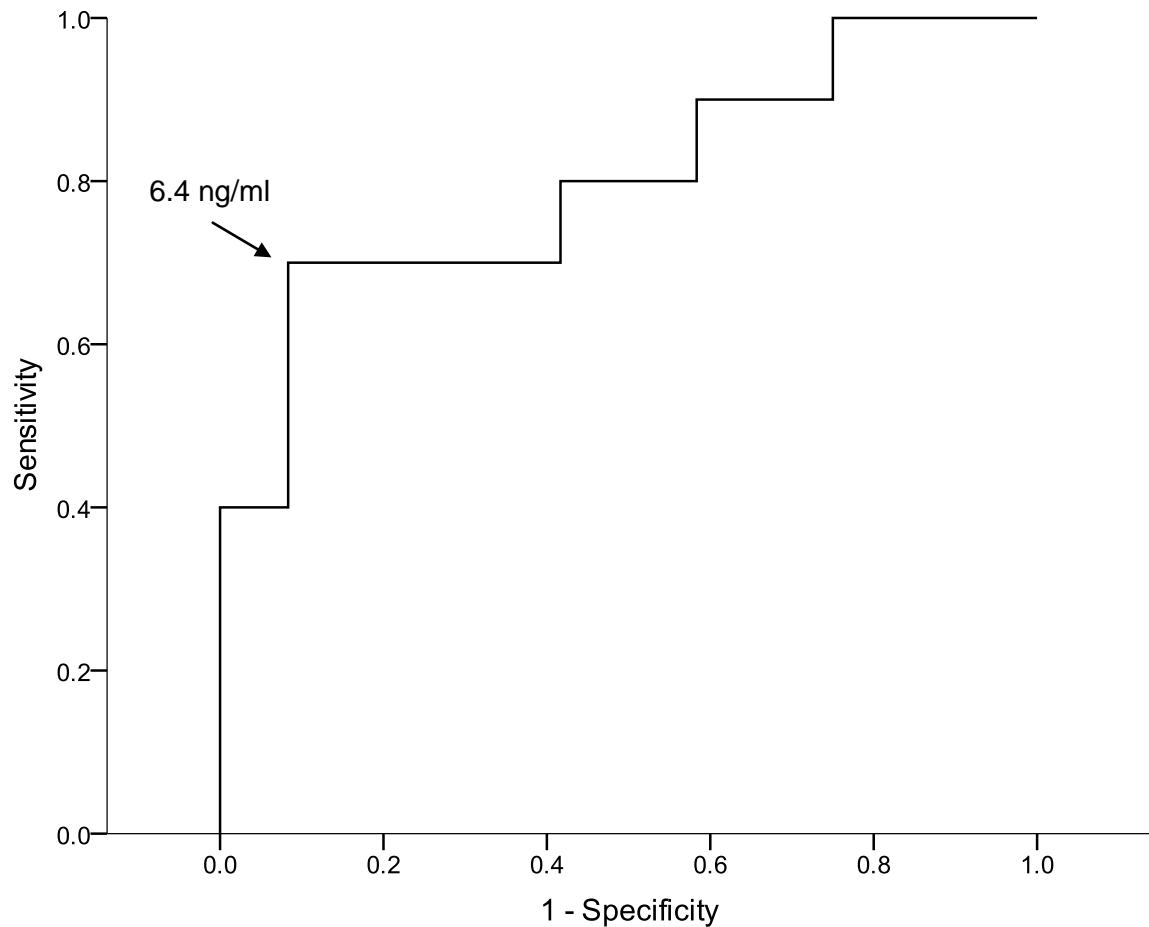
Fig 5.18 ROC curve of chymase measurement for confirming positive drug challenge. The concentration yielding greatest sensitivity and specificity was determined.
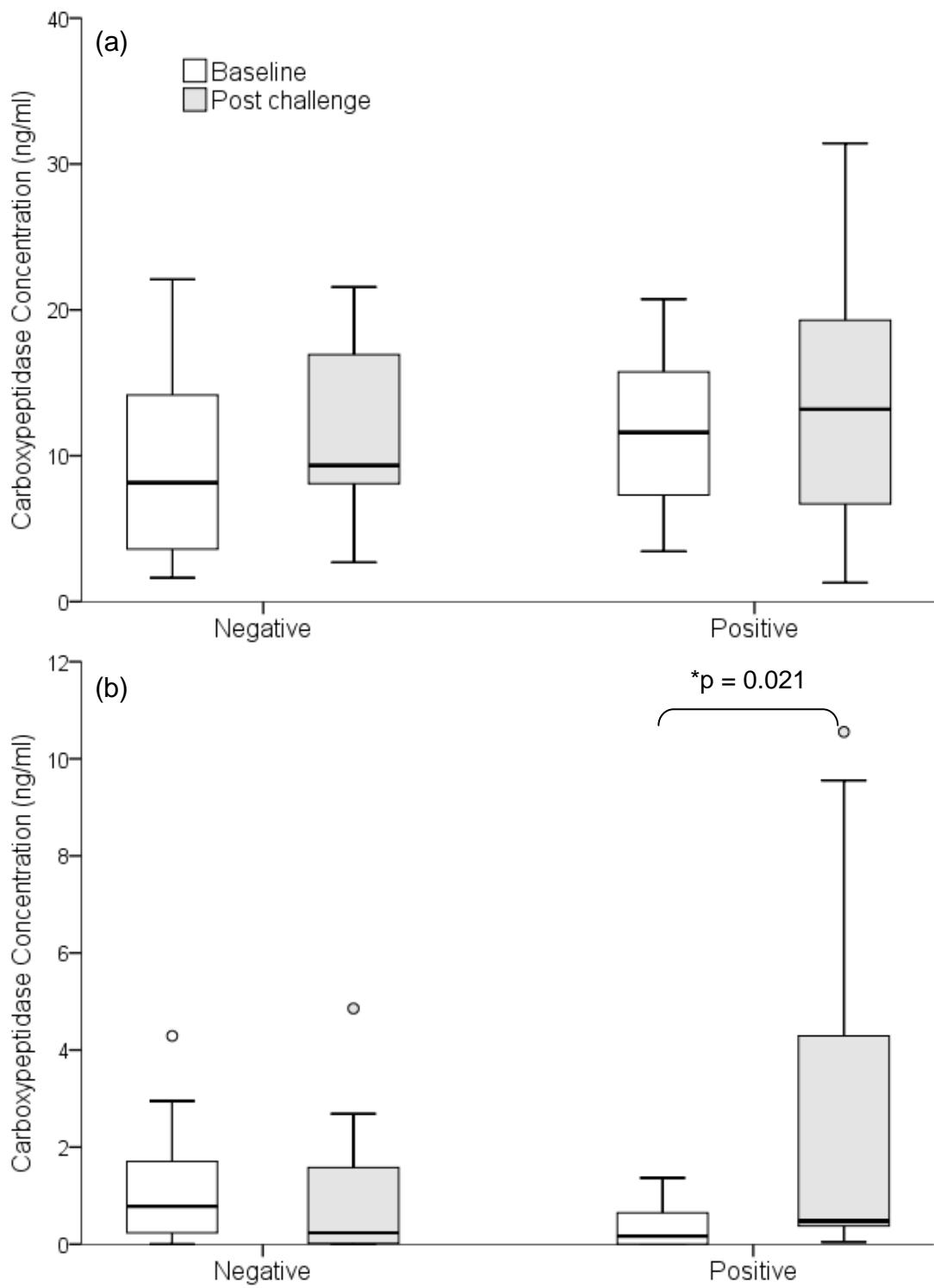
Fig 5.19 Baseline and post challenge levels of (a) serum and (b) saliva carboxypeptidase in positive and negative challenges. Levels were measured by ELISA in serum collected pre and post food challenge. Results are shown for positive (n = 15) and negative (n = 21) outcomes. Statistical analysis was performed using Mann-Whitney U Test.
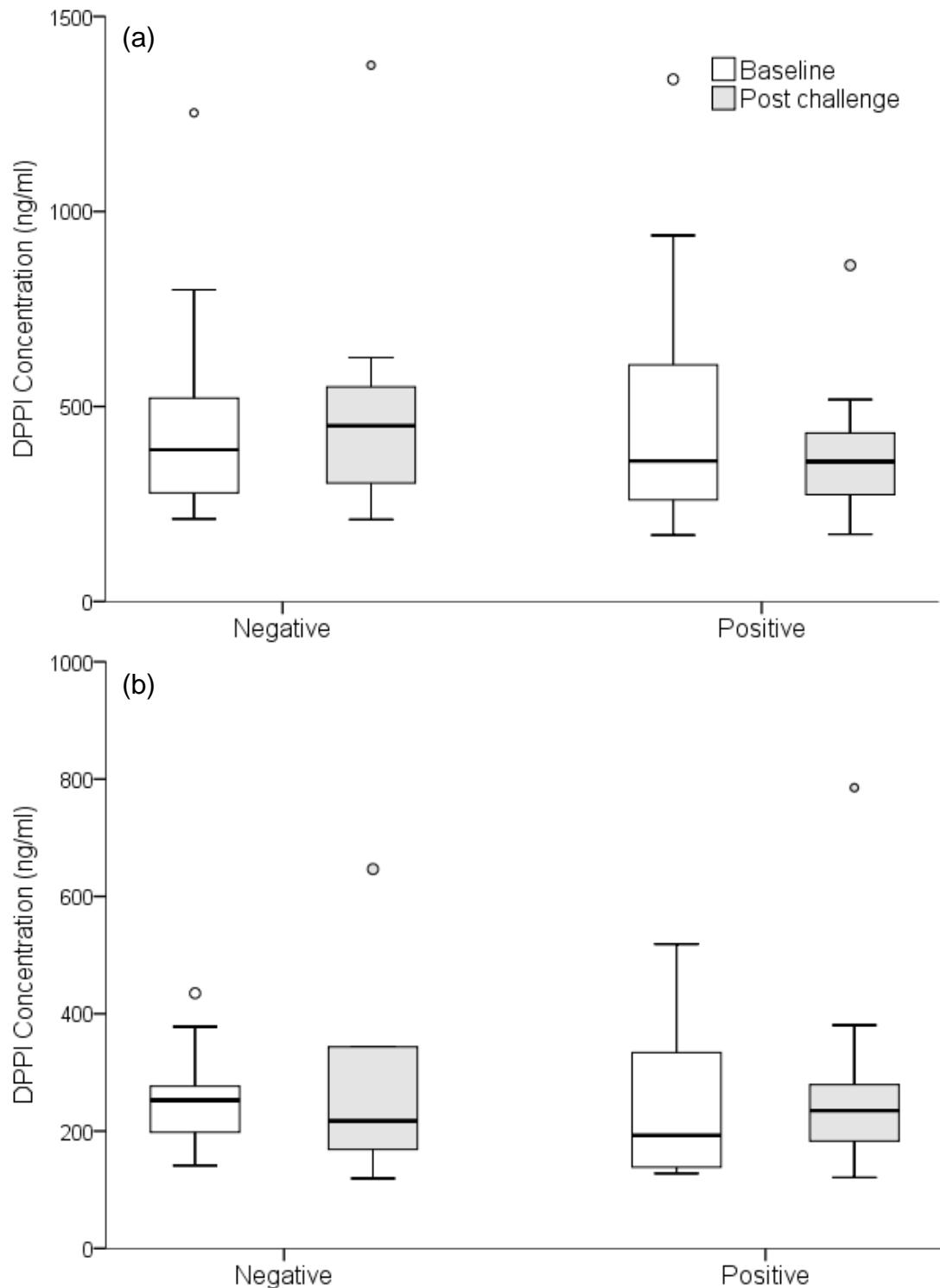
Fig 5.20 Baseline and post challenge levels of (a) serum and (b) saliva DPPI in positive and negative challenges. Levels were measured by ELISA in serum collected pre and post food challenge. Results are shown for positive (n = 15) and negative (n = 21) outcomes. Statistical analysis was performed using Mann-Whitney U Test.

ACE

As seen with tryptase levels, concentrations of serum ACE did not differ significantly between patients with positive or negative outcomes in drug challenge, either at baseline or after challenge. Furthermore, levels did not increase over the course of the challenge in either group.

Correlations between markers

In patients with positive skin testing, serum tryptase levels post challenge correlated with saliva tryptase (p = 0.009, r_s = 0.742, n = 11) and serum DPPI levels (p = 0.033, r_s = 0.57, n = 14). An association was also seen between concentrations of salivary tryptase and serum DPPI after challenge (p = 0.019, r_s = 0.507, n = 21). Post challenge serum chymase and salivary carboxypeptidase levels were significantly correlated (p = 0.006, r_s = 0.668, n = 15), and serum and salivary carboxypeptidase levels were negatively associated (p = 0.005, r_s = -0.802, n = 10). ACE levels did not correlate with those of any other mediator.

Anaphylaxis and allergic asthma exacerbation

Ten adult patients presenting at the Accident and Emergency Department with anaphylaxis or allergic asthma exacerbation, were recruited. Reactions were to a range of stimuli, including foods, drugs, insect venom or other allergens. Patients recruited from the Accident and Emergency department were grouped together with those experiencing severe allergic reactions in allergen challenge, and compared with those experiencing milder reactions or no reaction upon challenge. Fourteen healthy adult volunteers with no allergic history, or with a history of mild allergy to stimuli other than foods, drugs or insect venom, were recruited to serve as additional controls.

Serum markers

Serum levels of tryptase, chymase, carboxypeptidase, DPPI and ACE in patients with severe reactions following allergen exposure were compared with levels in patients with no reaction or less severe reactions. Concentrations were also compared with baseline concentrations in atopic and non-atopic control subjects.

Tryptase

Tryptase levels did not differ significantly between patients with anaphylaxis or allergic asthma exacerbation and those with no reaction or less severe reactions (Fig 5.21(a)). Concentrations were also similar in patients with reactions compared to baseline levels in atopic and non-atopic control groups.

Chymase

Serum levels of chymase were significantly higher in patients with non-anaphylactic reactions than in those with anaphylaxis or allergic asthma exacerbation (p = 0.045; Fig 5.21(b)). Concentrations were also higher than those in control subjects after negative allergen challenge (p = 0.031).

Carboxypeptidase

Serum carboxypeptidase levels were similar in patients with and without allergic reactions following allergen exposure (Fig 5.22(a)). Furthermore, there was no significant difference in concentrations between patients with anaphylaxis or allergic asthma exacerbation and those with milder reactions. Levels post allergen exposure did not differ from baseline levels in either atopic or non-atopic subjects.

DPPI

Levels of serum DPPI were significantly higher in patients with anaphylaxis or allergic asthma exacerbation than in healthy control subjects both at baseline ($p < 0.001$) and following negative allergen challenge ($p < 0.001$; Fig 5.22(b)). DPPI concentrations were also higher following anaphylaxis than less severe reactions ($p = 0.009$).

ACE

Similarly to tryptase and carboxypeptidase, ACE levels did not differ significantly between subjects with anaphylaxis or severe asthma exacerbation and those with less severe reactions. Concentrations were also not significantly different from the control groups, either at baseline or following allergen exposure.

Correlations between markers

In all subjects, serum DPPI levels were significantly correlated with those of carboxypeptidase ($p < 0.001$, $r_s = 0.323$, $n = 128$) and chymase ($p < 0.001$, $r_s = 0.343$, $n = 114$). Following an allergic reaction, i.e. when excluding baseline levels and those following negative allergen challenge, serum tryptase and carboxypeptidase levels were also associated ($p = 0.021$, $r_s = 0.443$, $n = 27$). Concentrations of ACE within serum did not correlate with those of any other marker.
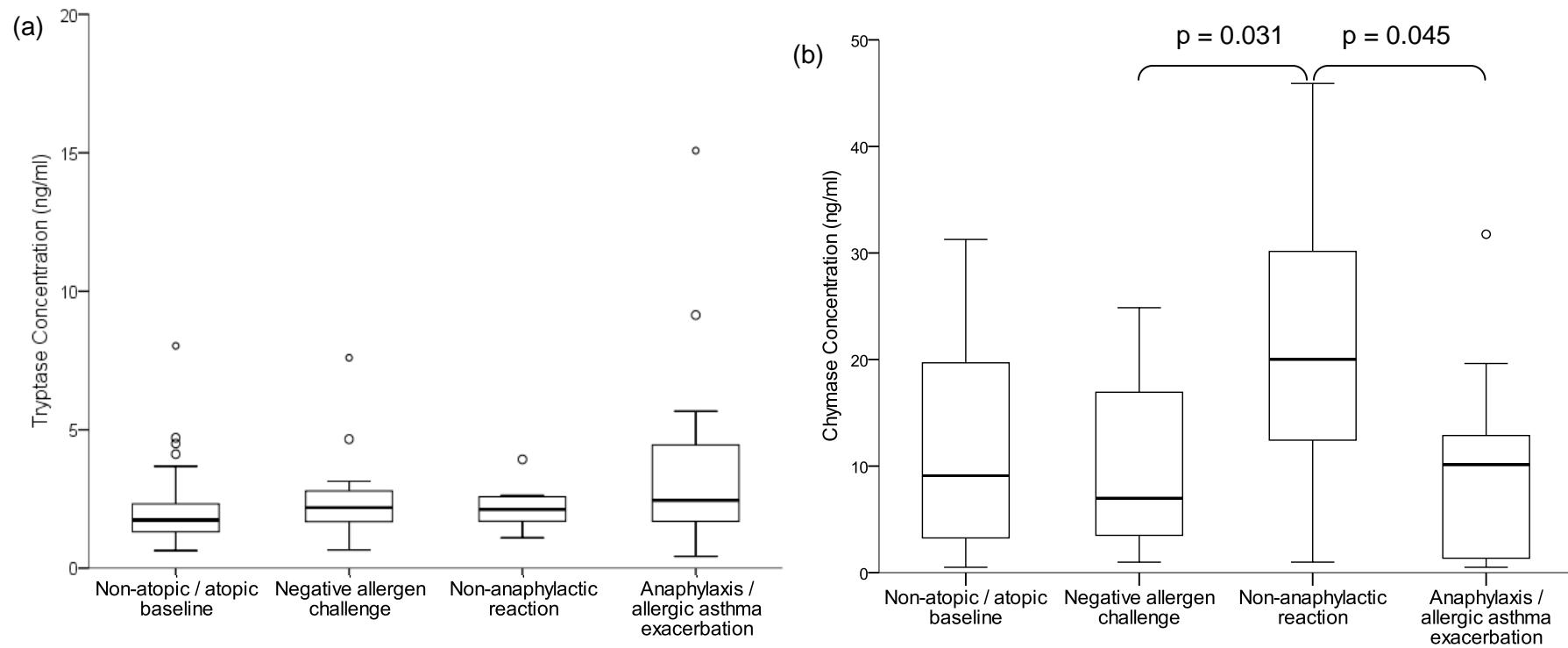
Fig 5.21 Serum (a) tryptase and (b) chymase levels in anaphylaxis/allergic asthma exacerbation. Levels in patients with anaphylaxis (n = 18) were compared with those in subjects with no reaction (n = 40) or less severe reactions (n = 24) in allergen challenge, and also with baseline levels in atopic/non-atopic control subjects (n = 72). Results were analyzed by Kruskal Wallis and Mann Whitney U Test.
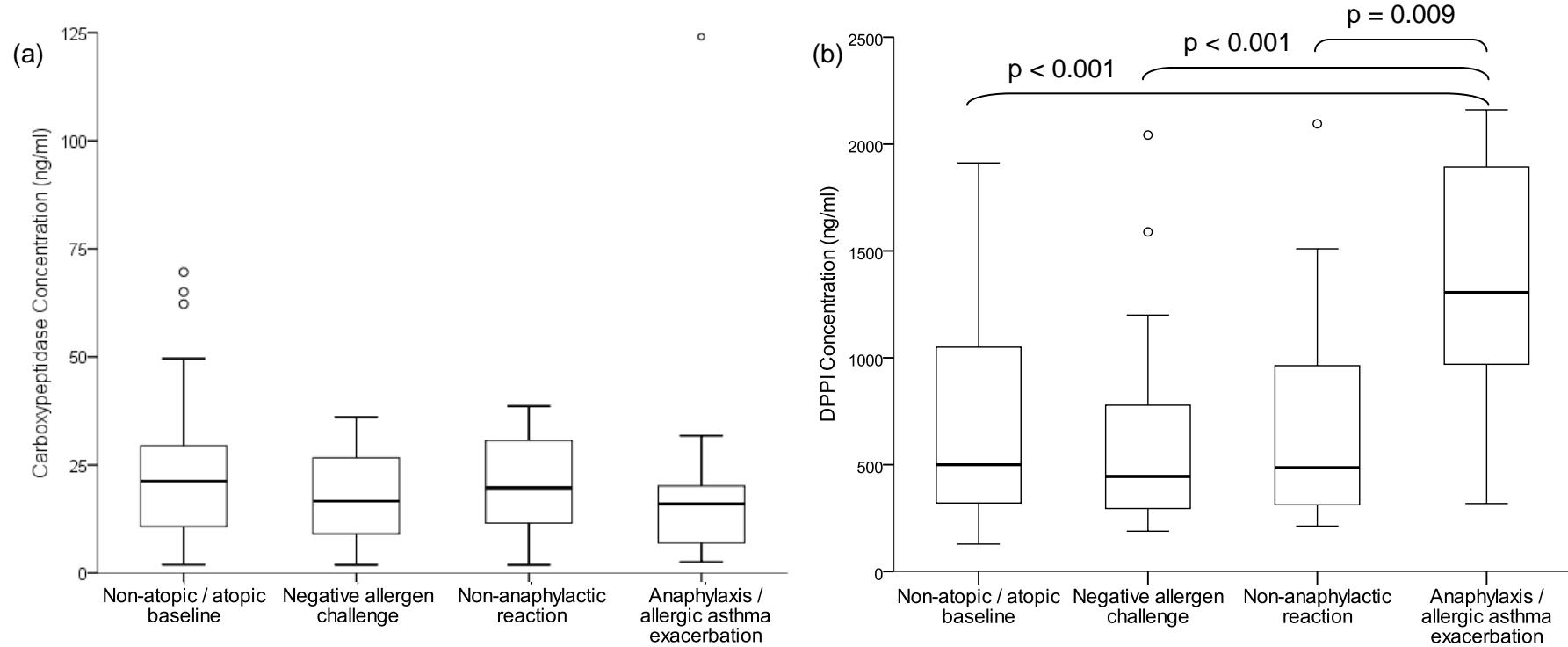
Fig 5.22 Serum (a) carboxypeptidase and (b) DPPI levels in anaphylaxis/allergic asthma exacerbation. Levels in patients with anaphylaxis (n = 18) were compared with those in subjects with no reaction (n = 40) or less severe reactions (n = 24) in allergen challenge, and also with baseline levels in atopic/non-atopic control subjects (n = 72). Results were analyzed by Kruskal Wallis and Mann Whitney U Test.

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5.4 Discussion

We have found evidence for increases in levels of mast cell proteases in serum and saliva during allergic reactions, thus supporting measurement of these markers in diagnosis of the condition. Our major findings have been illustrated in Fig 5.23 for food allergy and Fig 5.24 for drug allergy. The aim of the studies within this chapter was to identify confirmatory markers; and the predictive potential of baseline levels is discussed in Chapter 6.

Tryptase

In the current study, no differences in serum or saliva tryptase levels were observed over the course of the food challenges in children with positive (or negative) outcomes. In a previous study, serum tryptase levels were found to increase after reaction to bee sting (Schwartz *et al.*, 1989); and measurement of the protease is recommended for diagnosis of anaphylaxis by the UK Resuscitation Council (1999), with high levels confirming a positive diagnosis. However, consistent with our findings, Vila *et al* (2001) did not observe an increase in serum tryptase levels during food challenge in 75 % of patients with a positive outcome. Saliva tryptase was detected in only one patient, and levels did not increase after positive food challenge. Results of the current study and that by Vila *et al* (2001) suggest that serum tryptase is not a useful marker of food-induced reactions.

Interestingly, we did find tryptase levels to be elevated in saliva fifteen minutes after initial allergen exposure in children who experienced isolated oral allergy in food challenge. This effect was not seen in those with systemic symptoms. Thus perhaps increased levels are detectable only locally in food allergy; the oral mucosa is the first point of contact for the food. This may explain the failure to see increased levels after reaction in serum, as the allergen must firstly undergo the digestive process prior to entering the blood stream. Indeed, increased tryptase levels have been detected previously in the jejunum, but not serum, of food-allergic patients following localised allergen provocation (Santos *et al.*, 1999).
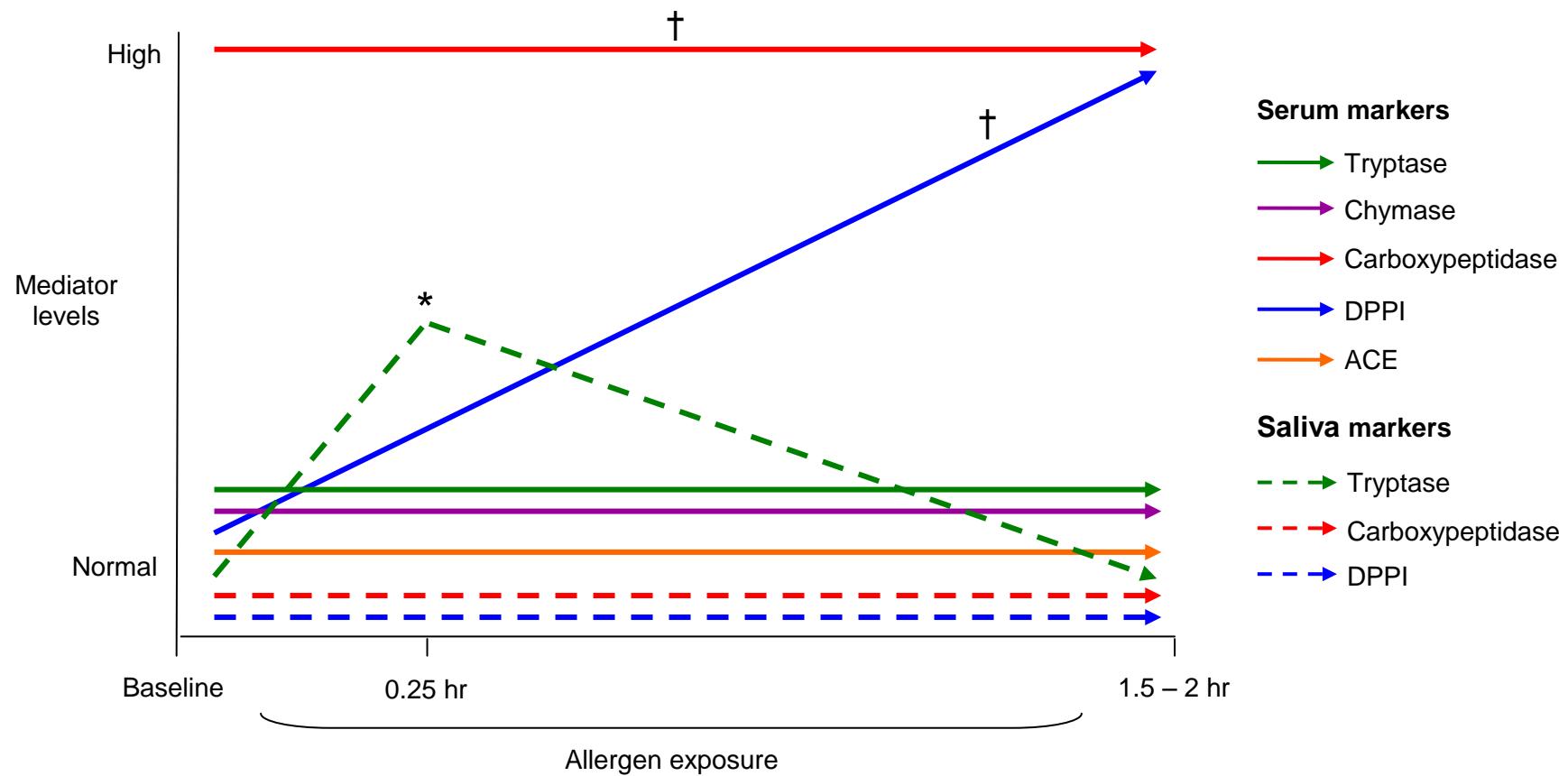
Fig 5.23 Mediators of allergic reactions to food. Serum levels of tryptase, chymase, carboxypeptidase, DPPI and ACE, and saliva levels of tryptase, carboxypeptidase and DPPI, are shown at baseline and during a <u>positive</u> food challenge. (* indicates cases of isolated oral allergy only, and † is indicative of moderate to severe reactions)
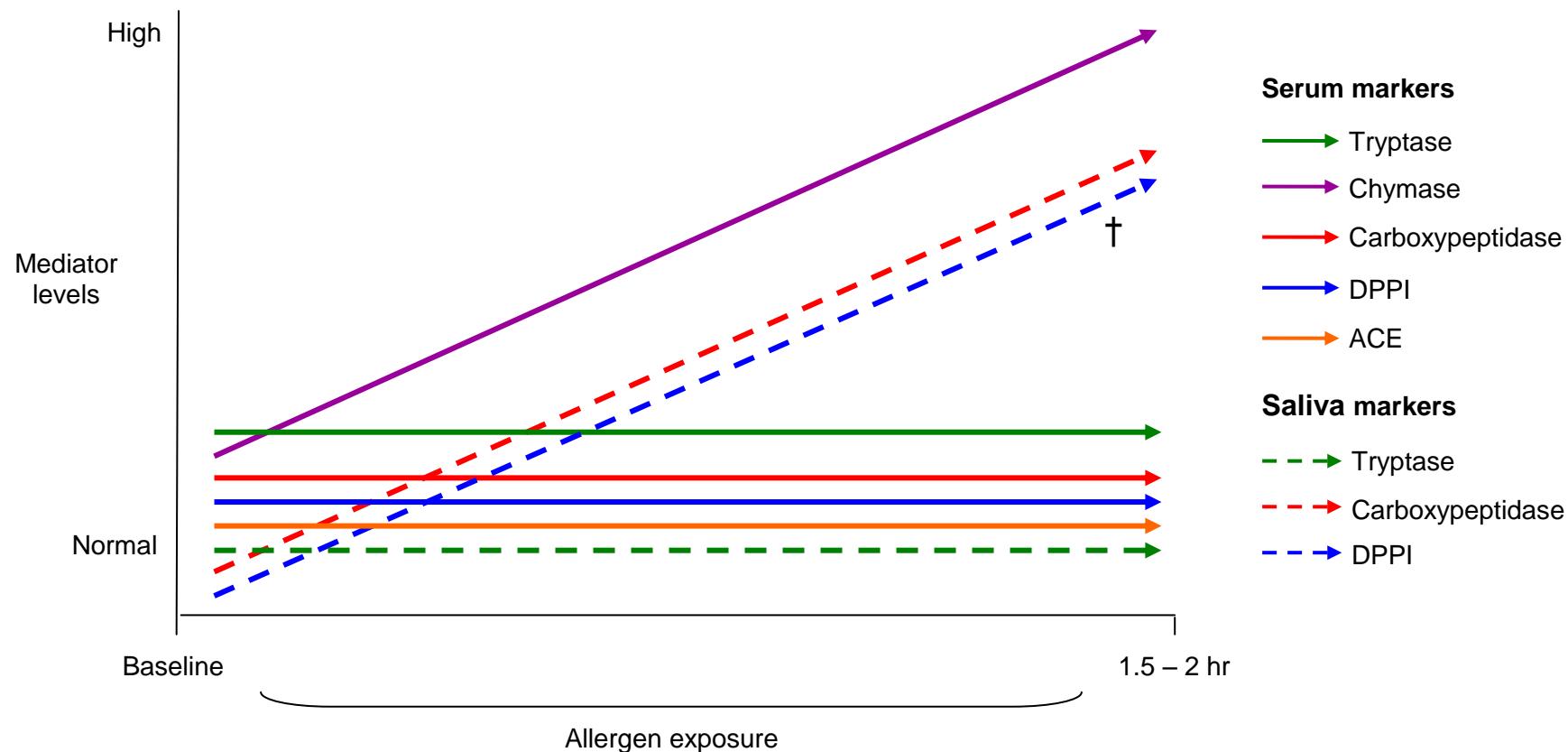
Fig 5.24 Mediators of allergic reactions to drugs. Serum levels of tryptase, chymase, carboxypeptidase, DPPI and ACE, and saliva levels of tryptase, carboxypeptidase and DPPI, are shown at baseline and after <u>positive</u> skin testing to drugs. († indicates cases in which allergic symptoms presented)

We also observed no change in levels of serum or salivary tryptase in adults undergoing drug challenge. However, as these challenges involved only skin testing (as described in Chapter 2), significant allergic reactions rarely occurred. If drugs were given via the normal route of administration (as foods are in allergen challenge) some patients may have experienced systemic reactions, and alterations in serum tryptase levels would perhaps have been observed. In a previous study, an increase in levels of the protease were observed in over half of patients with anaphylaxis in anaesthesia (Dybendal *et al.*, 2003); and measurement of serum tryptase (alongside urinary histamine) was reported to be useful in confirming allergic reactions to a range of therapeutic agents (Fernandez *et al.*, 1995).

In the current study, levels of serum tryptase were not significantly different in patients with anaphylaxis or allergic asthma exacerbations to foods, drugs, insect venom or other allergens than in those with no reaction or less severe reactions. Previous studies concerning general reactions (rather than those to a given allergen) yield conflicting results. The proportion of patients with high serum tryptase levels following acute allergic reactions has ranged from less than 10 % (Treudler. *et al.*, 2008) to almost 90 % (Yunginger *et al.*, 1991; Pumphrey and Roberts, 2000). A large number of patients with raised serum tryptase concentrations following anaphylaxis were also found to have mastocytosis (Treudler. *et al.*, 2008).

It is possible that, in the current study (and previous studies with similar findings), serum tryptase levels were not yet increased, or had declined, at the time of sampling. Previously, concentrations have been reported to be elevated in anaphylaxis from 30 minutes after wasp sting, peaking at two hours (Schwartz *et al.*, 1989). Similarly, in reactions to drugs, the optimal time for tryptase measurement was determined to be two hours after exposure; and it was recommended that tests are done no later than six hours (Laroche *et al.*, 1991). In the current study blood was taken upon arrival at hospital, and collection occurred within the 30 minute to six hour window.

Chymase

In the current study, we found no evidence that serum chymase levels are altered during allergic reactions to food. Previous work carried out within our group showed that serum levels of chymase were higher in patients with anaphylaxis than in healthy blood donors (Zhou *et al.*, 2011). However, baseline data was not available for the anaphylactic patients, and so there was no direct evidence that levels were increased during the allergic event.

There are several other key points that may account for the discrepancy with our current and previous results. Firstly, the majority of samples in the previous studies were from adults, whereas the food challenges were performed only in children. There is presently no data comparing levels of the protease in biological fluids from adults and children. Secondly, the majority of children undergoing food challenges did not experience anaphylaxis, as allergen was introduced gradually and treatment was provided as soon as symptoms presented. Thus insufficient time may have been allowed for chymase to accumulate in the blood. Finally, in the previous study reactions were experienced in response to a range of different allergens (mostly therapeutic agents), whereas all reactions in children recruited onto our study were to foods.

Interestingly, and in compliance with the unpublished observations described above, serum chymase concentrations were higher after drug challenge in patients with positive skin tests than in those with negative tests. There was no difference in baseline levels between the two groups, suggesting that levels increased during the challenge. However, tests revealed no statistical change within individuals. Thus we have found further evidence that serum chymase levels are high in patients with reactions to therapeutic agents. Whether this represents higher constitutive levels, or whether levels are increased during a reaction, remains to be deduced.

In support of our work (both in the current and previous studies), Nishio *et al.* (2005) detected chymase in serum collected at post mortem from all of eight victims of fatal anaphylaxis to therapeutic agents, but only in two of 104 non-anaphylactic fatalities. Chymase levels were considered high in six of the eight allergic reactions. As for the previous studies carried out within our laboratory (described above), baseline levels of the protease had not been measured. It can only therefore be an assumption that levels were increased during the reaction.

In the current study, using a cut off value of 6.4 ng/ml, chymase measurement was determined to have a sensitivity of 70 % and specificity of 92 % in confirming a positive result in drug challenge. Thus, the test would detect most cases of positive challenges; and few false positive results are likely to occur. Assuming the higher levels after positive challenges are due to an increase during the challenge, this suggests that chymase measurement post drug challenge may be a useful tool in confirming allergy.

Interestingly, when studying patients with reactions to foods, drugs, insect venom or other allergens, serum chymase levels were found to be higher in those with mild or moderate symptoms compared to those with anaphylaxis or allergic asthma exacerbations. Concentrations were also higher than in negative allergen challenges, and compared to baseline levels in non atopic or atopic subjects. The reason for lower levels in anaphylaxis compared to mild or moderate reactions requires investigation.

Carboxypeptidase

Serum levels of carboxypeptidase were higher in children who reacted in food challenge than in those who did not, both at baseline and after challenge; but levels did not change over the course of the challenge. This suggests that levels of the protease in serum may be predictive of clinical reactivity rather than useful for confirming a reaction to food has occurred. The potential for serum carboxypeptidase as a predictive marker is discussed in Chapter 6.

As in the food challenges, serum carboxypeptidase levels did not increase in adults undergoing diagnostic drug challenge. However, a rise in salivary levels was observed in those with a positive outcome. This suggests that carboxypeptidase may be a useful marker of reactions to drugs. The lack of increased serum levels may be due to the lack of symptoms in many positive challenges, and the mild nature of the majority of symptomatic reactions that did occur. Previous data from within our group has shown high levels of carboxypeptidase in serum following anaphylaxis to a range of allergens, but mostly therapeutic agents (Zhou *et al.*, 2006).

It is interesting that increased carboxypeptidase concentrations were detected in the saliva of adults with positive drug challenges given its remoteness from the area of skin testing. However, when symptoms were reported, they were often confined to the oral mucosa (such as oral pruritus). Thus measurement of salivary carboxypeptidase may be useful as a confirmatory marker of these very subjective reactions. Unfortunately we found no similar evidence for salivary carboxypeptidase in food allergy. This is perhaps unexpected given that the oral mucosa is often the first point of contact for the eliciting allergen, and that oral symptoms are common. This may reflect differences in the mechanisms involved in reactions to different types of allergens.

DPPI

In the current study we have demonstrated that DPPI is released into the circulation during allergic reactions to food in children. The protease was initially considered to act intracellularly, but evidence was provided for its release from dog mastocytomas (Wolters *et al.*, 1998). Furthermore, recent work has demonstrated release from a human mast cell-like cell line (LAD-2) and from human tonsil mast cells upon activation with anti-IgE or calcium ionophore (Zhou *et al.* Unpublished observation). This suggests that DPPI may act extracellularly as well as intracellularly. The current study is the first to show that DPPI is released from cells

in vivo and to provide evidence for the protease as a useful biomarker in confirming allergic reactions to food.

The increase in serum DPPI levels measured in food challenge was most prominent during moderate to severe reactions. Furthermore, concentrations of the protease were associated with reaction severity scores. This was true for both baseline and post challenge levels, but the correlation was strongest for the latter. In addition, DPPI levels were higher in patients who reported throat tightness. This suggests that the extent of DPPI release during a reaction may determine the severity of symptoms experienced. Thus perhaps a genetic disposition to low DPPI levels may be protective against anaphylaxis. In any case, our results indicate that DPPI may be most useful as a marker of *severe* reactions to food.

Using a cut off value of 1210 ng/ml, DPPI measurement post challenge had a high specificity (94%) in confirming a reaction to food. However, the sensitivity was low (45 %), which would result in an unacceptable false negative rate. The sensitivity was increased significantly (without a corresponding drop in specificity) when using the test to confirm only moderate to severe objective reactions. The specificity and sensitivity for this were 93 % and 62 %, respectively.

DPPI concentrations did not increase in the serum of patients who reacted in drug challenge. However, as described previously, a positive skin test was taken to indicate allergy, and symptomatic allergic reactions did not occur in all of these patients. Furthermore, in cases where patients did experience symptoms, these were generally mild. Interestingly, salivary DPPI levels were higher after challenge in patients who experienced symptoms than in those who did not. Many of these symptoms were confined to the oral mucosa, and thus it appears that DPPI release may have reflected the localised nature of the symptoms.

When all patients were grouped, including those undergoing drug and food challenges and those presenting at the Accident and Emergency Department with reactions to foods, drugs, insect venom or other allergens, serum DPPI levels were

significantly higher in subjects with anaphylaxis than in those with either no reaction or milder reactions following allergen exposure. Levels were also higher than at baseline in healthy atopic or non-atopic controls. These results indicate that serum DPPI may be a useful marker of severe allergic reactions not only to food, but to any allergen.

Interactions between DPPI and other mast cell proteases

Mast cells are one of the main cell types involved in the immediate allergic response and, as described, DPPI release from mast cells has been demonstrated (Wolters *et al.*, 1998; Whitworth *et al.*, 2010). It is therefore likely that this is the major source of DPPI detected in the current study. Associations observed between serum levels of DPPI and concentrations of tryptase, chymase and carboxypeptidase in study subjects provide evidence that the proteases are released from the same cell type.

DPPI has previously been shown to activate tryptase and chymase *in vitro* (McGuire *et al.*, 1993; McEuen *et al.*, 1998). However, other than using purified cells or cell lines, there is little evidence for this role in humans. Addition of exogenous DPPI to serum taken prior to food challenge in a subgroup of children from the current study was found to cause an increase in chymase activity (Zhou *et al.*, 2011). This effect was not observed, however, when using serum from the same children following a reaction in food challenge. This suggests that chymase present in the pre-challenge samples has the capacity to be activated, and is therefore in its pro-form. Chymase present in the post-challenge samples, on the other hand, appears to be already activated. Indeed, *in vivo* chymase activity was determined to be higher in (untreated) serum after challenge compared to at baseline. These studies provide further evidence that chymase activation is a key role of DPPI in humans.

Due to the increase in levels of DPPI, and also its roles in activation of tryptase and chymase, a corresponding increase in levels of activated tryptase and chymase

may have been expected during the reactions to food. Although high levels of serum chymase were detected following positive drug challenge, we did not observe an increase in tryptase or chymase concentrations after positive food challenge. Our assays for these proteases measure total levels, whereas measurement of proportions of pro and active forms may have been useful. Nevertheless, an association was observed between serum DPPI and chymase concentrations after food challenge in children; and serum DPPI and tryptase levels were correlated following drug challenge in adults.

ACE

In the current study, we found no evidence of changes in serum ACE levels during positive (or negative) challenges to either foods or drugs. Furthermore, levels were not significantly different in patients with anaphylaxis or allergic asthma exacerbation to foods, drugs, insect venom or other allergens compared to those with either no reaction or less severe reactions. Concentrations were also no different from baseline levels measured in atopic or non-atopic control subjects.

Plasma levels of angiotensin II have previously been shown to increase within five minutes of anaphylactic shock to bee sting (Van der Linden *et al.*, 1993), and levels were associated with a reduction in blood pressure. The rise in levels must have been due to increased conversion from angiotensin I; and this is typically thought to occur via the actions of ACE (Erdös *et al.*, 1976; Campbell *et al.*, 2004). However, chymase (Wintroub *et al.*, 1986) and carboxypeptidase (Goldstein *et al.*, 1989) also have angiotensin-converting ability, and have been shown to carry out this role in synergy in mice (Lundequist *et al.*, 2004). Our results provide no evidence for ACE release into the circulation during an allergic reaction. The increase in plasma angiotensin II levels previously detected during anaphylaxis are therefore perhaps likely to have resulted from release (and and thus increased action) of mast cell proteases such as chymase and carboxypeptidase.

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Limitations of study

The mast cell proteases that we have measured have all been shown to be stored within mast cell granules and released upon cell activation *in vitro*. Thus it is perhaps surprising that we did not observe more consistent changes in serum levels of markers other than DPPI. It is possible that the time points at which levels of the mediators were measured do not reflect those at which they are likely to be increased. This highlights a limitation in our study whereby blood was mostly taken at just two time points (one of which was at baseline). Ideally levels would have been measured at more times. However, as patients were not routinely cannulated, only two blood samples could be obtained for ethical reasons. As described previously, the recommended time for sample collection for tryptase is after 30 minutes and within six hours. This was adhered to within our study.

A limitation of studying reactions occurring in food challenge is that symptoms would not be allowed to progress. Food was given very gradually, and as soon as a reaction became apparent the patient was treated. For these reasons, the reaction seen in clinic may not be representative of a reaction occurring within the community. Indeed, challenge scores for reactions in food challenge were not similar to those for previous reactions to the same food (data not shown). For this reason, mast cell proteases may not have had time to accumulate within the circulation sufficiently for increased levels to be measured.

Summary

Our studies, for the first time, provide evidence of a role for DPPI measurement in confirming diagnosis of severe allergic reactions to foods and other allergens. Furthermore, the extent of DPPI release was associated with the severity of symptoms. Serum chymase may be a useful marker of reactions to drugs; but no evidence was found to support its use as a confirmatory marker of food allergy. Salivary carboxypeptidase may have use in confirming subjective oral reactions to drugs; whereas salivary tryptase may fulfil this role in cases of suspected reactions

to food. The potential for use of these mediators in confirming reactions, based on the findings of the current study, is presented in table 5.3.

Table 5.3 Potential use of mediators as confirmatory markers in allergy. The potential for use of serum/saliva measurement of tryptase, chymase, carboxypeptidase, DPPI or ACE in confirming an allergic reaction, based on the results of this study, is presented.

Mediator	Biological fluid	Use as confirmatory marker
Tryptase	Serum	None
	Saliva	Isolated oral allergy to food
Chymase	Serum	Reaction to drugs
Carboxypeptidase	Serum	None
	Saliva	Reaction to drugs
DPPI	Serum	Severe reaction to any allergen
	Saliva	Subjective/oral reaction to drugs
ACE	Serum	None

CHAPTER 6: PREDICTIVE MARKERS OF SEVERE ALLERGIC REACTIONS

6.1 Introduction

The range of symptoms that can present in IgE-mediated allergic reactions is vast. Some patients experience only mild symptoms, such as pruritus or localised urticaria. However, others can have much more severe, potentially life threatening reactions. These cases usually involve respiratory or cardiovascular compromise (Sampson *et al.*, 2006). There is currently little means of establishing which patients may be at risk of severe reactions. Some evidence has been presented of the risk factors implicated, the most important of which are a history of severe reactions and co-existing asthma (Bock *et al.*, 2007). However, development of a laboratory test that is effective in predicting risk of severe reactions would be of immense value to allergy practitioners and their patients. The aim of the current study was to investigate baseline levels of various allergic markers with respect to severity of allergic reactions and susceptibility to specific symptoms.

6.2 Methods

Methods are described in detail in chapter 2. In brief, baseline levels of serum tryptase, chymase, carboxypeptidase, DPPI and ACE, and saliva levels of tryptase, carboxypeptidase and DPPI, were investigated with respect to outcome of allergen challenge to foods and drugs. In addition concentrations of the markers were studied according to severity of reactions experienced in allergen challenge and within the community.

Paediatric food-allergic population

Concentrations of the markers described above were measured in baseline serum and saliva samples from 92 children presenting for diagnostic food challenge. Challenge outcome was recorded; and presenting signs/symptoms and causative dose were documented for subjects who reacted. For the purpose of analyses, results of only one challenge were included for each subject. Where children presented more than once, positive challenges were included preferentially. If children reacted in food challenge two or more times, results for the most severe reaction were used. In cases where a child had more than one negative challenge, but no positive challenges, the first challenge was selected.

Detailed information was also collected on the children's most severe historical allergic reaction to food within the community, including the causative allergen, nature of exposure, and symptoms experienced. Information on community reactions was recorded as described by the children and their parents, with support from medical notes where necessary. Reactions experienced in food challenge and within the community were assigned scores of severity based on the degree of allergen exposure and the resulting symptoms.

Results of skin prick tests and specific IgE measurements carried out previously for medical purposes were recorded for foods tested in allergen challenge. In addition,

information on co-existing atopic conditions, including asthma, eczema, hay fever and allergies to allergens other than foods, were noted.

Adult drug-allergic population

Levels of the markers described above were measured in baseline serum and saliva samples from 32 adults presenting for allergen testing to drugs. Results of skin prick and intra-dermal testing, and details of any presenting signs or symptoms, were recorded. In addition, information on most severe historical reactions experienced by subjects was documented, including the causative agent and symptoms experienced. As information on the degree of allergen exposure, i.e. the amount administered, was not easily obtainable, the scoring system used for food reactions could not be utilized. Instead reactions were grouped into those with mild, moderate or severe symptoms. Co-existing conditions, including asthma, eczema and hay fever, were also documented.

Analysis

The distribution of data for the markers was skewed, and thus non-parametric tests were used for analysis. For comparison of continuous variables between un-paired groups, Mann Whitney U Test or Kruskal Wallis Test was employed. Associations between continuous data sets were investigated using Spearman's Rank Correlation Coefficient.

6.3 Results

Food allergy

Ninety two children presenting for food challenge were recruited. The majority of challenges were to peanut, tree nuts, egg or milk. Eighty five of the children had previously experienced allergic reactions to foods. Saliva was collected from all children, but some chose not to provide blood samples. Information on the subjects and food challenges are presented in table 6.1, and historical reactions are presented in table 6.2.

Table 6.1 Patients and food challenges. Ninety two children attending for diagnostic food challenge were recruited.

Age	Range: 7 – 18 years; Mean: 12 years
Gender	Male: 62; Female: 30
Foods tested in allergen challenge	Peanut: 19; Tree nuts: 34; Egg: 23; Milk: 8; Seafood: 3; Fruit/veg: 1; Seeds: 1; Additives: 3
Food challenge outcome	Positive: 38; Negative: 54
Co-existing conditions	Asthma: 64; Eczema: 68; Hay fever: 65; Severe allergy to non-food allergen: 11

Table 6.2 Patients and historical reactions. Eighty five children had previously experienced allergic reactions to food.

Age	Range: 7 – 17 years; Mean: 12 years
Gender	Male: 59; Female: 26
Causative foods of historical reactions	Peanut: 33; Tree nuts: 9; Egg: 16; Milk: 13; Seafood: 4; Fruit/veg: 7; Seeds: 1; Soya: 1; Additives: 1

Food challenges

The average and range of baseline serum levels of tryptase, carboxypeptidase, chymase, DPPI and ACE, and saliva levels of tryptase, carboxypeptidase and DPPI, are presented in table 6.3. Concentrations were compared in children who did and did not react in food challenge, and also investigated with respect to reaction severity and specific symptoms experienced. Results of skin prick test and IgE measurement were also studied according to challenge outcome, and severity and symptoms of reactions.

Table 6.3 Baseline levels of serum and saliva mediators in children presenting for food challenge.

	Mean	Median	Range
Serum tryptase	2.24	1.96	0.64 – 8.03
Saliva tryptase	0.46	0.39	0.00 – 1.93
Serum chymase	13.3	5.70	0.78 – 65.1
Serum carboxypeptidase	21.9	20.7	1.93 – 69.6
Saliva carboxypeptidase	4.25	0.19	0.00 – 32.0
Serum DPPI	657	481	128 – 1910
Saliva DPPI	120	0.00	0.00 – 814
Serum ACE	467	454	201 – 873

Tryptase

Baseline serum and saliva tryptase levels were not significantly different in children who did and did not react in food challenge (Fig 6.1). A correlation was observed between baseline serum tryptase levels and reaction severity scores in children with systemic symptoms ($p = 0.037$, $r_s = 0.525$, $n = 16$) but not isolated oral

symptoms (Fig 6.2). Baseline salivary tryptase levels were significantly higher in children who reported oral pruritus in than in those who did not (p = 0.007). Many subjects who did not experience this symptom had concentrations below the lower limit of detection of the assay used. Serum and saliva levels of tryptase were not associated with any other symptom in reactions to food during allergen challenge.

Chymase

Levels of chymase measured in serum at baseline were no different in children with positive or negative food challenges (Fig 6.3) No associations were observed between concentrations of the protease and either reaction severity (Fig 6.4) or specific symptoms experienced.

Carboxypeptidase

Baseline serum levels of carboxypeptidase were significantly higher in children who reacted to food challenge than those who did not (p = 0.033; Fig 6.5). Salivary concentrations of the protease were no different in the two groups. ROC curve analysis was used to assess the usefulness of serum carboxypeptidase measurement in predicting positive food challenge. Using a cut off value of 29.4 ng/ml, the specificity was 90 %, but the sensitivity only 46 % (Fig 6.6). This resulted in positive and negative likelihood ratios of 4.6 and 0.6, respectively. Baseline concentrations of carboxypeptidase were correlated with severity scores of reactions occurring in challenge (p = 0.034, r_s = 0.501, n = 18; Fig 6.7), particularly in systemic reactions (p = 0.015, r_s = 0.706, n = 11). No associations were seen between levels of the protease in either serum or saliva and any specific symptoms occurring in food challenge.
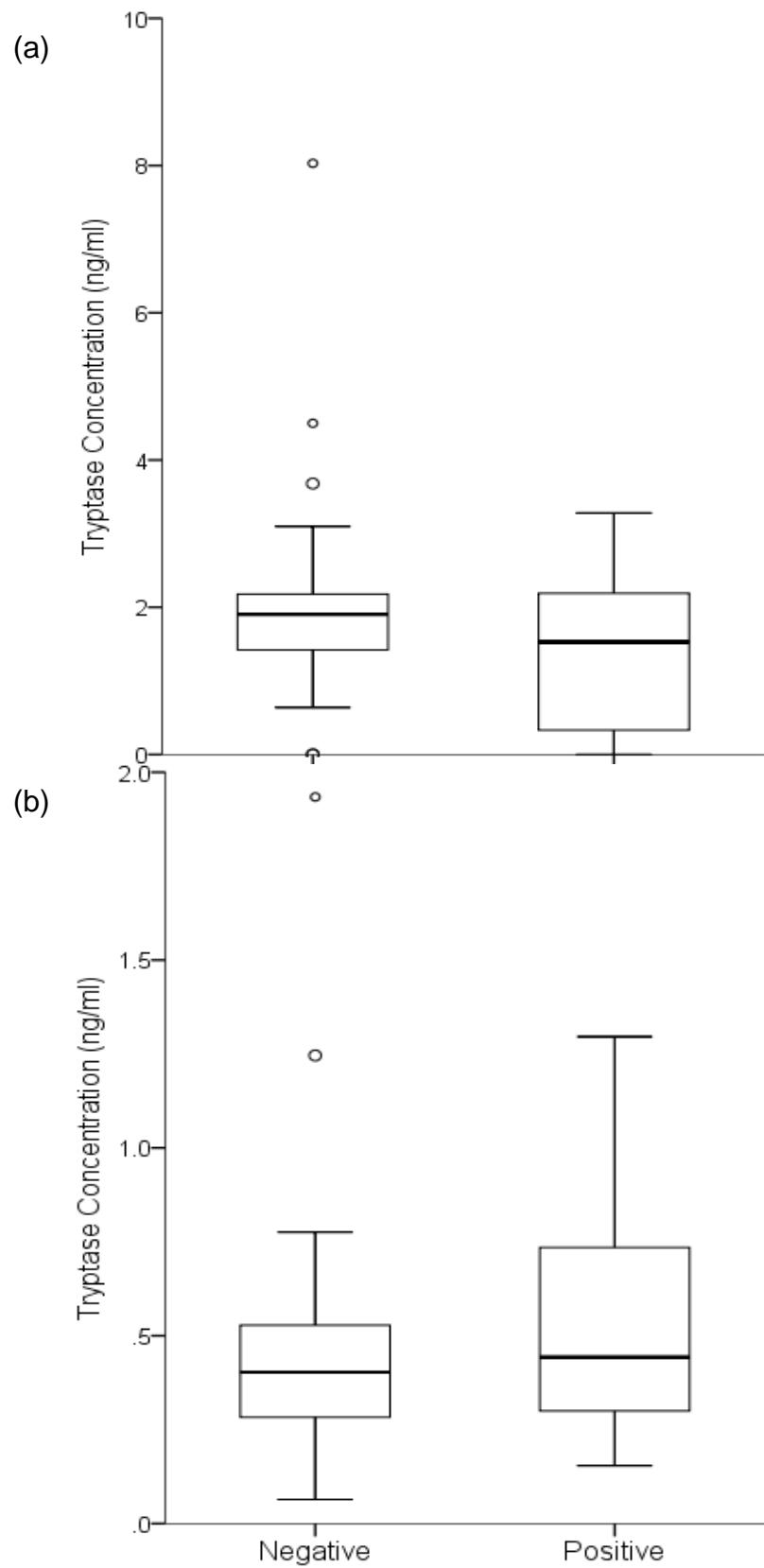
Fig 6.1 Baseline (a) serum and (b) saliva levels of tryptase in children with positive (n = 38) and negative (n = 54) food challenge. Levels were measured by ELISA, and analysis performed by Mann Whitney U Test.
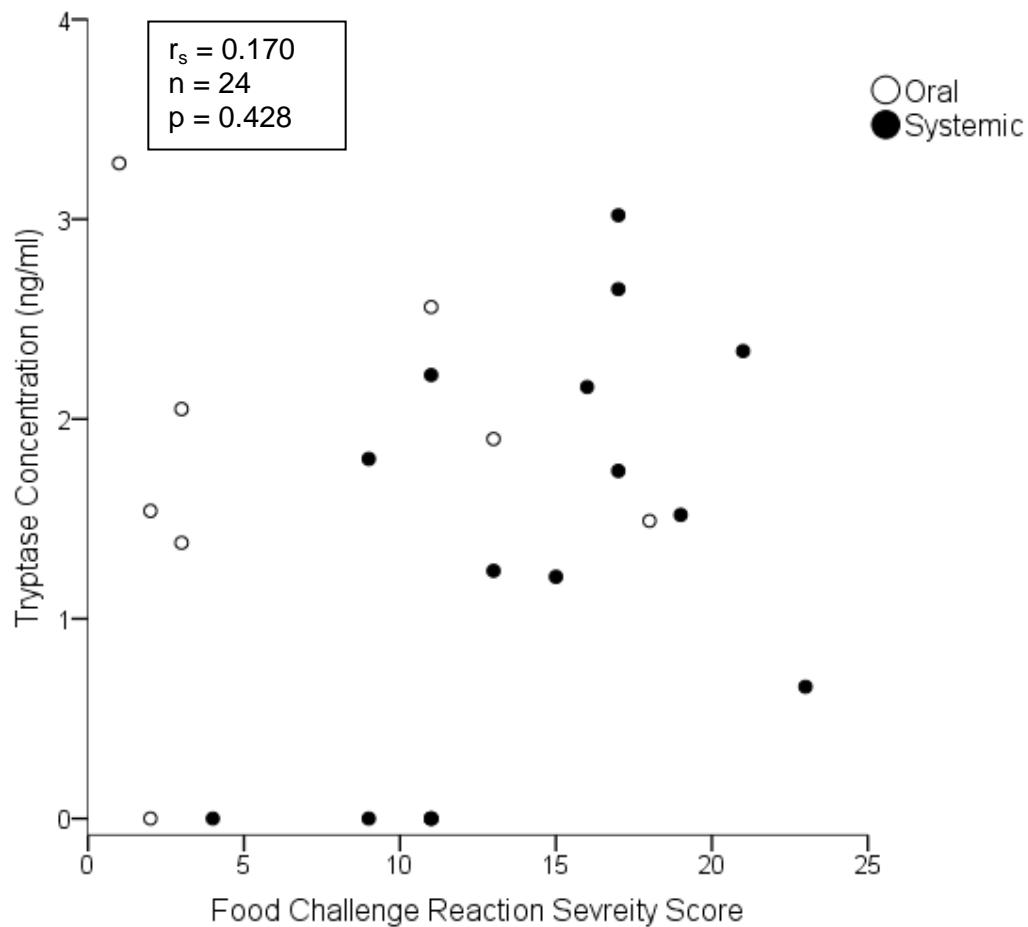
Fig 6.2 Association between baseline serum levels of tryptase and severity scores of reactions experienced in food challenge. Results are shown for reactions involving systemic and isolated oral symptoms separately. Analysis was performed using Spearman's Rank Correlation Coefficient.
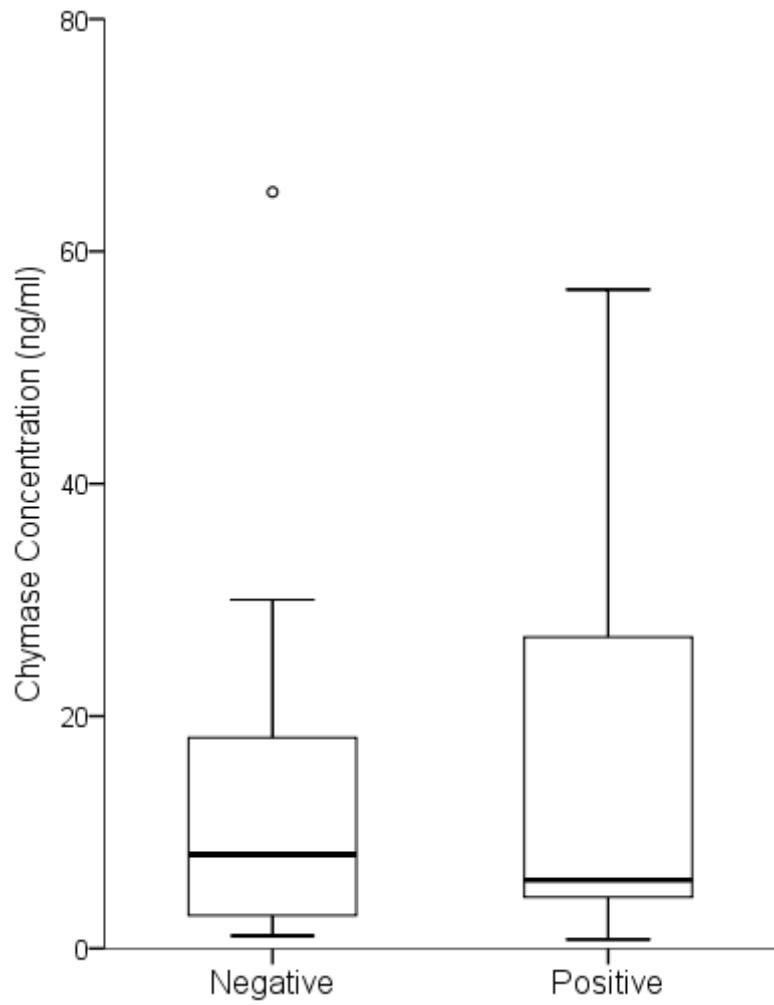
Fig 6.3 Baseline serum chymase levels in children with positive (n = 12) and negative (n = 29) food challenge. Levels were measured by ELISA, and analysis performed by Mann Whitney U Test.
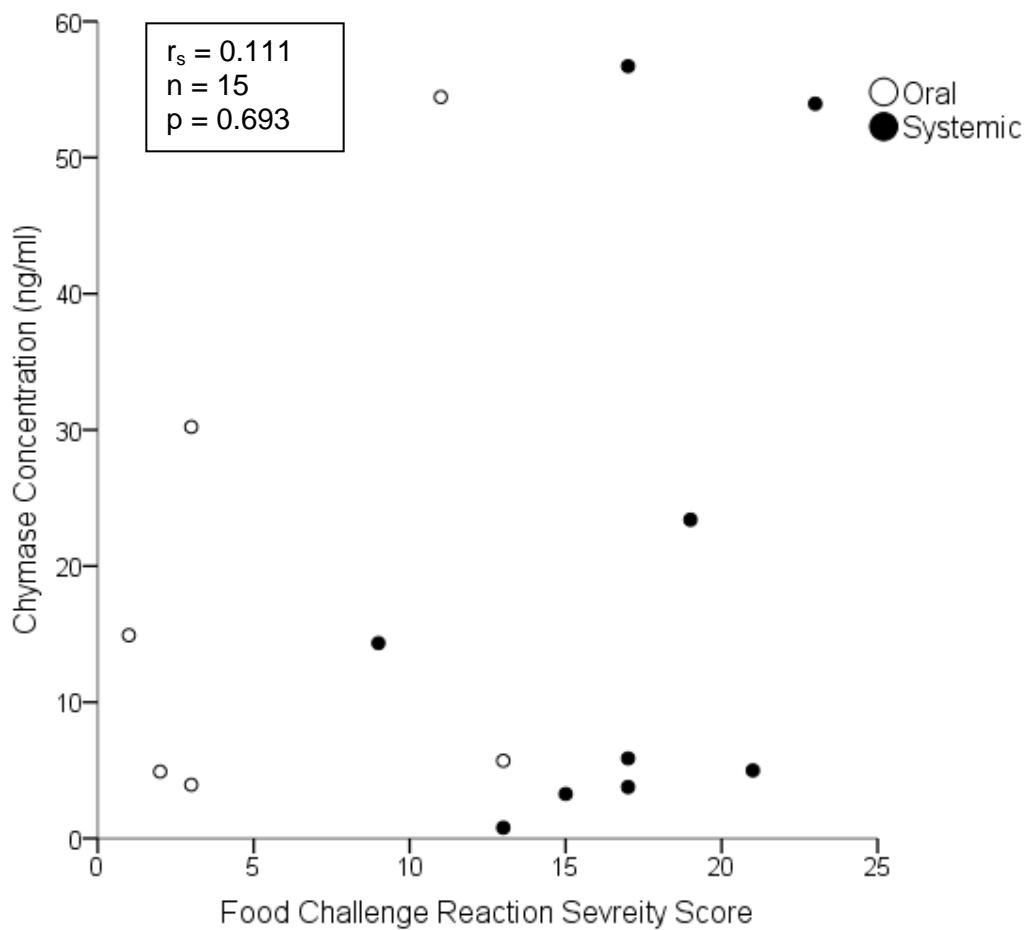
Fig 6.4 Association between baseline serum levels of chymase and severity scores of reactions experienced in food challenge. Results are shown for reactions involving systemic and isolated oral symptoms separately. Analysis was performed using Spearman's Rank Correlation Coefficient.
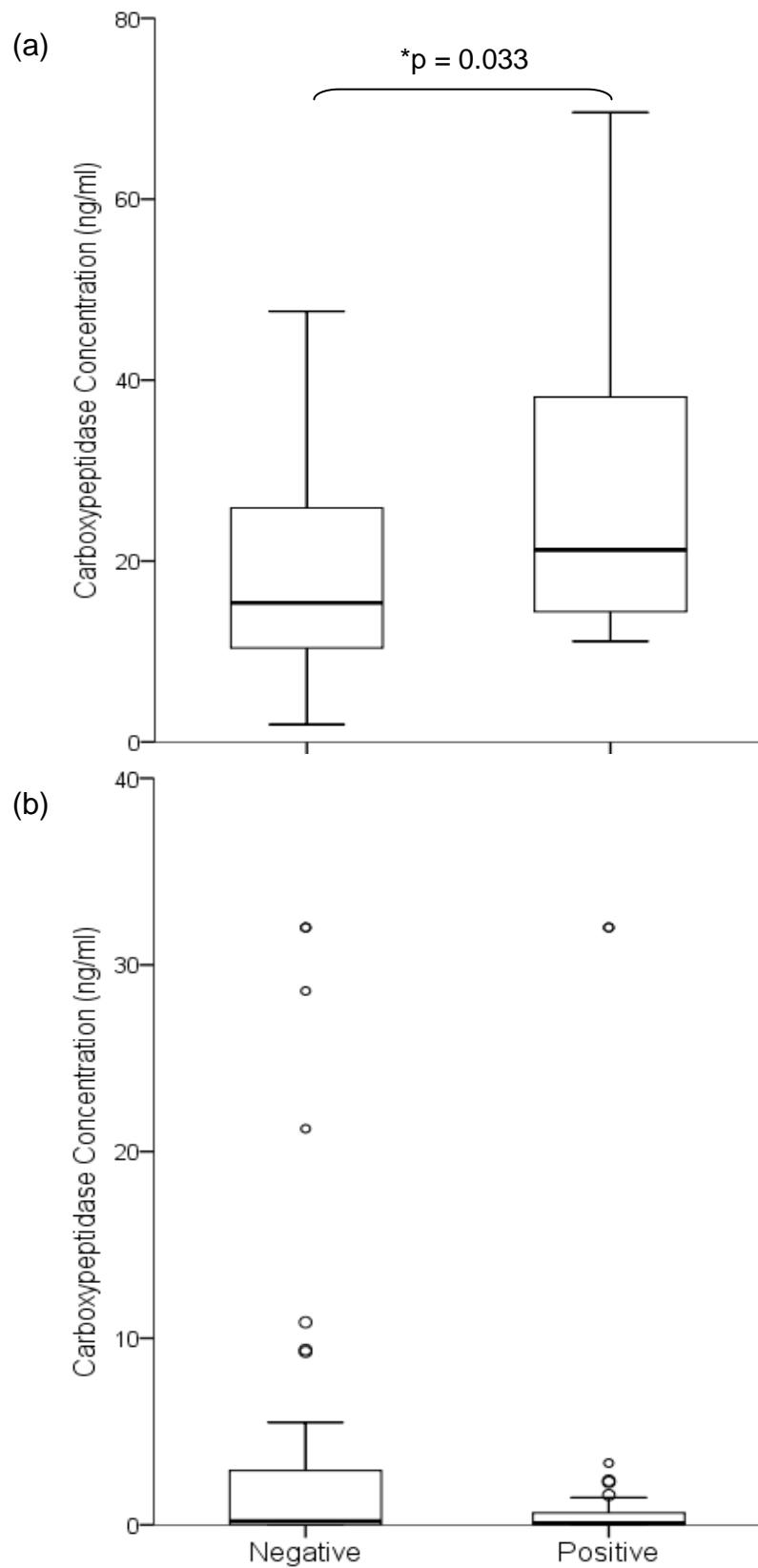
Fig 6.5 Baseline (a) serum and (b) saliva levels of carboxypeptidase in children with positive (n = 38) and negative (n = 54) food challenge. Levels were measured by ELISA, and analysis performed by Mann Whitney U Test.

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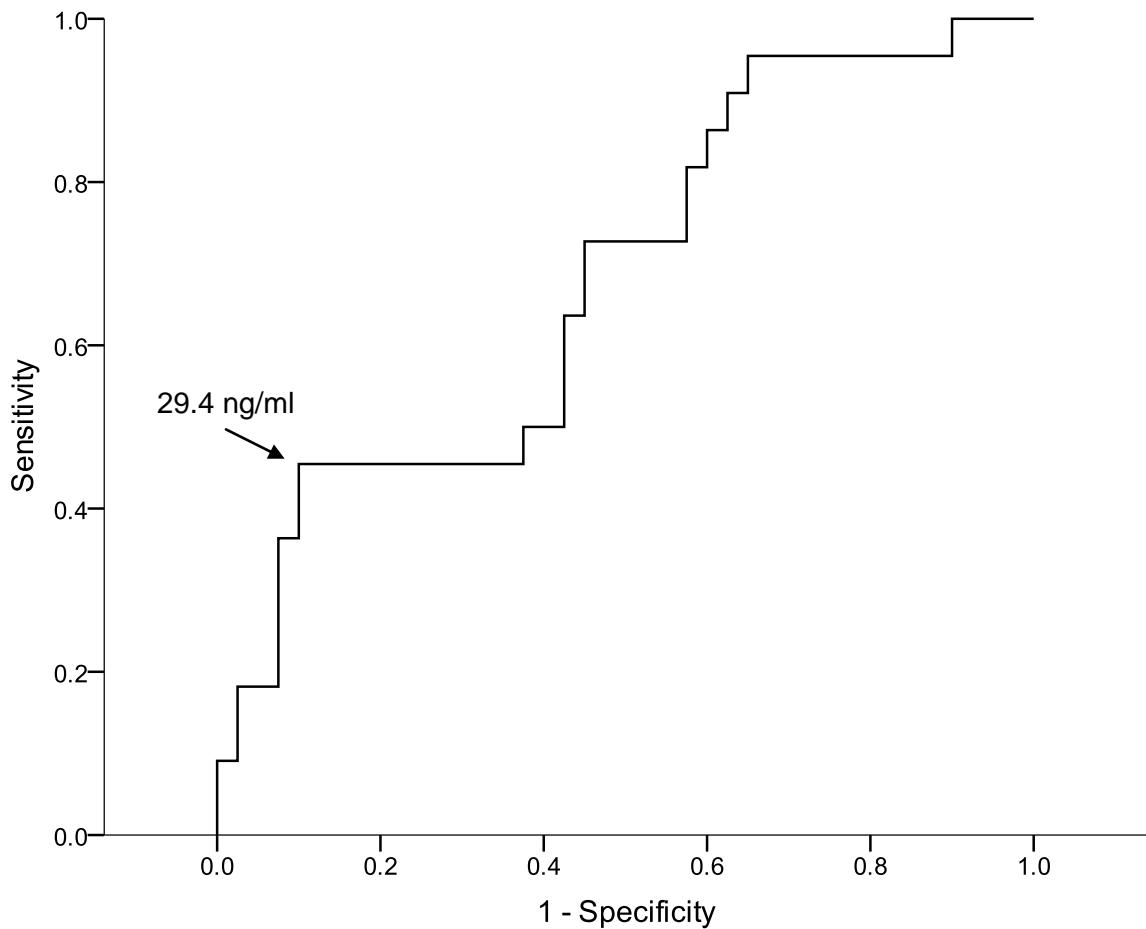
Fig 6.6 ROC curve analysis for determining specificity and sensitivity of baseline carboxypeptidase measurement in predicting positive food challenge. The concentration with optimal specificity and sensitivity was determined.
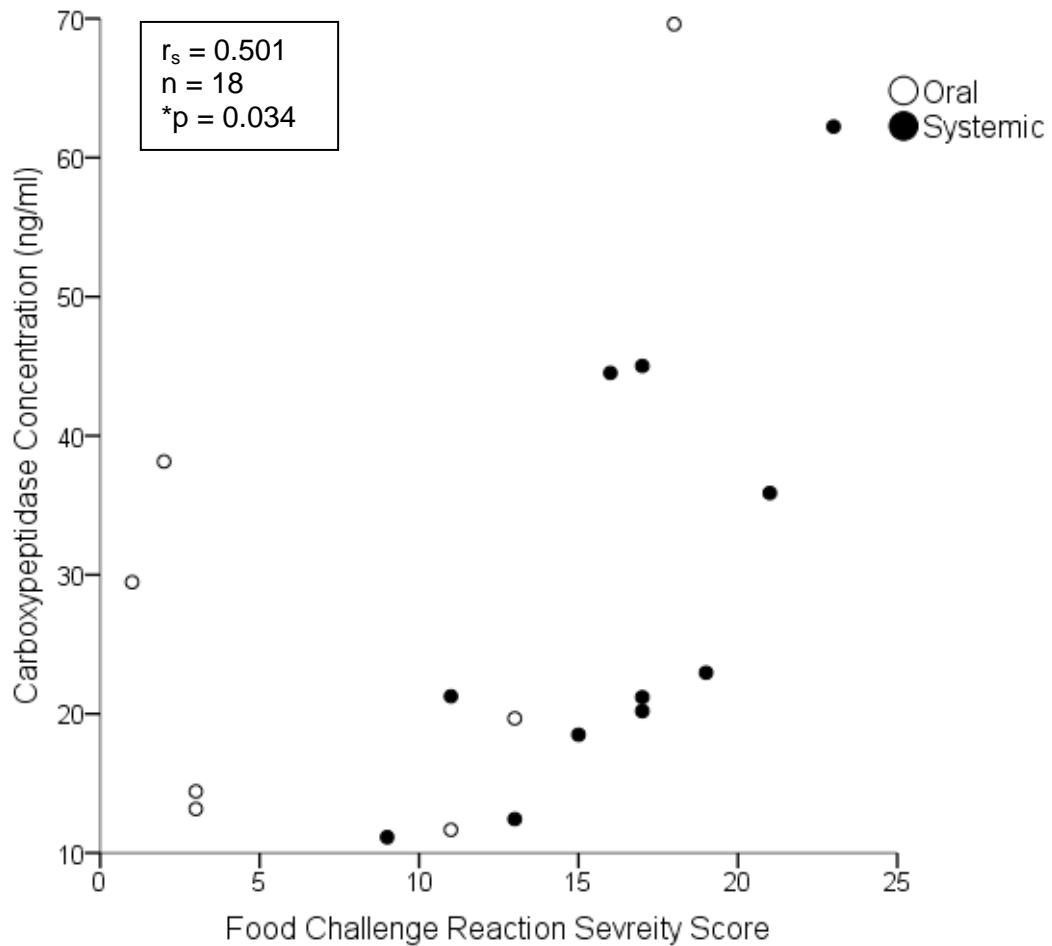
Fig 6.7 Association between baseline serum levels of carboxypeptidase and severity scores of reactions experienced in food challenge. Results are shown for reactions involving systemic and isolated oral symptoms separately. Analysis was performed using Spearman's Rank Correlation Coefficient.

DPPI

There was a trend for higher baseline serum levels of DPPI in children who reacted to food challenge that those who did not, but this was not statistically significant (p = 0.102; Fig 6.8). However, concentrations of the protease were correlated with severity scores of reactions in subjects with a positive outcome (p = 0.022, r_s = 0.535, n = 18; Fig 6.9). In addition, serum levels of DPPI were higher at baseline in children with throat tightness during food challenge than in those without this symptom (p = 0.01). Baseline concentrations of DPPI in saliva were similar in children with positive and negative outcomes in food challenge. Levels were not associated with severity of reactions or specific symptoms experienced.

ACE

Serum levels of ACE at baseline were not significantly different in children who reacted and did not react in food challenge (Fig 6.10); and concentrations were not associated with severity of reactions in subjects who tested positive (Fig 6.11). However, levels were significantly higher in patients who experienced oral oedema in food challenge than those who did not (p = 0.024).Concentrations were not associated with any other symptom.

Skin prick test and specific IgE measurement

Skin prick test (SPT) diameter was significantly larger in patients who reacted in food challenge than those who did not (p < 0.001; Fig 6.12(a)). Furthermore, SPT diameter was correlated with reaction severity scores (p < 0.001, r_s = 0.420, n = 83). However, no association was seen when excluding negative challenges. Levels of specific IgE were associated with SPT diameter for the same food allergen (p = 0.001, r_s = 0.397, n = 62). However, levels were not significantly different in children who had positive or negative food challenges (Fig 6.12(b)). In addition, levels were not associated with reaction severity scores.
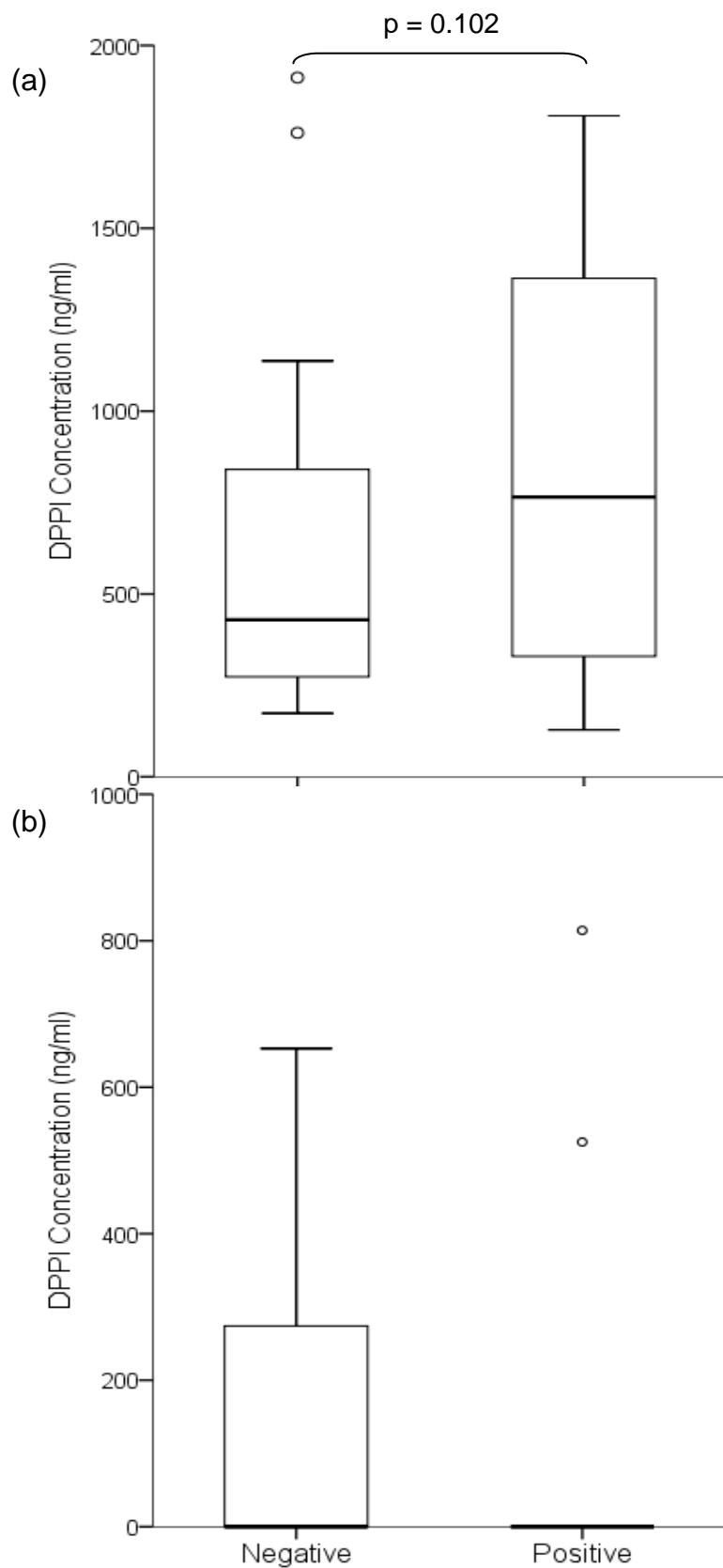
Fig 6.8 Baseline (a) serum and (b) saliva levels of DPPI in children with positive (n = 38) and negative (n = 54) food challenge. Levels were measured by ELISA, and analysis performed by Mann Whitney U Test.
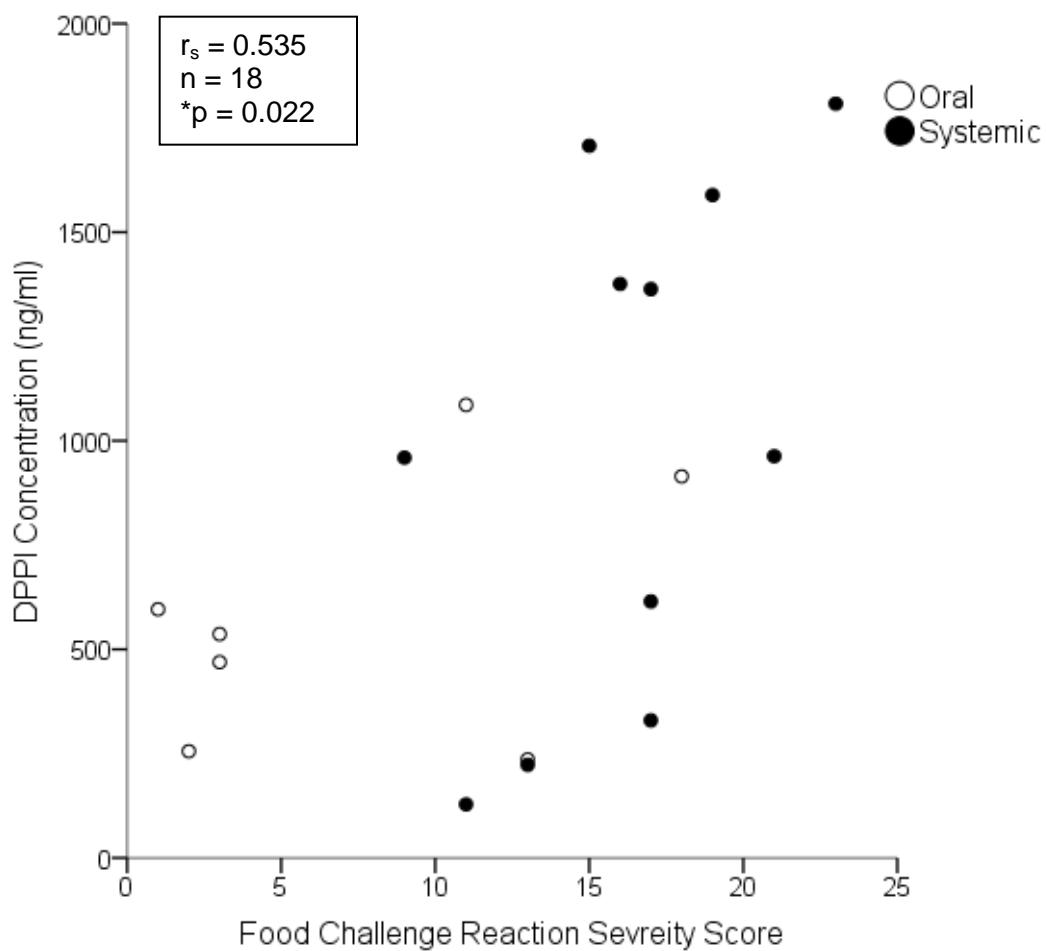
Fig 6.9 Association between baseline serum levels of DPPI and severity scores of reactions experienced in food challenge. Results are shown for reactions involving systemic and isolated oral symptoms separately. Analysis was performed using Spearman's Rank Correlation Coefficient.
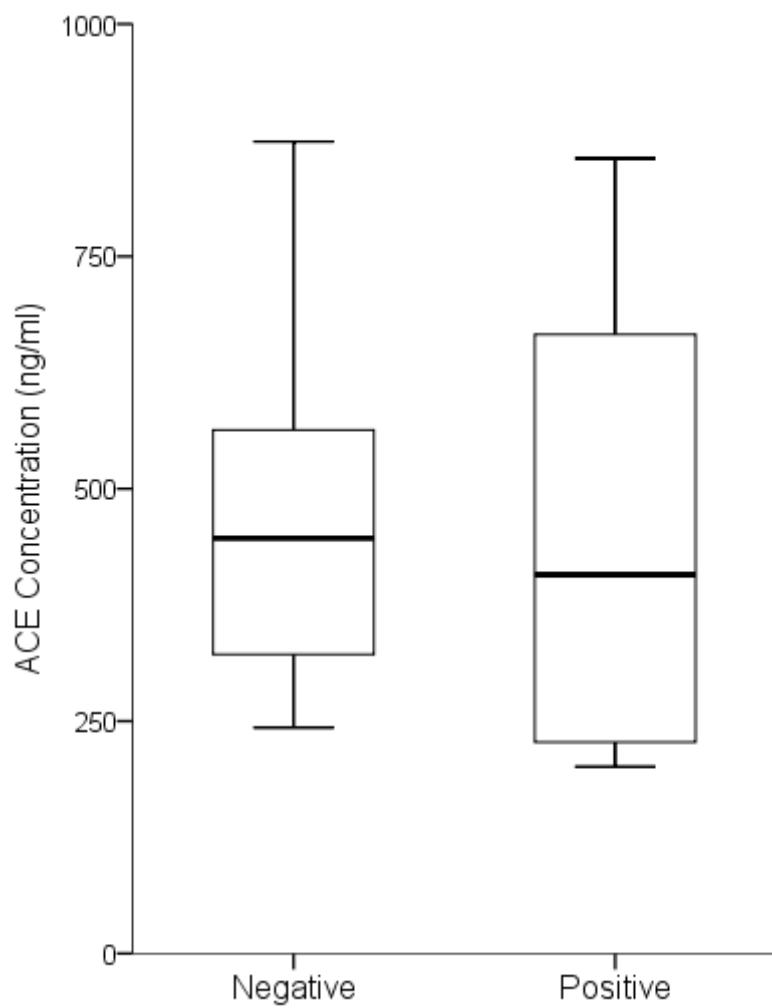
Fig 6.10 Baseline serum levels of ACE in children with positive (n = 18) and negative (n = 32) food challenge. Levels were measured by ELISA, and analysis performed by Mann Whitney U Test.

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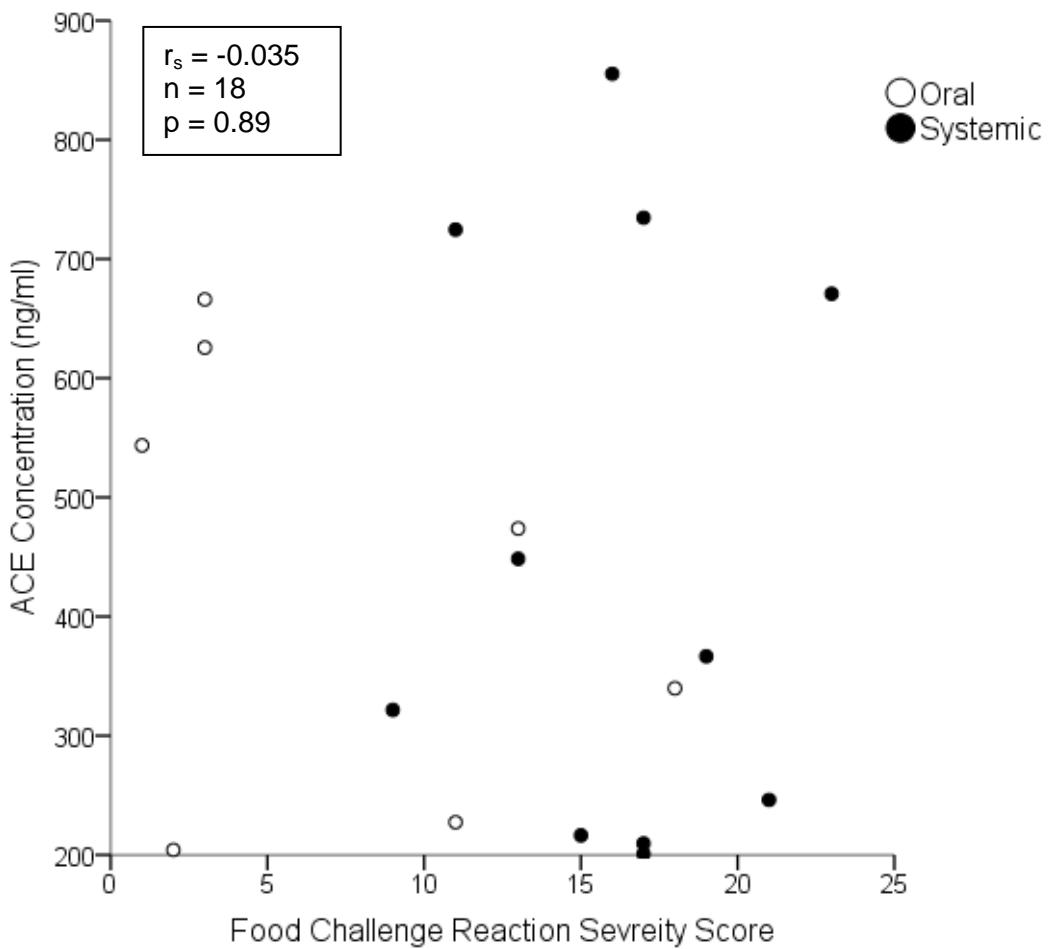
Fig 6.11 Association between baseline serum levels of ACE and severity scores of reactions experienced in food challenge. Results are shown for reactions involving systemic and isolated oral symptoms separately. Analysis was performed using Spearman's Rank Correlation Coefficient.
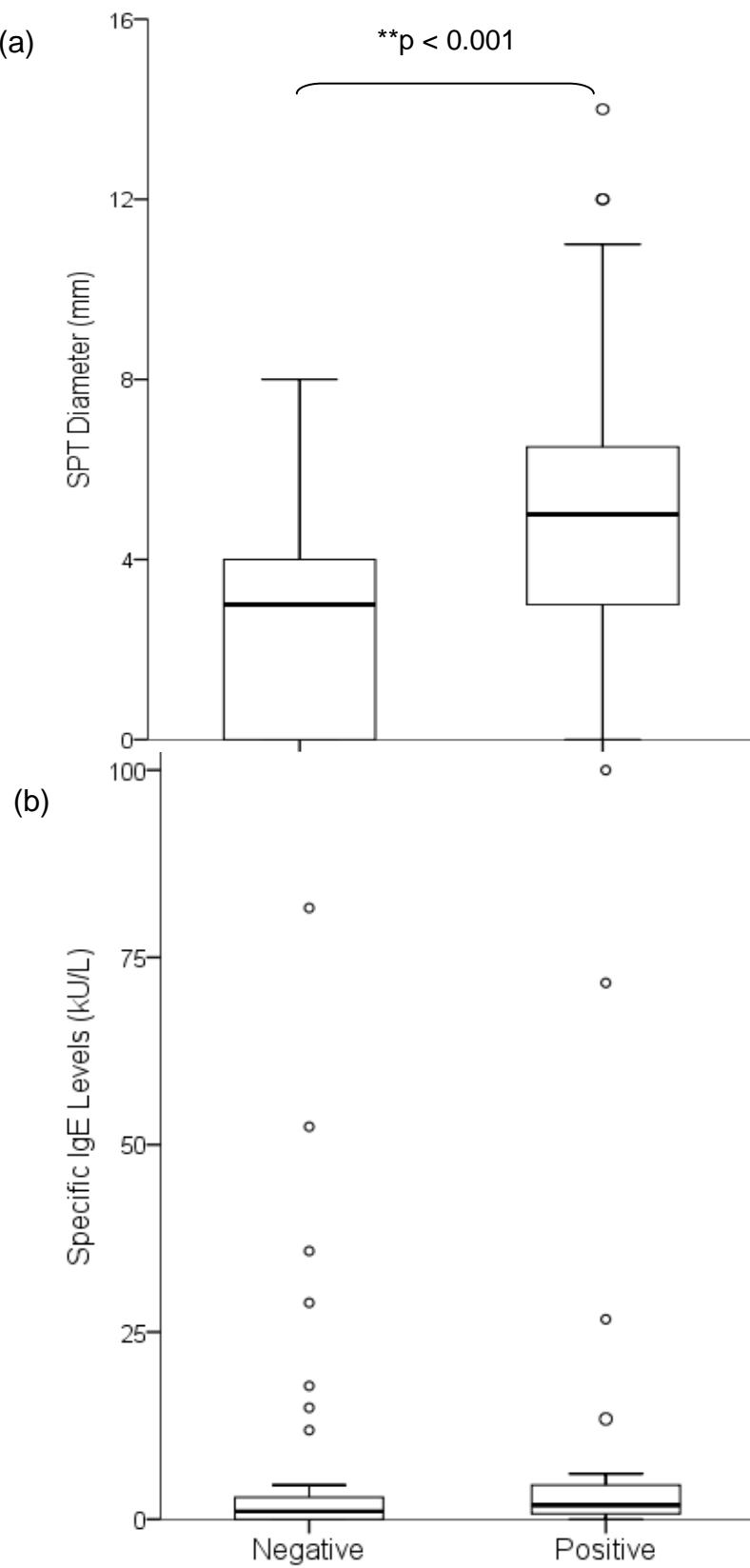
Fig 6.12 (a) SPT diameter and (b) specific IgE levels in children with positive (n = 38) and negative (n = 54) food challenge. Analysis performed by Mann Whitney U Test.

Historical reactions to food

Baseline serum levels of tryptase, carboxypeptidase, chymase, DPPI and ACE, and saliva levels of tryptase, carboxypeptidase and DPPI, were investigated with respect to reaction severity and specific symptoms experienced in children's worst historical reactions to foods. Results of skin prick test and IgE measurement were also studied according to severity and symptoms of these reactions.

Tryptase

No association was seen between baseline levels of serum or saliva tryptase and severity of worst historical allergic reactions to foods (Fig 6.13). Concentrations in serum were significantly higher in children who reported experiencing urticaria during reactions to food than in those who did not ($p = 0.032$). However, levels were not related to any other symptoms. Saliva tryptase concentrations were not associated with any symptoms experienced in historical reactions.

Chymase

Baseline levels of serum chymase were not associated with severity of historical allergic reactions to food (Fig 6.14). However, concentrations were higher in those who reported cyanosis than those who did not ($p = 0.021$).

Carboxypeptidase

Baseline serum carboxypeptidase levels were associated with severity scores of children's worst historical reactions to food ($p = 0.009$, $r_s = 0.382$, $n = 46$; Fig 6.15(a)). Levels were higher in those who reported experiencing oedema than those who did not ($p = 0.033$). This effect was true specifically for oedema of the oral mucosa ($p = 0.039$). In addition, there was a trend for higher baseline serum carboxypeptidase levels in children who experienced throat tightness than those who did not ($p = 0.063$). However, this did not reach statistical significance.
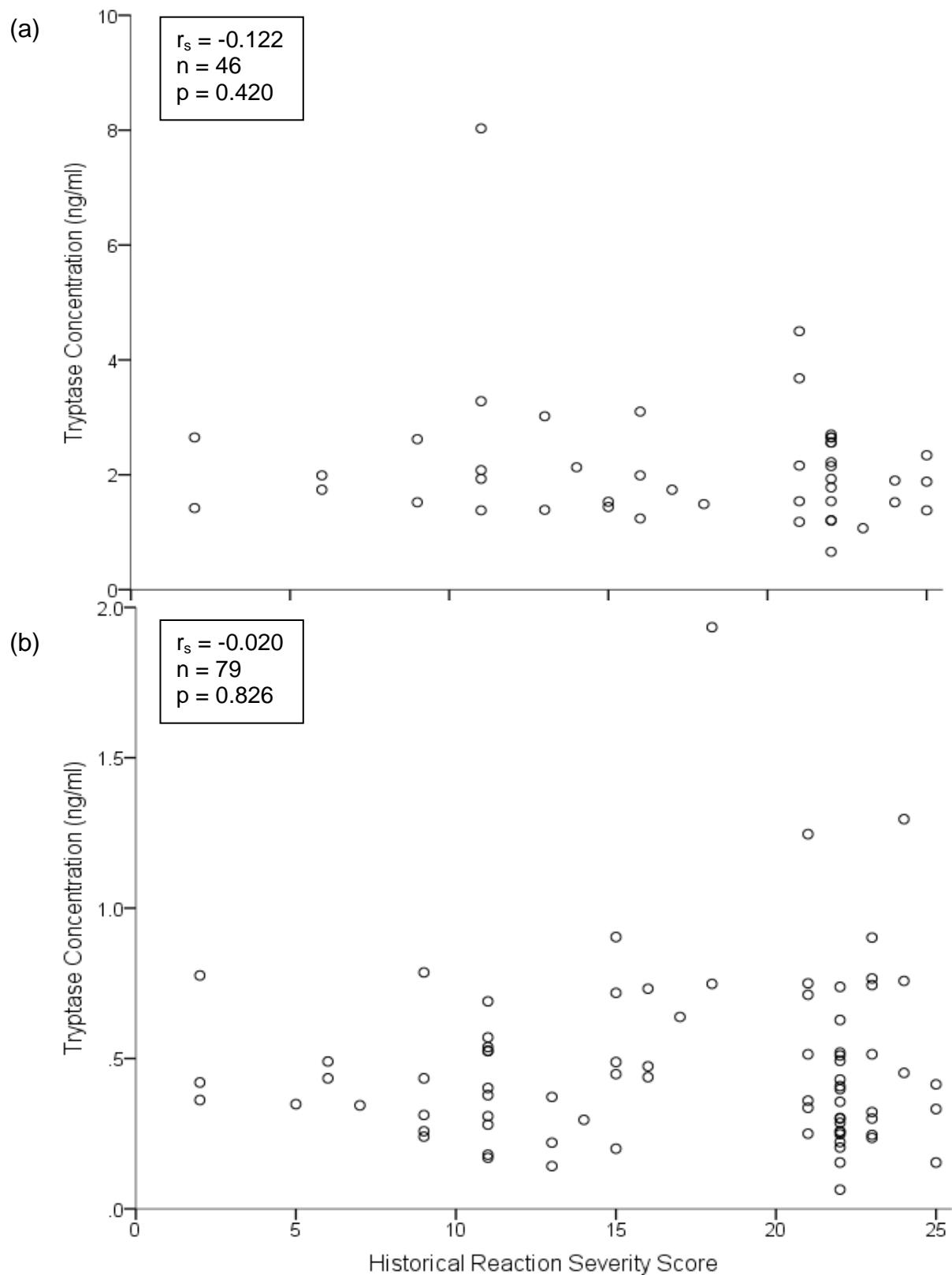
Fig 6.13 Association between baseline (a) serum and (b) saliva levels of tryptase and severity scores of worst historical reactions. Levels were measured by ELISA, and scores are based on severity of symptoms and nature of allergen exposure. Analysis was performed using Spearman's Rank Correlation Coefficient.

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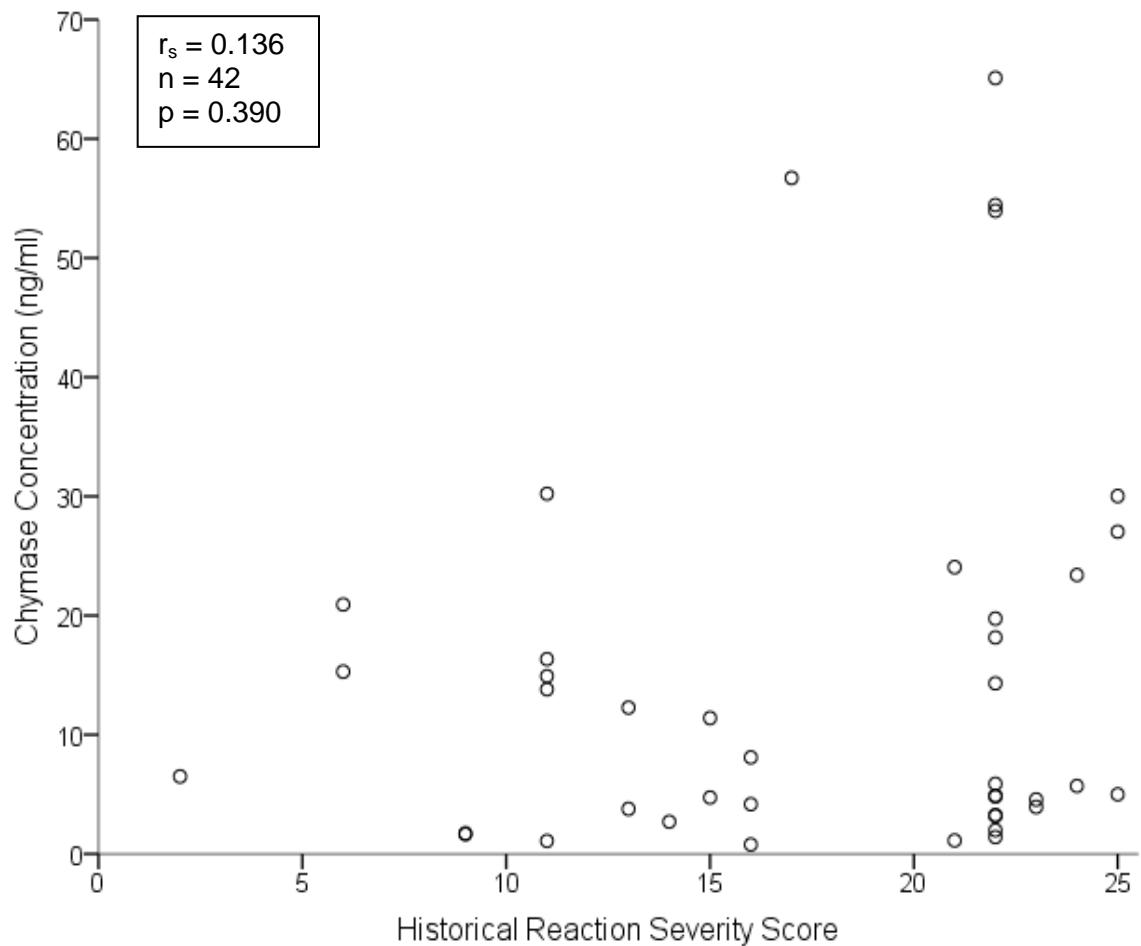
Fig 6.14 Association between baseline serum levels of chymase and severity scores of worst historical reactions. Levels were measured by ELISA, and scores are based on severity of symptoms and nature of allergen exposure. Analysis was performed using Spearman's Rank Correlation Coefficient.
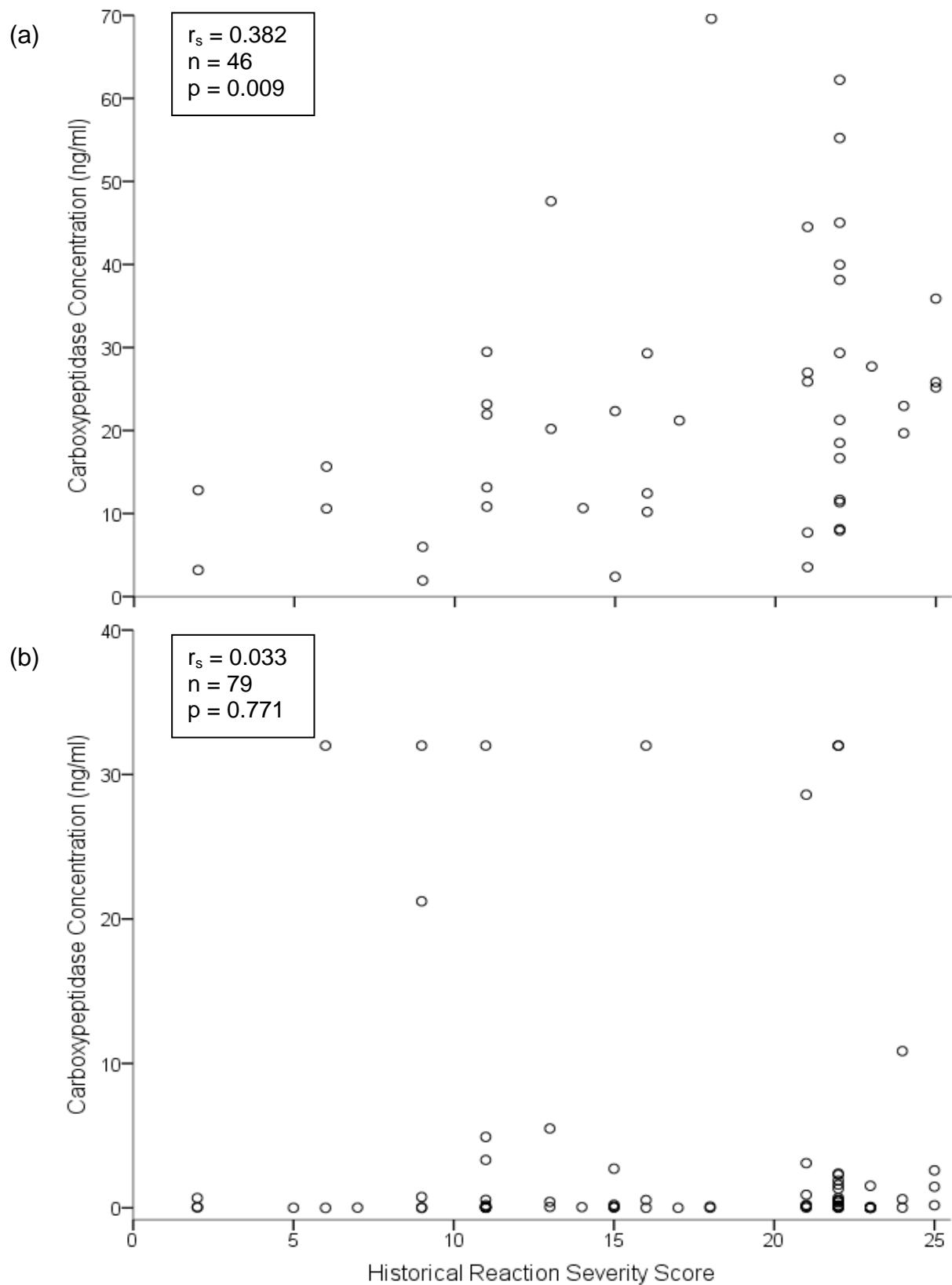
Fig 6.15 Association between baseline (a) serum and (b) saliva levels of carboxypeptidase and severity scores of worst historical reactions. Levels were measured by ELISA, and scores are based on severity of symptoms and nature of allergen exposure. Analysis was performed using Spearman's Rank Correlation Coefficient.

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Similarly, there was a non-significant trend for higher concentrations in patients with wheeze than in children without this symptom (p = 0.1). No associations were observed between baseline levels of carboxypeptidase in saliva and severity of, or symptoms experienced in, historical reactions (Fig 6.15(b)).

DPPI and ACE

Serum concentrations of DPPI and ACE at baseline did not correlate with severity scores of children's worst historical reactions (Fig 6.16 and 6.17, respectively). Furthermore, levels were not related to any specific symptoms. Similarly, baseline salivary DPPI concentrations were not associated with reaction severity or specific symptoms experienced historically.

Co-existing atopic conditions

Eighty five (93 %) of the children recruited reported currently or previously having co-existing atopic conditions. These included asthma, eczema, hay fever and/or allergies to allergens other than foods. Associations between the presence of co-existing atopic conditions and reaction severity/symptomatology were examined. In addition, baseline levels of the markers listed above were compared in those who reported having the conditions and those who did not.

Asthma

Seventy six per cent of children had asthma. Severity scores for historical reactions to food were significantly higher in subjects with coexisting asthma than in those without (p = 0.009; Fig 6.18(a)). Similarly, the percentage of children with asthma was highest in the group with severe reactions to food; and lowest in those with mild reactions (p = 0.031). Children with asthma more often experienced respiratory difficulties during reactions to food (p = 0.011), including wheeze (p = 0.039). However, baseline levels of tryptase, chymase, carboxypeptidase, DPPI or ACE in serum or saliva were no different in children with and without asthma.

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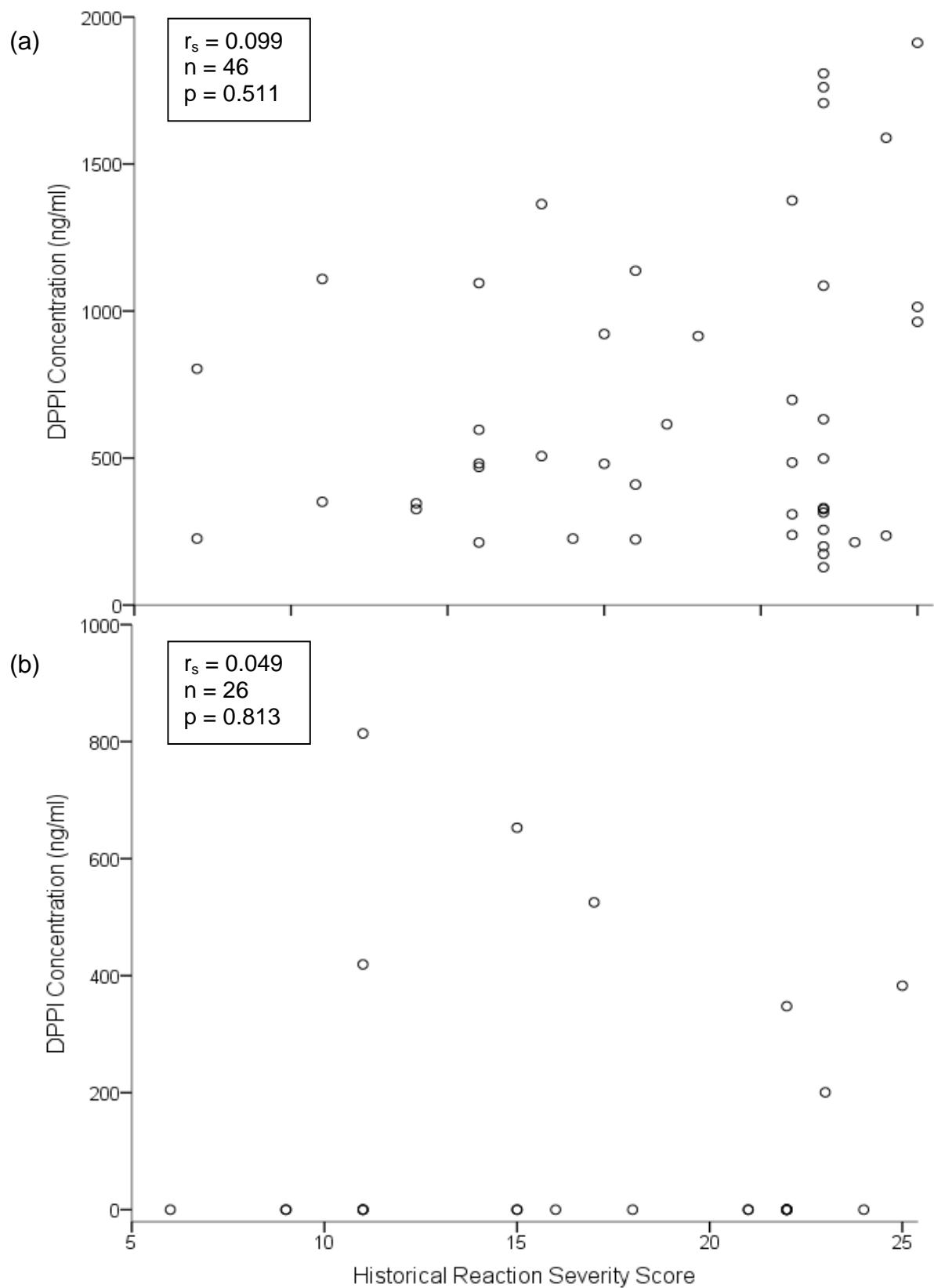
Fig 6.16 Association between baseline (a) serum and (b) saliva levels of DPPI and severity scores of worst historical reactions. Levels were measured by ELISA, and scores are based on severity of symptoms and nature of allergen exposure. Analysis was performed using Spearman's Rank Correlation Coefficient.
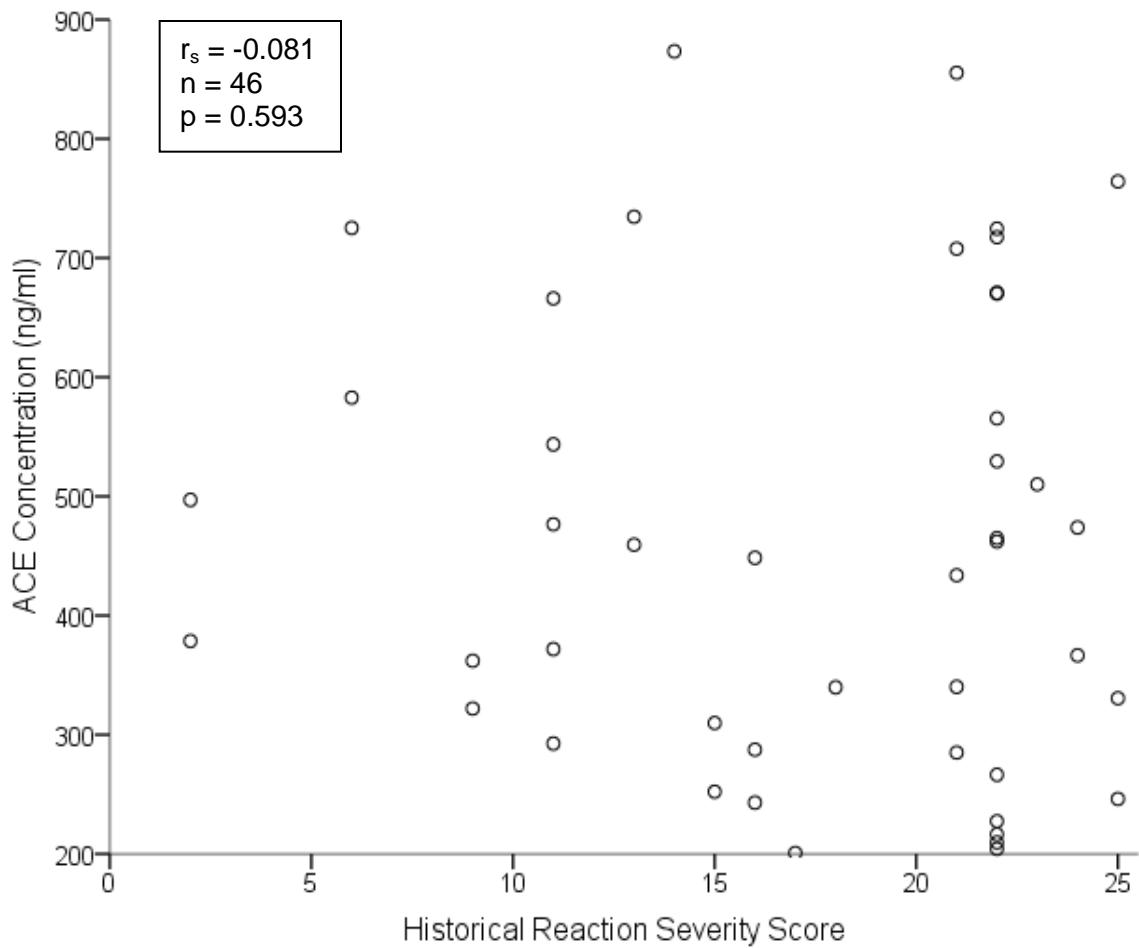
Fig 6.17 Association between baseline serum levels of ACE and severity scores of worst historical reactions. Levels were measured by ELISA, and scores are based on severity of symptoms and nature of allergen exposure. Analysis was performed using Spearman's Rank Correlation Coefficient.
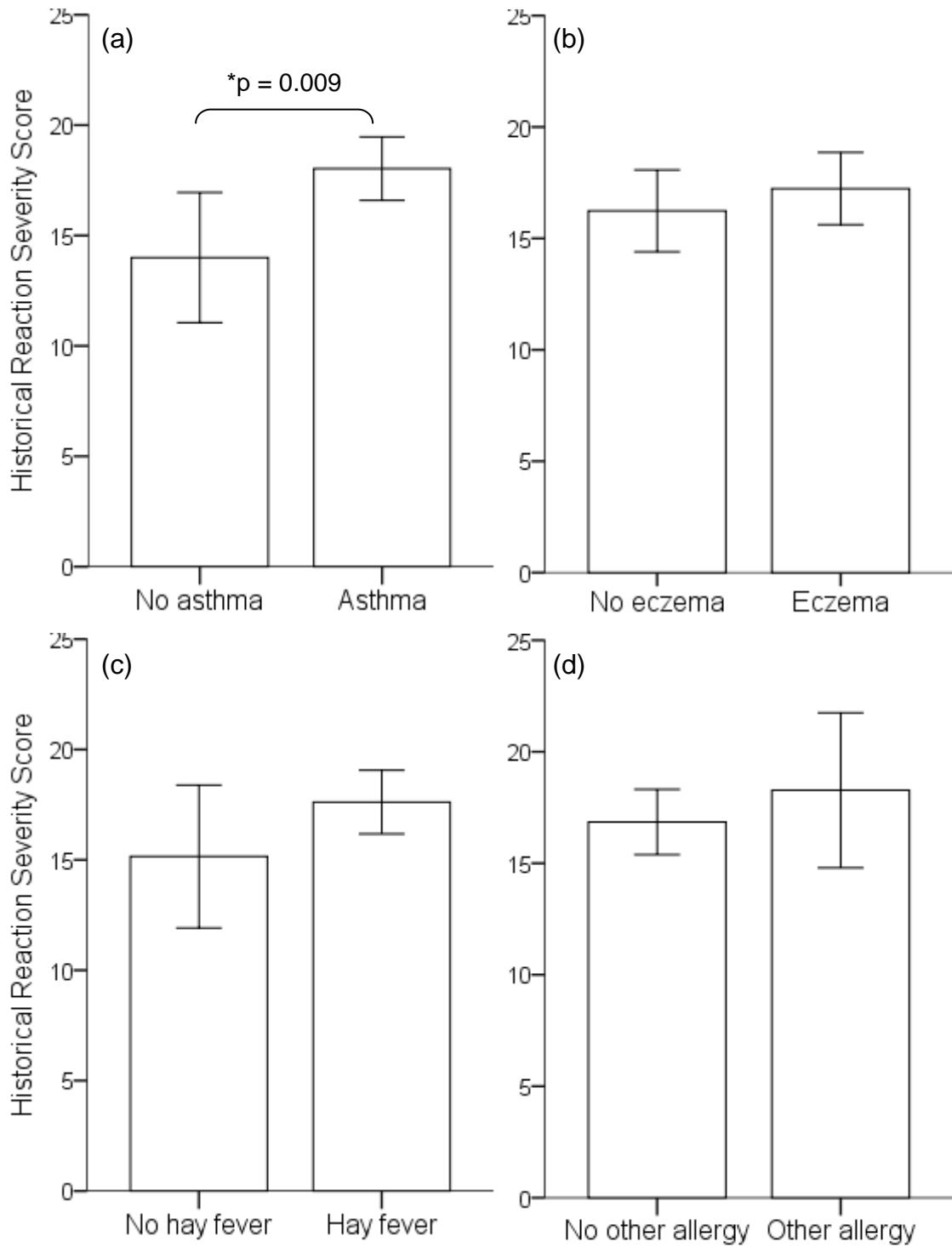
Fig 6.18 Severity scores of historical reactions in children with and without a history of (a) asthma, (b) eczema, (c) hay fever and (d) previous severe reactions to non-food allergens. Graphs show mean scores and standard error. Statistical analysis was performed using Independent Samples T Test.

Eczema

Eighty one per cent of the children recruited on to the study had experienced eczema. No association was observed between co-existing eczema and either severity of reactions or specific symptoms experienced in response to foods (Fig 6.18(b)). There were no differences in serum levels of any of the markers in children with and without the condition. However, children with a history of eczema had higher baseline levels of salivary carboxypeptidase (but not tryptase, chymase, DPPI or ACE) than those without (p = 0.041).

Hay fever

Seventy seven per cent of subjects reported having experienced hay fever. Severity scores for reactions to food were not significantly different in children with and without co-existing hay fever (Fig 6.18(c)). There was a trend for an association between hay fever and the symptom pruritus during reactions to food. However, this did not reach statistical significance (p = 0.083). Baseline serum and saliva levels of carboxypeptidase were significantly higher in children with a history of hay fever than in those without (p = 0.008 and 0.024, respectively). No association was seen between serum or saliva levels of tryptase, chymase, DPPI or ACE and the condition.

Reactions to other allergens

Thirteen per cent of subjects had allergies to allergens other than foods, and thirteen had previously experienced severe reactions to drugs, animals, dust mites or pollens. There was no significant difference in severity scores of reactions to food in children with and without other (non-food) allergies (Fig 6.18(d)). Furthermore, scores were no different in those who had previously experienced severe symptoms to allergens other than food. Baseline serum/saliva levels of the markers described above were not significantly different in children with and without allergies to non-food allergens.

Correlations between markers

Baseline serum tryptase concentrations did not correlate with levels any other marker. However, serum DPPI levels were associated with those of both chymase ($p < 0.001$, $r_s = 0.566$, $n = 42$) and carboxypeptidase ($p = 0.006$, $r_s = 0.345$, $n = 64$) at baseline. Levels of DPPI and carboxypeptidase were also correlated after food challenge (described in Chapter 5). However, the level of significance was greatest between baseline levels of carboxypeptidase and post challenge levels of DPPI ($p < 0.001$, $r_s = 0.49$, $n = 51$). As for tryptase, baseline serum ACE concentrations were not associated with those of any other marker. Similarly, no associations were seen between markers in saliva.

Drug allergy

Thirty two adults presenting for drug challenge were recruited. The majority of challenges were to antibiotics and non-opiate analgesics. Twenty seven subjects had previously experienced allergic reactions to drugs. Information on the subjects, drug challenges and historical reactions are presented in tables 6.4 and 6.5.

Table 6.4 Patients and drug challenges. Thirty two adults attended for diagnostic drug challenge were recruited. NMBA: neuromuscular blocking agent.

Age	Range: 17 – 82 years; Mean: 50 years
Gender	Male: 6; Female: 26
Number of visits	One: 29; Two: 4
Number of drugs tested	One: 5; Two: 11; Three: 8; Four: 5; Five: 3
Drugs challenged	NMBA: 8; Hypnotic: 9; Opiate: 9; Antibiotic: 23; Non-opiate analgesic: 18; Antiseptic: 9; Other: 10
Outcome of skin testing	Positive: 15; Negative: 21
Additional symptoms	Present: 9; Not present: 6
Co-existing conditions	Asthma: 8; Eczema: 2; Hay fever: 8

Table 6.5 Patients and historical reactions. Twenty seven adults had previously experienced reactions to drugs.

Age	Range: 17 – 82 years; Mean: 49 years
Gender	Male: 6; Female: 21
Drug tested in allergen challenge	Opiate: 1; Antibiotic: 13; Non-opiate analgesic: 5; Antiseptic: 3; Other: 5

Drug challenges

The average and range of baseline serum levels of tryptase, carboxypeptidase, chymase, DPPI and ACE, and saliva levels of tryptase, carboxypeptidase and DPPI are presented in table 6.6. Concentrations were compared in adults with positive and negative skin testing in drug challenge. Levels were also investigated according to whether symptoms were experienced (in addition to positive skin test).

Table 6.6 Baseline levels of serum and saliva mediators in adults presenting for drug challenge.

	Mean	Median	Range
Serum tryptase	1.78	1.75	0.00 – 5.63
Saliva tryptase	0.00	0.00	0.00 – 0.14
Serum chymase	5.62	5.90	0.00 – 13.1
Serum carboxypeptidase	10.2	9.26	1.64 – 22.1
Saliva carboxypeptidase	1.84	0.40	0.00 – 25.1
Serum DPPI	475	401	212 – 1253
Saliva DPPI	260	251	128 – 519
Serum ACE	191	191	99.3 - 346

Tryptase and chymase

There was no significant difference in serum levels of either tryptase or chymase in patients with positive and negative drug challenges. Furthermore, concentrations were no different in subjects who experienced symptoms in addition to responding in skin test. Saliva tryptase levels were similar in all patients.

Carboxypeptidase

Baseline serum and saliva levels of carboxypeptidase were no different in patients who tested positive and negative in drug challenge. However, concentrations of the protease in saliva were significantly higher in subjects who experienced symptoms in addition to responding in skin test ($p = 0.022$). More specifically, levels were higher in patients with oral pruritus than in those without ($p = 0.014$).

DPPI and ACE

Baseline serum and saliva levels of DPPI, and serum ACE concentrations, were similar in patients with positive and negative outcomes in drug challenge. Levels were also no different in those who experienced symptoms in addition to responding in skin test.

Historical reactions to drugs

Baseline serum levels of tryptase, carboxypeptidase, chymase, DPPI and ACE, and saliva levels of tryptase, carboxypeptidase and DPPI, were investigated with respect to reaction severity and specific symptoms experienced in subject's worst historical reactions to drugs.

Tryptase and chymase

No association was observed between baseline serum levels of tryptase or chymase and severity of historical reactions to drugs (Fig 6.19(a) and (b), respectively). Furthermore, concentrations were not related to any symptoms experienced during previous allergic reactions. Similarly, baseline salivary tryptase levels were not associated with severity or symptomatology of historical reactions.

Carboxypeptidase

Serum baseline levels of carboxypeptidase were significantly associated with severity of historical reactions to drugs ($p = 0.008$; Fig 6.20(a)). Concentrations were higher in subjects with a history of severe symptoms than in those with mild or moderate symptoms ($p = 0.003$ and 0.021, respectively). There was also a trend for lower levels in patients with mild compared to moderate reactions. However, this did not reach statistical significance ($p = 0.082$). Baseline serum carboxypeptidase levels were significantly higher in subjects with cardiovascular symptoms in worst historical reactions to drugs than in those without ($p = 0.01$). More specifically, concentrations were higher in patients with hypotension, as defined by a blood pressure below 100/60 mmHg ($p = 0.003$), and there was a trend for higher levels in those with collapse ($p = 0.055$; non-significant). No association was observed between baseline levels of carboxypeptidase in saliva and severity of worst historical reactions to drugs. Furthermore, concentrations were not related to occurrence of specific symptoms.

DPPI and ACE

As for tryptase and chymase, baseline levels of DPPI (Fig 6.20(b)) and ACE in serum were no different between subjects with historical reactions of differing severity. Similarly, concentrations were not associated with specific symptoms experienced. Baseline DPPI concentrations in saliva were also similar in patients with reactions of differing severity or symptomatology.
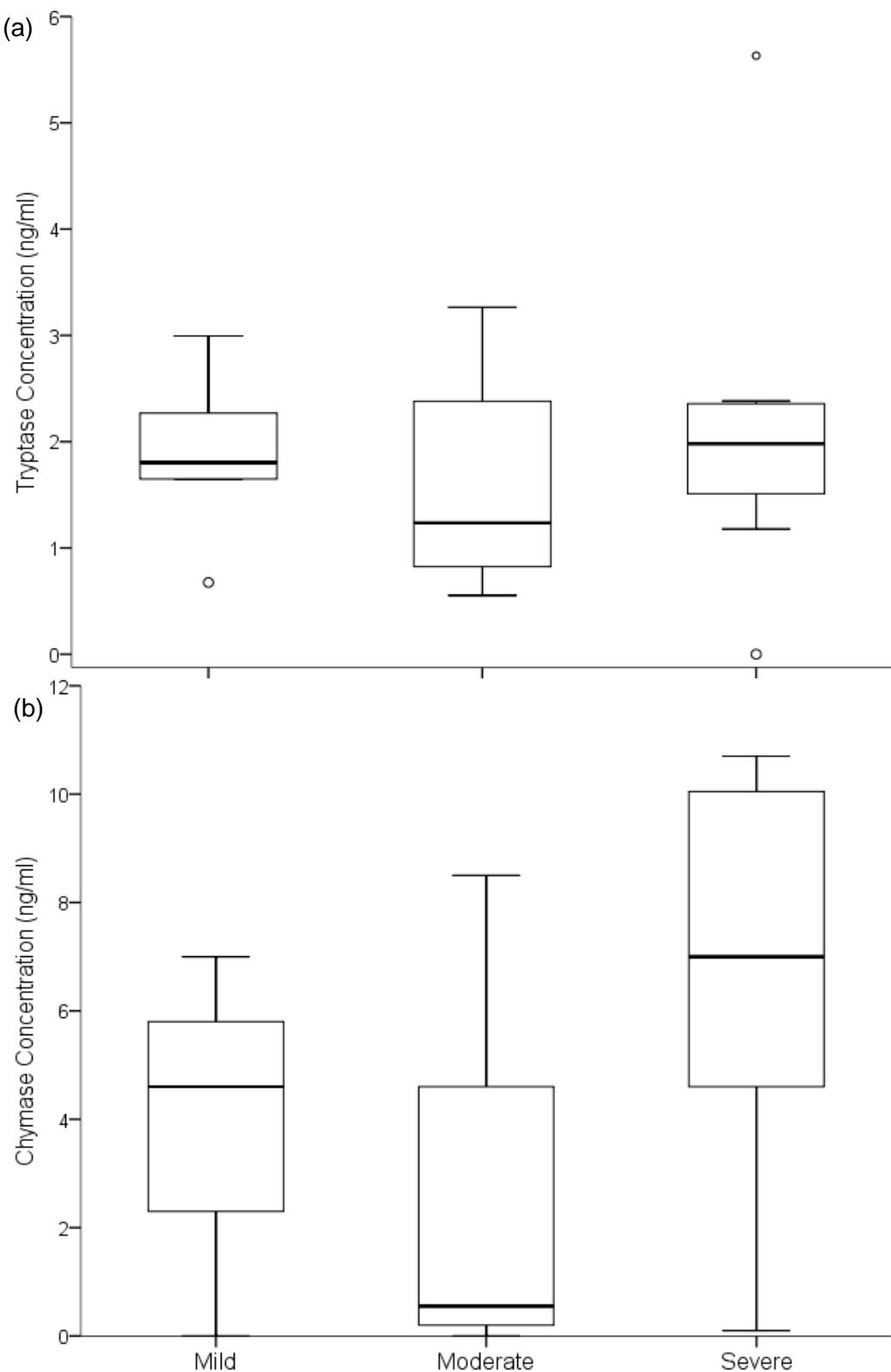
Fig 6.19 Baseline serum levels of (a) tryptase and (b) chymase and severity of historical reactions to drugs. Subjects were divided into those who had experienced mild (n = 7), moderate (n = 10) or severe (n = 10) reactions. Statistical analysis was performed using Mann Whitney U Test.
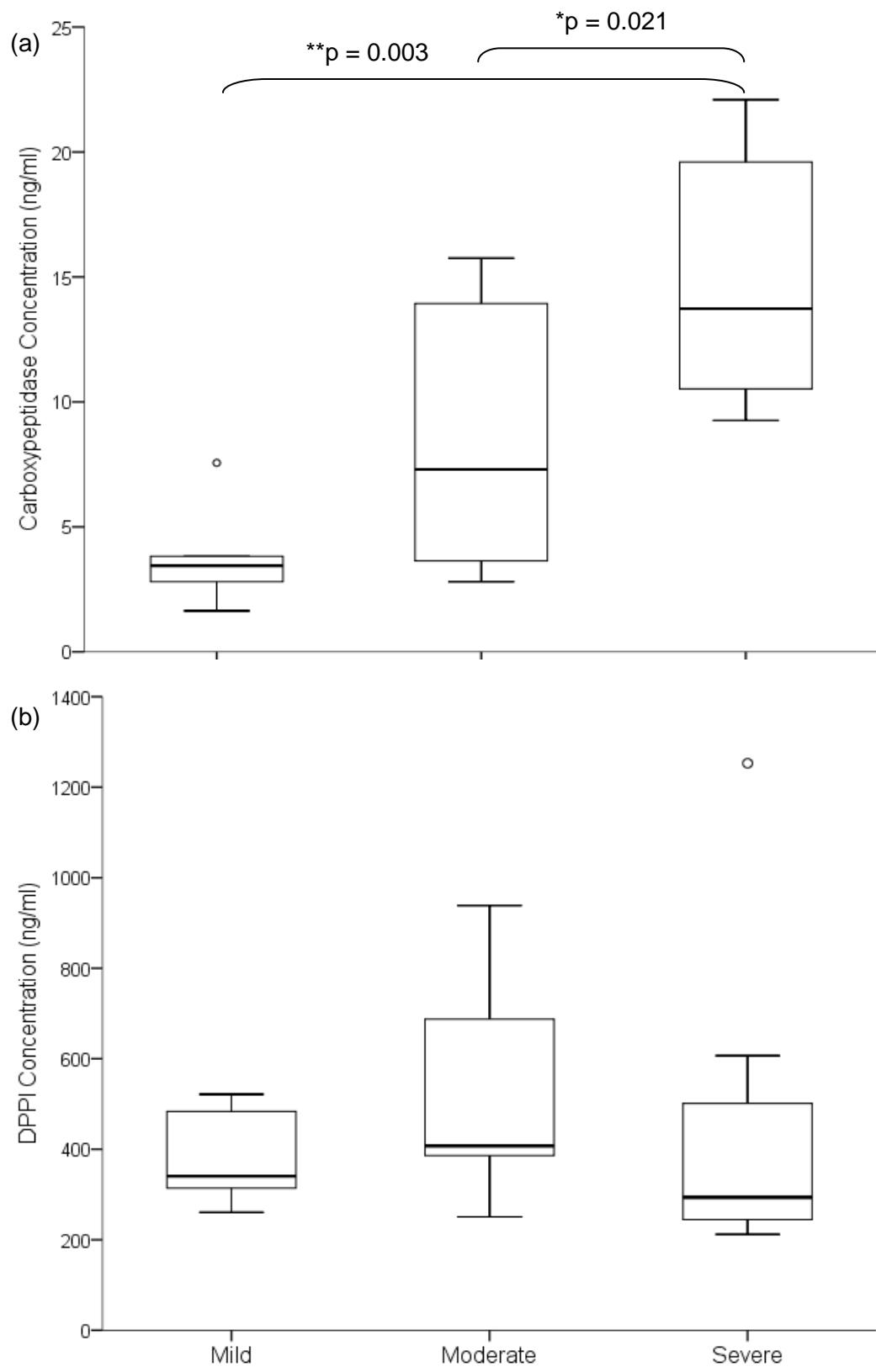
Fig 6.20 Baseline serum levels of (a) carboxypeptidase and (b) DPPI and severity of historical reactions to drugs. Subjects were divided into those who had experienced mild (n = 7), moderate (n = 10) or severe (n = 10) reactions. Statistical analysis was performed using Mann Whitney U Test.

Co-existing atopic conditions

Ten subjects had co-existing atopic conditions, including atopic asthma, eczema or hay fever. Information on allergies to allergens other than drugs was not available. Associations between the presence of co-existing atopic conditions and severity or symptomatology of historical reactions were examined. In addition, baseline levels of the markers listed above were compared in those who reported having the conditions and those who did not.

Atopic asthma

Eight subjects had atopic asthma. Severity of reactions to drugs was no different between subjects with and without this condition. Similarly, presence of atopic asthma was not associated with specific symptoms experienced. However, baseline levels of both carboxypeptidase and DPPI in saliva were significantly higher in patients with a history of atopic asthma compared to those who did not ($p = 0.013$ and 0.01, respectively). This relationship was not seen for saliva tryptase, chymase or ACE concentrations, or for serum levels of any of the markers.

Eczema and hay fever

Eight subjects reported experiencing hay fever, and two had eczema. Co-existing eczema and hay fever were not associated with severity of reactions to drugs or with specific symptoms experienced. There were also no differences in baseline serum or saliva levels of tryptase, chymase, carboxypeptidase, DPPI or ACE levels between subjects with and without the conditions.

Correlations between markers

Serum levels of tryptase at baseline were significantly correlated with those of DPPI ($p = 0.039$, $r_s = 0.415$, $n = 25$). No other associations were observed between any other markers.

6.4 Discussion

We have found evidence that baseline levels of mast cell proteases may be predictive of severity of allergic reactions to foods and drugs. Furthermore, concentrations of the proteases were associated with specific symptoms experienced. Of particular interest was carboxypeptidase. Findings are collectively illustrated in Fig 6.21.

Tryptase

In the current study, we found no evidence that either serum or saliva concentrations of tryptase at baseline were associated with severity of reactions to foods in children or drugs in adults. Baseline levels of the protease in serum have frequently been linked with severity of allergic reactions to insect venom; with higher levels measured in patients with more severe reactions (Ludolph-Hauser *et al.,* 2001; Haeberli *et al.,* 2003; Kucharewicz *et al.,* 2007; Potier *et al.*, 2009). To our knowledge, this concept has only ever been reported with respect to venom allergy. Similarly to our study, Bonadonna *et al.* (2009) found high baseline levels of serum tryptase in only nine of 137 patients with food or drug allergy.

Previous studies on venom allergy found that baseline serum tryptase levels were associated with age. Concentrations within the circulation were higher in older subjects (Guenova *et al.*, 2010; Kucharewicz *et al.*, 2007). Thus perhaps tryptase content within the mast cell increases with age. In support of this, all of mast cells within infant foreskin, but only five to 15 % of mast cells in adult lung, foreskin or bowel, were determined to be immature; and mast cell granules became more numerous and larger during cell maturation (Craig *et al.*, 1989). This could explain why we found no association of baseline tryptase levels and severity of reactions to foods in our paediatric population. Indeed, levels were low in all children. Possible associations between mast cell proteases and age are discussed further in Chapter 8.
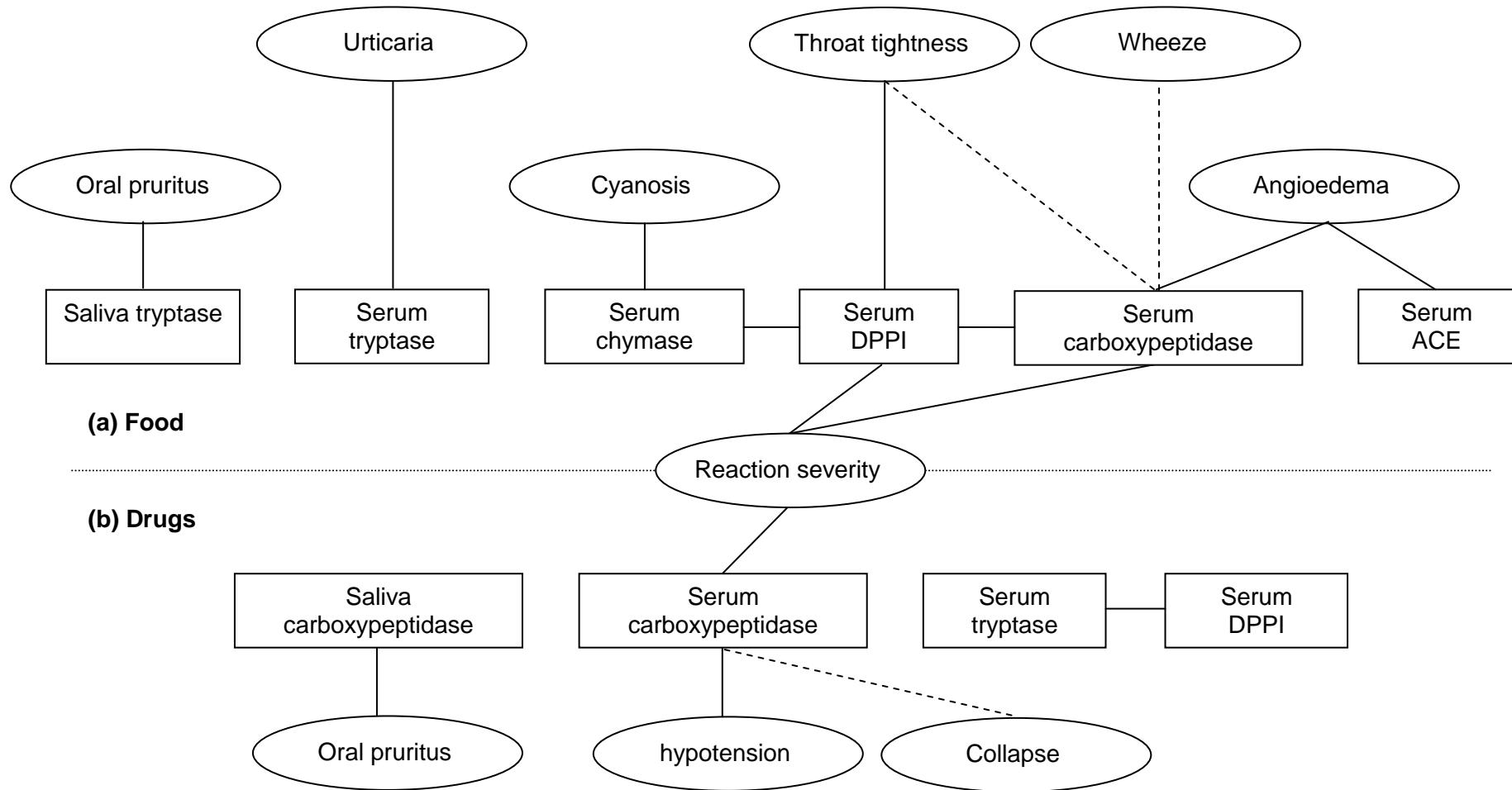
Fig 6.21 Serum and saliva markers of severity and specific symptoms of allergic reactions to (a) food and (b) drugs. Solid lines indicate associations; and dashed lines indicate trends for associations. The central dotted line divides associations in reactions to food and drugs. Markers that were not associated with reaction severity or symptomatology are not shown.

In the present study, we found higher baseline serum tryptase levels to be associated with presence of urticaria during reactions to food in children. Tryptase concentrations were recently reported to be increased in patients with chronic urticaria compared to healthy atopic and non-atopic subjects (Ferrer *et al.*, 2010). However, levels were higher in patients who were symptomatic compared to those who were asymptomatic at the time of blood collection, suggesting that levels were increased in the presence of urticaria. All of our patients were symptom free when baseline samples were taken.

Potier *et al* (2009) found that patients with high baseline serum tryptase levels were less likely to experience urticaria during reactions to insect venom than patients with normal levels of the protease. This appears to be at variance to our data, and may reflect differences in mechanisms in sensitivity to different allergens. Alternatively, the discrepancy may result from differences in paediatric and adult populations. We found no link between baseline serum levels of tryptase and urticaria during reactions to drugs in our adult subjects.

Detectable tryptase within saliva at baseline was found to be predictive of experiencing oral pruritus in food challenge. Serum levels were no different in children with and without this symptom, suggesting that it resulted due to localised effects. Tryptase has previously been shown to activate tonsil and synovial mast cells, and to stimulate histamine release from these cells (He *et al.*, 1998; He *et al.*, 2001). Thus, perhaps the presence of tryptase within saliva at baseline amplifies mast cell activation and mediator release, resulting in oral symptoms.

Baseline saliva tryptase levels were not associated with experiencing oral pruritus in historical reactions to food. This may be due to greater *in vivo* variability of protease levels in saliva than in serum. Alternatively, it may reflect the recall of symptoms by patients. For example, they may be more likely to report more severe and objective symptoms such as oedema, wheeze or vomiting. In addition, historical reactions had often occurred during infancy and could not be remembered by the patient. In these cases symptoms were described by the

parent/guardian who may not have been aware of subjective symptoms such as oral pruritus.

Chymase

In the current study we found that higher baseline serum chymase levels were associated with experiencing cyanosis during most severe historical reactions to food in children. Cyanosis is indicative of deoxygenated haemoglobin, which may result from respiratory or cardiovascular compromise. Although chymase concentrations did not correlate with reaction severity scores, they were associated with carboxypeptidase levels (which were linked with reaction severity).

The ELISA used for chymase measurement detects all circulating protease. Thus it was not possible to distinguish between pro and mature forms. There is evidence that chymase is activated from its pro form by DPPI (McEuen *et al.*, 1998), and we have shown that DPPI is released during allergic reactions to food (Chapter 5). Thus perhaps high baseline chymase levels present a source that is readily available for activation upon release of DPPI during a reaction, allowing a fast and extensive response. In addition, chymase has previously been shown to promote mast cell activation (Urata *et al.*, 1990). High levels of activated protease could therefore cause exacerbation of the allergic response, leading to more severe reactions.

Carboxypeptidase

In this study, we have demonstrated for the first time that baseline serum carboxypeptidase measurement may be useful in predicting susceptibility to severe reactions. Levels were associated with severity of allergic reactions to food both in challenge and historically in children, and also historical reactions to drugs in adults. Serum levels of carboxypeptidase at baseline were not associated with outcome of drug challenge. However, many patients with positive skin test did not experience symptoms, and those who did generally had only minor reactions.

Interestingly, carboxypeptidase concentrations within saliva were higher at baseline in subjects who went on to experience symptoms in addition to responding in skin test. The symptoms experienced by these patients were frequently confined to the oral mucosa, including oral pruritus and angioedema.

Baseline carboxypeptidase levels within human biological fluids were also associated with the presence of other atopic conditions. Serum and saliva levels of the protease were higher in children who had experienced hay fever than in those who had not, and saliva concentrations were higher in children with a history of eczema. Carboxypeptidase levels in saliva were also associated with a history of atopic asthma in adults. This effect was not seen for children. However, the vast majority of children within our study had this co-existing condition.

It is interesting that similar findings were not observed for tryptase given the co-localization of the two proteases within mast cell granules. However, there is evidence for a mast cell population distinct from MCT and MCTC that contain chymase and carboxypeptidase, but not tryptase, in human tissues (MCCs) (Weidner and Austen, 1993). Furthermore, studies *in vitro* showed that mast cells expressing or deficient in tryptase can be induced from human umbilical cord and bone marrow progenitor cells depending on the cytokine environment (Li *et al*., 1996).

As well as being associated with severity of reactions to foods and drugs, baseline levels of carboxypeptidase also appeared to be predictive of specific symptoms. These included angioedema, respiratory difficulties, and cardiovascular compromise. These are discussed separately below.

Angioedema

We have found that high baseline carboxypeptidase levels were associated with angioedema in reactions to food in children. Oedema results from an abnormal accumulation of interstitial fluid; and this can result from increased capillary

leakage or reduced clearance of fluid. Body fluid balance is regulated in part by the renin-angiotensin system (RAS) (Abraham and Schrier, 1994; Mitchell and Navar, 1989; Harrison-Bernard, 2009). ATII increases water retention via sodium re-absorption and aldosterone secretion, and also via ADH release from the pituitary (Harrison-Bernard, 2009). It may also effect vascular permeability via mechanisms involving vascular endothelial growth factor (VEGF), as blockade or knockout of the angiotensin II type I receptor in mice was found to suppress vascular hyper-permeability mediated by VEGF (Sano *et al.*, 2006).

Carboxypeptidase and chymase have been implicated in ATII formation (Reilly *et al.*, 1985; Goldstein *et al.*, 1989), and there is evidence in mice that the two proteases may act in synergy in this role (Lundequist *et al.*, 2004). ATII production was inhibited in peritoneal cell cultures from chymase knock-out mice when carboxypeptidase inhibitors were added; but chymase knock-out or carboxypeptidase inhibition alone did not have this effect. Thus perhaps high carboxypeptidase levels induced susceptibility to oedema via increased production of ATII and the resulting mechanisms described above. Upon release of mast cell and basophil mediators such as histamine and tryptase during an allergic reaction, perhaps the resulting increase in vascular permeability (Guth and Hirabayashi, 1983; He and Walls, 1997) further contributes to leakage of excess fluid into interstitial spaces, causing oedema. Our finding of an association between serum carboxypeptidase and chymase levels supports the concept that the two proteases work in synergy.

Asthma and wheeze

Asthma affected the vast majority of children within our study, and symptoms of the respiratory system were common during allergic events, particularly during reactions within the community. Historical reactions to food were more severe in children with asthma; and the majority of anaphylactic reactions in these patients were considered severe due to respiratory rather than cardiovascular involvement.

The current study revealed a trend for higher baseline serum levels of carboxypeptidase in children who experienced wheeze during reactions to food than those who did not. Although respiratory involvement was not a key feature of reactions to drugs in adults, baseline saliva levels of carboxypeptidase were higher in those with a history of atopic asthma. This perhaps suggests a role for carboxypeptidase in incurring susceptibility to allergic bronchoconstriction.

As mentioned previously, it is interesting to observe these results for carboxypeptidase but not tryptase. However, there is evidence for mast cells that express chymase and carboxypeptidase but are deficient in tryptase (Weidner and Austen, 1993; Li *et al.*, 1996). In addition, a population of mast cells containing chymase but not tryptase was identified in the lungs in cases of fatal anaphylaxis (Perskvist and Edston, 2007). A recent study has provided evidence of a unique sub-population of mast cells that develop via a specific pathway and play a key role in allergic bronchoconstriction in mice (Cyphert *et al.*, 2010). Thus, perhaps there is a population of mast cells within the lungs that contain granules rich in chymase and carboxypeptidase, and that are key in mediating allergic bronchoconstriction. In such case, individuals expressing higher numbers of these cells may be at greater risk of allergic asthma and respiratory difficulties during allergic reactions.

Cardiovascular symptoms

Cardiovascular symptoms were a key feature of severe historical reactions to drugs in adults. Furthermore, baseline levels of carboxypeptidase were higher in patients with a history of cardiovascular involvement. This was particularly true for hypotension, perhaps suggesting a role for carboxypeptidase in contributing to vasodilatation during allergic responses.

As described above, carboxypeptidase may play a role in conversion of ATI to ATII. Although ATII is typically thought to cause vasoconstriction via the type 1 receptor (AT_1), studies in mice have demonstrated ATII-mediated vasodilatation via AT_2 and involving bradykinin-dependent activation of endothelial nitric oxide (NO)

synthase (Yayama and Okamoto, 2007). In addition, ATII has been shown to stimulate VEGF production (via AT_1) (Shi *et al.*, 2009); and VEGF can stimulate NO-mediated vasodilatation (Hood *et al.*, 1998; Dafni *et al.*, 2001). Thus perhaps high constitutive release of carboxypeptidase into the circulation predisposes to vasodilatation via increased ATII production.

Our results may also suggest involvement of cardiac mast cells. Genovese *et al.* (2010) demonstrated *in vitro* that human heart mast cells could be activated not only via IgE-mediated mechanisms, but also directly with therapeutic and diagnostic agents. Direct activation *in vivo* results in a serious allergic reaction. Thus perhaps a high burden of mast cells in the heart increases susceptibility to cardiovascular symptoms due to a greater capacity for activation and degranulation via IgE-dependant and independent mechanisms. In addition, intravenous drugs have almost instantaneous contact with cardiac tissue, and thus high numbers of cardiac mast cells may allow for vast and rapid degranulation.

Human heart mast cells were previously found to express and release a range of mediators, including unusually large amounts of chymase (Patella *et al.*, 1995). In our study we found levels of chymase to be associated with those of carboxypeptidase. Thus the finding of high constitutive carboxypeptidase release in patients with a history of cardiovascular compromise during reactions to drugs may perhaps indicate an increased cardiac mast cell burden.

DPPI

In the current study we found a non-significant trend for higher baseline levels of DPPI in serum in children who reacted in food challenge than in those who did not. Furthermore, concentrations were associated with severity of reactions, and in particular with the symptom throat tightness, occurring in challenge. This suggests that a higher level of constitutive release of the protease into the circulation may incur susceptibility to more severe reactions. This could result due to a readily

available source of DPPI present for activation of other mast cell proteases upon their release during IgE-mediated cell activation.

Baseline levels of serum DPPI were not associated of severity or symptomatology of historical reactions. This may reflect variation in levels of the protease *in vivo*. We have shown that, within a one year period, concentrations vary reasonably minimally (Chapter 4). However, to date there have been no studies on variation over a longer time. As many of the historical reactions reported occurred at least several years prior to blood sampling, it is possible that DPPI levels may have changed. Despite this, concentrations of the protease were consistently correlated with those of carboxypeptidase, which were associated with severity of reactions occurring both historically and in food challenge.

Baseline serum levels of DPPI were also not associated with severity of (or symptoms experience during) reactions to drugs in adults. The failure to see this during drug challenge was perhaps unsurprising, as not all patients with positive skin test experienced symptoms, and reactions that did present were generally mild. The lack of association with severity of historical reactions may be due to similar reasons as those described for the children with food allergy. Also, there may be differences in allergic mechanisms occurring in food and drug allergy, or between adults and children.

ACE

In the current study, ACE levels within biological fluids at baseline were not associated with reaction severity, or with specific symptoms experienced, to foods or drugs. A previous study linked low activity of serum ACE to severe reactions to tree nuts and peanuts. Low activity levels were specifically linked to severe pharyngeal oedema (Summers *et al.*, 2008). Similarly, ACE inhibitors have been suggested to be associated with susceptibility to anaphylaxis, due to their ability to cause angioedema (Stumpf *et al.,* 2006). We found no evidence to support this. In

fact, our study revealed that baseline ACE concentrations were higher in patients who experienced oedema in food challenge than those who did not.

ACE inhibitor-induced angioedema is thought to result from the reduction in degradation of bradykinin (Ferner *et al*., 1987), leading to vasodilatation (Bönner *et al*., 1985) and increased vascular permeability (Greaves and Shuster, 1967). However, there are several indications that different mechanisms may be involved in allergic and non-allergic angioedema. Firstly, whereas adrenaline is often effective in the treatment of allergic oedema, it is less effective in ACE inhibitor-induced angioedema. Additionally, urticaria often accompanies allergic but not ACE inhibitor-induced angioedema (Kaplan and Greaves, 2005). Thus, whereas a reduction in serum ACE levels caused by inhibitors may cause angioedema by one mechanism, a separate mechanism may incur predisposition of patients with high baseline concentrations to oedema during an allergic reaction.

Potential mechanisms whereby high levels of ACE at baseline could in fact incur susceptibility to oedema may involve increased generation of ATII, resulting in a greater circulation volume due to sodium and water retention. Release of mediators that cause increased microvascular permeability during an allergic reaction, such as histamine (Guth and Hirabayashi, 1983) and tryptase (He and Walls, 1997), may allow leakage of this fluid into interstitial spaces. There is also evidence that ATII may indirectly increase vascular permeability by inducing VEGF production (Sano *et al*., 2006).

Although high levels of ACE were found to be associated with angioedema occurring in food challenge, this effect was not seen for historical reactions. Blood samples were collected from children on the day of the challenge, but the historical reactions may have occurred years previously. This is a limitation of the current study, and is discussed further below. We found ACE levels to be relatively stable *in vivo* within up to a one year period (Chapter 3). However, concentrations have previously been suggested to reduce with age; higher levels were found in children than in adults (Bénéteau-Burnat *et al*., 1990). Thus perhaps only recent

measurement of ACE is useful in predicting susceptibility to angioedema during reactions to food.

We found no association between serum ACE levels at baseline and angioedema during allergic reactions to drugs in adults. This could reflect differences in mechanisms occurring in food and drug allergy, or perhaps between adults and children. Furthermore, we did not have information available on whether the adult subjects on our study were taking prescribed ACE inhibitors.

Skin prick test and specific IgE levels

In this study we have shown that SPT diameter was higher in subjects with positive than negative food challenges, supporting the use of skin prick testing in predicting clinical reactivity in food challenge. This is consistent with a study by Eigenmann and Sampson (1998) in which SPT diameter was greater in children with positive double blind, placebo-controlled food challenge to egg, milk, peanut, soy and wheat than in those with negative challenges to the foods.

We also found SPT diameter to be positively correlated with severity of reactions experienced in food challenge. Larger wheals were associated with more severe reactions. This implies that SPT measurement may be useful in predicting those at risk of severe reactions. However, these results are contradictory to previous findings, which showed no association between SPT diameter and reaction severity in peanut challenge (Hourihane *et al.*, 1998). In addition, in the current study, no correlation was observed following exclusion of negative challenges. Thus, although skin prick testing may have some use in predicting clinical reactivity to a food, it is likely to have little use in predicting severity of reactions.

SPT diameter and specific IgE levels in blood were significantly correlated, consistent with results previously found by Hourihane *et al* (2005) within children. Despite this, in the current study, specific IgE levels were not found to be predictive of challenge outcome. This is likely to be due to a large time gap between IgE

measurement and food challenge. Measurements recorded were those most recently taken for diagnostic purposes, but many of the tests were performed over one year prior to the food challenge. Thus levels may not be representative of those present at the time of food challenge. All recorded SPT diameters, on the other hand, were performed less than one year prior to the challenge.

Associations between markers

Baseline serum levels of carboxypeptidase, chymase and DPPI were correlated in the paediatric population with food allergy. However, no association was seen with tryptase concentrations. As carboxypeptidase and chymase have been localised to cell granules alongside tryptase, one would expect the proteases to be released simultaneously. Indeed, in adults, serum levels of DPPI and tryptase were correlated.

One explanation for the differences in baseline levels of mast cell proteases may be due to differential activation of mast cell subpopulations (discussed above). The finding of two major mast cell populations, MC_T and MC_{TC}, is well documented (Irani *et al.*, 1986; Schwartz *et al.*, 1987); but there is also evidence for a population of cells that expresses chymase and carboxypeptidase without tryptase (Weidner and Austen, 1993; Li *et al.*, 1996; Perskvist and Edston, 2007). In addition, a novel mast cell population has been identified in mice, which is essential for mediating allergic bronchoconstriction (Cyphert *et al.*, 2010). Diversity of protease composition of mast cell subtypes, and activation of these different populations during reactions, may account for discrepancies between levels of proteases.

Alternatively the failure to observe an association between levels of tryptase and those of other mast cell proteases may be related to different rates of migration through tissue or clearance from the circulation. Following allergic reactions, tryptase levels have been shown to peak at one to two hours, and decline thereafter (Schwartz *et al.*, 1989). Histamine, on the other hand, peaked by five to ten minutes and declined rapidly thereafter. Tryptase has been reported to have a

half life of two hours, whereas for histamine this was approximately five minutes. Preliminary results suggest that carboxypeptidase concentrations are also increased by two hours, but are raised for much longer periods of time following reaction (Zhou *et al*, 2006). Thus, there are clear differences in rates at which mast cell granular components reach the circulation, and at which they are degraded.

Limitations of study

In this chapter, levels of markers have been investigated in human biological fluids with regard to severity of reactions and specific symptoms experienced in food challenge and historically. There are pros and limitations of studying either type of reaction. In food or drug challenge, sampling can be carried out immediately prior to allergen exposure, and presenting signs and symptoms can be accurately documented. However, to ensure safety, patients are treated upon the onset of a reaction. This means that reactions occurring in allergen challenge are mostly very mild, and that symptoms do not progress as they may within the community. Indeed, in the present study, we found no association between severity of historical and food challenge reactions (data not shown). Similarly, the association between the severity of reactions experienced in response to peanut in the community and those occurring in DBPCFC has been reported to be poor (Hourihane *et al*., 2005).

When studying historical reactions it must be considered that many reactions reported in the current study occurred several years previous to sample collection, and it is therefore possible that baseline levels of the mediators may have changed. We have studied consistency of baseline serum levels of the markers in a number of subjects over the course of the study, and have found concentrations to vary relatively minimally within a one year period. However, there is evidence that ACE levels may decline with age, as lower levels have been reported in adults than children (Bénéteau-Burnat *et al*., 1990). Long term changes of the mast cell proteases have not been investigated. The capacity of levels of these markers to vary within subjects from childhood into adulthood therefore requires further investigation.

Another limitation of investigating levels of markers with respect to severity and symptomatology of historical reactions was that all reactions were self-reported. Thus accuracy of descriptions of reactions cannot be guaranteed. This is particularly the case for subjects who experienced the reported reaction many years previously. In order to minimize effects of this limitation, patients and/or their parents were asked in detail using a standardised questionnaire about the episode in order to obtain sufficiently accurate information. In some cases the patient had been admitted to hospital, and in most cases they had received medical attention soon after the event. Thus medical notes were utilised to ensure accuracy of descriptions. All scoring of paediatric reactions was carried out by a single observer (myself) to minimize variation in interpreting the data.

Within this study we have investigated the usefulness of measurement of individual markers in predicting susceptibility to severe reactions or specific symptoms. It may be even more useful to look at the predictive value of a number of mediators and other factors (such as SPT and history). Although multiple analyses were beyond the scope of the current study, this will be utilised in continuing research.

Summary

This study has found evidence that baseline serum levels of carboxypeptidase (and perhaps DPPI and chymase) may be useful in predicting susceptibility to severe reactions to food in children and drugs in adults. Furthermore, high baseline levels of different mediators may be useful in predicting susceptibility to types of symptoms experienced. The potential for use of biomarker measurement in predicting susceptibility to severe reactions or specific symptoms, based on our findings, are summarised in table 6.7.

Table 6.7 Potential use of baseline serum and saliva markers in predicting reaction severity and symptomatology of allergic reactions to foods and drugs. The potential for use of measurement of serum and saliva tryptase, carboxypeptidase and DPPI concentrations, and serum chymase and ACE concentrations are presented. Suggestions are based on the results of the current study. Where (?) is used, supporting evidence showed only a trend for an association.

		Foods		Drugs	
		Reaction severity	**Symptomatology**	**Reaction severity**	**Symptomatology**
Tryptase	**Serum**	No	Urticaria	No	None
	Saliva	No	Oral pruritus	No	None
Chymase	**Serum**	No	Cyanosis	No	None
Carboxypeptidase	**Serum**	Yes	Angioedema; Throat tightness (?); Wheeze (?)	Yes	Hypotension; Collapse (?)
	Saliva	No	None	No	Oral pruritus
DPPI	**Serum**	Yes	Throat tightness	No	None
	Saliva	No	None	No	None
ACE	**Serum**	No	Angioedema	No	None

CHAPTER 7: FURTHER DEVELOPMENT OF ASSAYS FOR TRYPTASE AND BASOGRANULIN

7.1 Introduction

In this chapter we have aimed to address the limitations of the current techniques for measurement of tryptase, including both our in-house ELISA and the commercially available assay (ImmunoCAP™ Tryptase, Phadia). There is a need for techniques that are more sensitive, and that can determine levels of variants of tryptase. The production of new monoclonal antibodies for such assays has been described.

A technique for measuring basophil activation in an allergic reaction has not been available. The advent of a new monoclonal antibody (Fawzy et al, 2011) against the basophil specific granular constituent (McEuen *et al.*, 1999; McEuen *et al.*, 2001), basogranulin, has opened the way for the development of a sandwich ELISA for this marker, and this has been explored here.

Requirement for further development of tryptase assay

A commercially available assay for measurement of tryptase is the only test presently recommended for confirming diagnosis of anaphylaxis (Project Team of The Resuscitation Council (UK), 1999). The majority of evidence supporting the use of the test is from patients with reactions to drugs or insect venom (Yunginger *et al.*, 1991; Pumphrey and Roberts, 2000; Schwartz *et al.*, 1989). However, research into the usefulness of serum tryptase measurement in reactions to food is less conclusive (Vila *et al.*, 2001).

In the present study, we have found no evidence for increased serum tryptase levels following allergic reactions to either food or drugs upon allergen challenge using our in-house tryptase ELISA (Chapter 5). This could be explained by the lack of severe reactions (particularly on drug challenge), or perhaps by the time point

for blood sampling selected. However, we also found that levels in the serum of patients presenting at the Accident and Emergency Department with anaphylaxis did not differ from those in healthy atopic or non-atopic subjects.

Sensitivity of ELISA for tryptase

Our in-house tryptase ELISA allowed detection of this protease in the range of 0.5 to 32 ng/ml. In many of our study participants, serum tryptase concentrations at baseline were low. In some cases tryptase levels were below the detection limits of the assay.

We also sought to investigate tryptase levels in saliva during reactions to foods or drugs. We did find evidence of increased concentrations in children with isolated oral allergy in response to foods (Chapter 5). However, salivary levels were very low, and often below the limits of detection. This has previously been reported in a study of salivary tryptase levels in food allergy: the protease was detected in only one of 12 subjects (Vila *et al.*, 2001). Thus development of a more sensitive ELISA for tryptase may allow for increased accuracy in these types of studies.

Measurement of tryptase subtypes

As discussed in Chapter 1, there are multiple subtypes of human tryptase. Our in-house ELISA and the commercially available assay for tryptase detect all forms of the protease within the circulation (α and β tryptase). These are closely related proteases; but the α tryptase pro-peptide is deficient of a basic amino acid required for activation (Sakai *et al.*, 1996). Although expression of active α tryptase has been performed in insect cells (Huang *et al.*, 1999), the potential for activation in humans is disputed. Activation of human mast cell β tryptase has been demonstrated with DPPI (Sakai *et al.*, 1996; Fajardo and Pejler, 2003; Fukuoka and Schwartz, 2004); and this form of tryptase was deemed to be more physiologically relevant in humans. Thus perhaps measurement of β tryptase levels would provide a better indication of mast cell activation than 'total' levels.

In a study by Soto *et al* (2002), it was found that approximately one third of the population may be genetically deficient in α tryptase. Evidence was presented that α tryptase alleles compete with some β alleles at one site of the human tryptase locus; and this resulted in a lack of α tryptase genes in some genomes. From these findings the authors hypothesized that expression of α tryptase genes may be protective against severe allergic reactions due to occupation of a site that that would otherwise contain genes expressing the β form. Thus perhaps measurement of α tryptase levels (or the ratio of α to β tryptase) may be of use in predicting those susceptible to severe reactions.

Requirement for a measure of basophil activation

There has been much speculation about the role of basophils during allergic reactions. These cells have been shown to play a key role in IgG-mediated systemic anaphylaxis in mice (Tsujimura *et al.*, 2008); and an *in vitro* basophil activation test (BAT) has proved useful in predicting clinical reactivity (Kucera *et al.*, 2010). However, to date there has been no reliable means of measuring basophil involvement in humans *in vivo*.

In 1999 a new monoclonal antibody, termed BB1, was raised against human basophils within our laboratory (McEuen *et al.*, 1999). The antigen to which this binds was found to be located within the cell granules, and was thus termed basogranulin (McEuen *et al.*, 2001). A dot blot procedure was developed using BB1 for detection; and this has been used to demonstrate basogranulin release from isolated peripheral blood basophils upon stimulation with various stimuli (Mochizuki *et al.*, 2003). However, development of an ELISA that utilizes a second antibody specific for the protease would allow for more sensitive, specific and reliable measurement of basogranulin within human biological fluids.

Aims

Because of the limitations described for existing assays for tryptase, our objective has been to produce a more sensitive assay, and to develop techniques that would allow detection of the major subtypes, α and β tryptase. Thus, in this study we aimed to prepare new monoclonal antibodies against tryptase for these purposes.

Production of a second monoclonal antibody against human basogranulin has recently been achieved within our laboratory (Fawzy *et al.*, 2011). We have aimed also to develop a novel ELISA technique for measurement of the basophil-specific protease using this new antibody and BB1.

7.2 Methods and results

Production of new monoclonal antibodies for human tryptase

New mouse monoclonal antibodies were produced against human tryptase with the help of Dr Laurie Lau and Dr Kathy Bodey. These were purified and biotinylated. Finally, use of the antibodies in sandwich ELISA was assessed.

Antibody production

In brief, antibody-producing spleen cells from mice injected with human tryptase were fused with myeloma cells to create hybridomas. The cells were cloned and sub-cloned until monoclonal, and binding of antibodies to tryptase was confirmed.

Mouse immunization and splenocyte preparation

Two mice were immunized in the tail vein with 7 ug recombinant α and β tryptase (a gift from Dr Chushim Somarhuff, Munich) in complete Freund's adjuvant (Sigma). On days 21 and 56 the mice were injected with 7 ug α and β tryptase in incomplete Freund's adjuvant and sterile saline, respectively. The mice were culled on day ..., and the spleens removed and put into 15 ml Dulbecco's Modified Eagles Medium (DMEM; Sigma) containing antibodies, glutamine and pyruvate (AGP). The splenocytes were dispersed using a 2 ml syringe to achieve a single cell suspension, and the volume of DMEM with AGP was increased to 50 ml. The cells were centrifuged three times at 500 x g for 10 minutes at 22 ºC, replacing the supernatant in between. The cells were re-suspended in 15 ml DMEM with AGP and transferred to an incubator at 37 °C.

Fusion of splenocytes with NS-1 cells

The same procedure (described above) was carried out with NS-1 cells as for the splenocytes; 50 ml DMEM with AGP was added and the cells centrifuged three

times at 500 x g for 10 minutes at 22 ºC. The cells were re-suspended in 15 ml DMEM with AGP. The splenocytes and NS-1 cells were fused by mixing at a ratio of 2:1; and the volume was increased to 50 ml with DMEM containing AGP. The cells were centrifuged for 5 mins at 500 x g, and the supernatant removed. One ml polyethylene glycol (PEG) solution (1 g/ml PEG (Sigma) in DMEM) was added drop-wise over one minute, and the cells were suspended by mixing for 90 seconds. Three ml DMEM containing AGP was added gradually over two and a half minutes whilst mixing, and another 7 ml was added over the next two and a half minutes. The cells were centrifuged at 80 x g for five minutes and the supernatant removed. Twenty five ml standard growth medium (SGM), containing 20 % hypoxanthine aminopterin thymidine (HAT; Sigma), was added, and the pipette used to break up the cell pellet by aspiration. The fused cells (hybridomas) were placed in an incubator at 37 °C for 30 minutes.

Fusion plates were prepared by adding three drops 15 % hybridoma enhancing supplement (HES; Sigma) in HAT medium to each well of 10 flat-bottomed 96 well plates using a Pasteur pipette. The hybridomas were dispersed by flushing using a pipette, and one drop was added per well of each plate. One hundred and twenty ul supernatant was removed from each well and replaced with fresh HAT medium every two to three days.

Screening antibodies for binding to α and β tryptase

After one week the cells were screened by direct ELISA to determine whether they were producing antibodies against tryptase. Assays were performed for α and β tryptase individually. Ninety-six well plates were coated with α or β tryptase, diluted in carbonate bicarbonate buffer (15 ng/ml). The plates were refrigerated at 4 °C overnight, and then blocked with 2 % BSA in PBS at 22 ºC for 90 minutes. Cell culture supernatant from each well of the fusion plates, diluted 1:2 in PBS, was added, and the plates incubated at 22 ºC for 90 minutes. The anti-tryptase antibody AA5 was used as a positive control, and SGM was used for a negative control. Rabbit anti-mouse HRP conjugated antibody, diluted 1:1000 in PBS, was

added, and the plates incubated at 22 ºC for 90 minutes. Substrate solution was added and colour allowed to develop. The reaction was stopped with H_2SO_4, and the optical density determined at 450/595 nm using a spectrophotometer.

Sub-cloning of antibody-producing hybridomas

Twenty hybridomas testing positive for antibodies against α and/or β tryptase in ELISA, and also appearing to be growing from a single colony under microscope, were selected for cloning. Cells were re-suspended in supernatant in their wells, and transferred to bijou pots containing 15 % HES and 2 % HAT in SGM. Two hundred ul from each fusion well was added to the top wells of half a 96 well plate, and 150 ul medium was added to the wells below. The top wells were mixed and 50 ul transferred to the next row of wells. These wells were mixed and 50 ul transferred to the next row. This was continued to the bottom row of wells, resulting in a 1:4 dilution of cells in each row. Dilution resulted in a single cell (or very few cells) per well in the lower rows of wells, and therefore a greater chance of achieving monoclonal antibodies. The plates were stored in the incubator for eight days. When necessary, supernatant was removed and fresh medium added.

The supernatants from the cloned cells were screened for binding to tryptase a further three times. Each time the cells testing positive for antibodies against tryptase and appearing to be growing from single colonies were sub-cloned. In initial screening rounds, clones producing antibodies specific for both α and β tryptase, and also those specific for either α or β tryptase, were detected. With subsequent rounds of sub-cloning and screening, however, it appeared that the numbers of clones producing antibodies against only one of the two subtypes of tryptase was reduced. Some cells began to produce antibodies against both α and β tryptase. Fig 7.1 shows results of the final screen. Most antibodies bound to both forms of tryptase, but with a greater affinity for α tryptase. Two antibodies bound to α tryptase only; and one antibody bound to β but not α tryptase.
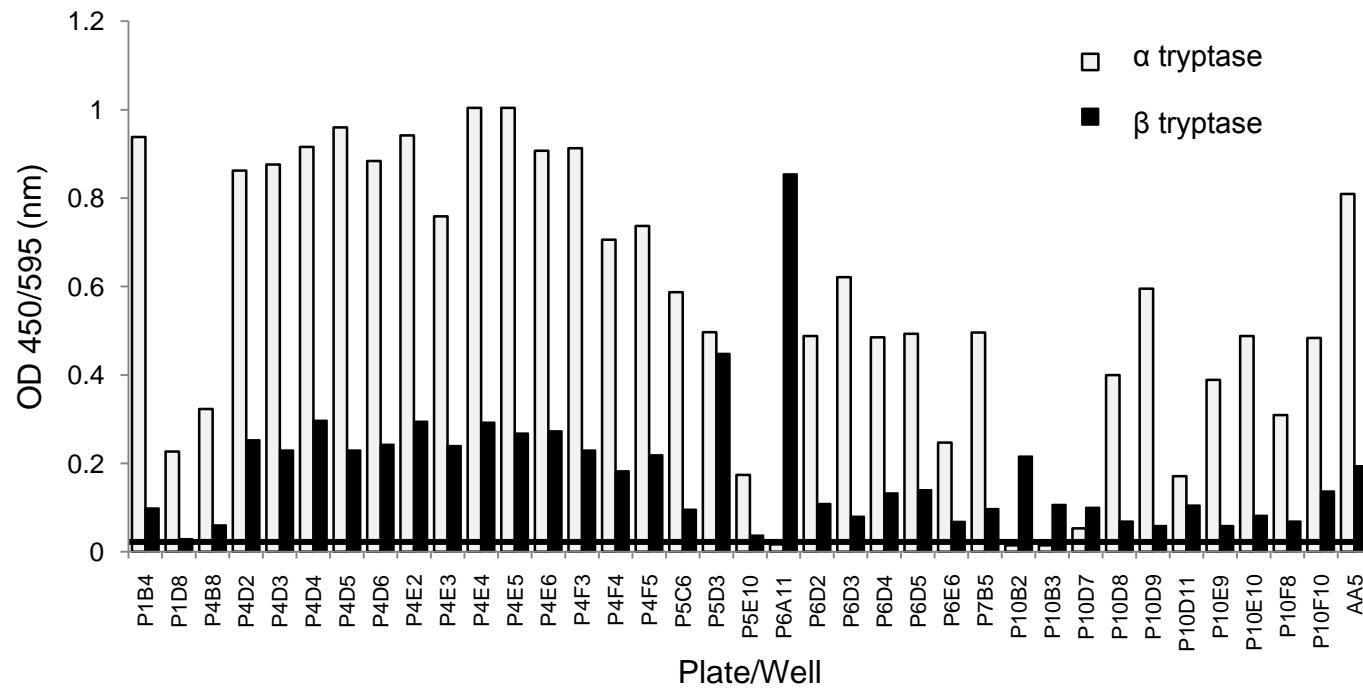
Fig 7.1 Binding of new monoclonal antibodies to α and β tryptase in direct ELISA. Hybridoma supernatants were tested for binding to both α and β tryptase individually by ELISA. The antibody AA5 was used as a positive control; and the black line represents background levels (no antibody control).

Expanding hybridomas and collecting supernatants

Ten hybridomas that appeared to be growing in a single colony and that
produced antibodies that bound to α and/or β tryptase in screening were
selected for expansion. The selected hybridomas were expanded by
transferring to small flasks with 10 ml medium, then adding a further 20 ml
medium three days later. As the cells expanded they were transferred to large
flasks, increasing volumes of medium as necessary until approximately 400 ml
supernatant was obtained. Cells were allowed to grow for one week at 37 ºC
and then centrifuged at 170 x g for 20 minutes in falcon tubes. The antibody-
containing supernatants were collected and frozen at -20 ºC. The remaining cell
pellets were discarded.

At various points during the process of hybridoma expansion, some cells from
each flask were collected and stored for future use. Approximately 25 ml
supernatant was collected and centrifuged at 225 x g for five minutes. The cells
were re-suspended in 1 ml complete medium with 10 % DMSO, and frozen at -
70 °C before transferring to liquid nitrogen.

Antibody purification and biotinylation

Purification

The mouse anti-tryptase monoclonal antibodies were purified by affinity
chromatography using a protein G column (Thermo Scientific). Protein G binds
immunoglobulins of the IgG isotype. The column was washed with PBS for one
hour (0.5 ml/minute). Culture supernatant (1:2 in PBS) was pumped through the
column at 0.5 ml/minute. The column was again washed with PBS for one hour.
Elution buffer (0.1 M glycine (Sigma), pH 2.5) was pumped through the column
at 0.5 ml/min, and the eluant collected in fractions of 2 ml. The eluate was
immediately neutralized with 100 µl neutralization buffer (2 M Tris (Sigma), pH
8.0) per fraction.

A protein assay, according to the Coomassie blue binding method (BioRad),
was used to determine protein concentrations in the eluted fractions. Known

concentrations of γ-globulin were used for standards. The antibody fractions that contained the highest protein concentrations were pooled and then dialyzed overnight against PBS at 4 ºC.

Biotinylation

Antibodies were dialyzed against $NaHCO_3$ (Sigma) buffer overnight, and then concentrated by centrifuging in Microcon centrifugal filter devices (Millipore) at 14,000 x g for 30 minutes at 4 ºC. Protein assay was carried out to determine antibody concentrations. Twenty μl of biotinylation reagent (N-hydroxysuccinimidyl-6-(biotinamido)hexanoate; Sigma), diluted 25 mg/ml in DMSO, was added to 1 ml of concentrated antibody. The tube was wrapped in parafilm to ensure no leakage and mixed for two hours on a roller. The reaction was stopped by adding 11 mg glycine. The tube was again wrapped in parafilm and mixed for a further 15 minutes. The biotinylated antibody was dialyzed twice against PBS at 4 °C, and stored at -20 ºC.

Use of antibodies in sandwich ELISA

Use of the antibodies was assessed in sandwich ELISA. A plate was coated with rabbit anti-tryptase polyclonal antibodies (1:5000) in carbonate bicarbonate coating buffer, and then blocked with 2 % BSA in PBS. Varying concentrations of purified human lung tryptase were added (0 – 16 ng/ml) in duplicate, and the new antibodies were added for detection (1:500 in 0.5 % BSA in PBS). Following incubation of the plate with Extravidin-HRP (1:5000 in 0.5 % BSA in PBS), it was developed with sodium acetate buffer. The reaction was stopped, and the optical density measured.

Two antibodies produced sensitive and specific concentration-response curves with increasing tryptase levels. These were termed AA8 and AA9. Standard curves using these antibodies are shown in Fig 7.2.
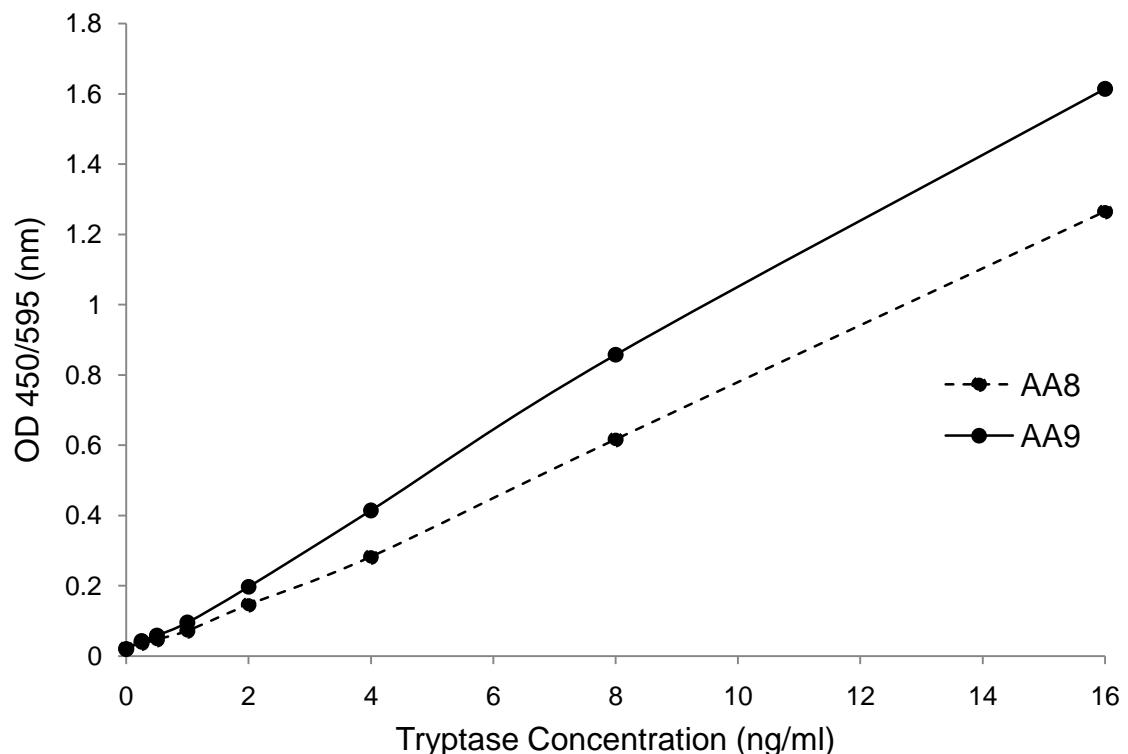
Fig 7.2 New mouse monoclonal antibodies in tryptase ELISA. A sandwich ELISA was carried out as previously, using new anti-tryptase monoclonal antibodies AA8 and AA9 for detection.

Development of novel ELISA for basogranulin

Previously, a mouse monoclonal antibody has been raised against the basophil specific protein, basogranulin (McEuen *et al.*, 1999; McEuen *et al.*, 2001). This antibody was termed BB1, and it is of the IgG2a isotype. Recently a second mouse monoclonal basogranulin-specific antibody, of the IgM isotype, has been produced and characterized (Fawzy *et al.*, 2011). This has been termed BB5.

We have used these new antibodies to develop a prototype ELISA for measurement of basogranulin. Due to the technical difficulties and time involved in purifying basogranulin, and also the low yields, basophil lysates were used as the standard for development purposes. The basophils used were isolated from peripheral blood collected from healthy volunteer adults following informed consent. Binding of BB1 and BB5 to the basophil lysate was confirmed by direct ELISA. The antibodies were then partially purified and assessed for use in an ELISA. Ethical approval was obtained for these studies.

Isolation and lysis of human basophils

Isolation of basophils was carried out using a MACS® cell separation kit (Miltenyi Biotech), which involves a negative selection process. In brief, cells other than basophils in peripheral blood mononuclear cells (PBMCs), isolated from whole blood, were magnetically labelled. Following application of a magnetic field, only basophils were able to pass through. The cells were lysed by repeated freeze-thawing.

Isolation of peripheral blood mononuclear cells

Peripheral blood was collected in tubes coated with ethylenediaminetetraacetic acid (EDTA; Sigma). Blood was diluted 1:3 in PBS containing 2 M EDTA and layered on top of 15 ml Ficoll-Paque (Lymphoprep). The blood was centrifuged at 400 x g for 30 minutes at 20 °C (without acceleration or braking), and the upper layer aspirated using a Pasteur pipette. The mononuclear cell layer (containing lymphocytes, monocytes and thrombocytes) was collected, and cells from 20-30 ml blood were added to 50 ml PBS-EDTA. The cells were

centrifuged at 300 x g for 10 minutes at 20 °C and the supernatant removed. The cell pellet was re-suspended in 50 ml MACS buffer. The cells were centrifuged a further two times at 200 x g for 10 minutes at 20 °C, each time discarding the supernatant and re-suspending the cell pellet in MACS buffer. After the final centrifugation, the buffer volume was reduced to 5 ml and a cell count performed.

Magnetic labelling

The cell suspension was centrifuged at 300 x g for 10 minutes and the supernatant aspirated. The cell pellet was re-suspended in 30 µl buffer with 10 µl MACS FcR blocking reagent and 10 µl MACS basophil biotin-antibody cocktail, per 10^7 total cells. After cooling for 10 minutes at 4 °C, 30 µl MACS buffer and 20 µl MACS anti-biotin micro-beads were added per 10^7 total cells. The solution was cooled at 4 °C for 15 minutes. Cells were washed with 2 ml MACS buffer per 10^7 total cells and then centrifuged at 300 x g for 10 minutes. The supernatant was aspirated completely and the cells re-suspended in 500 µl buffer per 10^8 total cells.

Magnetic separation

Cells were divided by magnetic separation with an LS column, placed in the magnetic field of a MACS separator. The column was washed with 3 ml MACS buffer, and the cell suspension loaded onto the column. The labelled cells were attracted to the column, and the effluent (basophil fraction) was collected. The column was washed a further three times with 3 ml MACS buffer and the effluent collected in order to obtain any remaining basophils.

Lysing of basophils

Isolated basophils were lysed by freezing at -80 ºC and thawing. This was repeated three times. Due to the potential for enzymes within the lysate to degrade basogranulin, the cell lysate was stored at -20 ºC until use.

Binding of BB1 and BB5 to basophil lysate

Binding of the antibodies to the human basophil lysate was confirmed by direct ELISA. A 96 well micro-plate was coated at 4 ⁰C overnight with 50 µl basophil lysate, diluted 1:10, 1:20 and 1:50 in carbonate bicarbonate buffer. Lysate of LAD2 cells (a mast cell-like cell line that stains positive for basogranulin) was also used as a positive control, and buffer containing no cell lysate was used as a negative control. Non-specific binding sites were blocked with 100 µl 3 % BSA and PBS at 22 ⁰C for two hours.

Antigen was detected with BB1 or BB5, diluted 1:10 in 1 % BSA in PBS, for two hours at 22 ⁰C. The plate was incubated with 50 µl secondary antibody specific for mouse immunoglobulins (1:1000 in 1 % BSA in PBS) for two hours at 22 ⁰C, and then developed using TMB substrate. The reaction was stopped with H_2SO_4, and optical density measured at 450/595 nm on a spectrophotometer.

Both antibodies bound to LAD2 and basophil lysates, but not the negative control. Binding occurred in a concentration dependent manner with increasing concentrations of basophil lysate.

Antibody purification and development of prototype ELISA

Antibodies were partially purified by ammonium sulphate precipitation. Combinations of the antibodies, both purified and unpurified, were assessed for their use in ELISA. Different secondary antibodies were also tested for suitability in the assay.

Antibody purification

A volume of each of BB1 and BB5 were partially purified by ammonium sulphate $((NH_4)_2SO_4)$ precipitation as described previously for DPPI antibodies. In brief, 2.7 g $(NH_4)_2SO_4$ was added per 10 ml culture supernatant. The solution was mixed on a roller for 3 h at 4 °C, and then ultra-centrifuged at 3,000 x g for 20 minutes at 4 °C. The supernatant was discarded and the pellet re-suspended in 5 ml PBS. The partially purified antibody was dialyzed against PBS at 4 °C

overnight; and against fresh buffer for 3 h. Binding of partially purified antibodies to the basophil lysate was confirmed as described above. The antibodies were then frozen at -20 ºC until required for use.

Prototype ELISA

A prototype ELISA for measurement of basogranulin was developed by assessing different combinations of the new antibodies and various secondary antibodies. Use of either BB1 or BB5 for capture and detection were assessed. In addition, antibodies were used in both purified and partially purified forms and at different concentrations. A secondary antibody against murine IgG was added when using BB1 for detection; and an antibody against murine IgM was added when using BB5.

A close association between increasing concentrations of basophil lysate and optical density was achieved when using BB5 for capture, BB1 for detection, and an anti-mouse IgG secondary antibody. The plate was coated overnight with 50 μl unpurified BB5, diluted 1:10 in carbonate bicarbonate buffer. Non-specific binding sites were blocked with 100 μl 3 % BSA in PBS. Fifty μl basophil lysate was added at concentrations of 1:20, 1:100 and 1:500 in PBS; and PBS was used as a blank control. Antigen was detected with 50 μl partially purified BB1, diluted 1:20 in 1 % BSA in PBS. The plate was incubated with 50 μl anti-mouse IgG secondary antibody, diluted 1:5000 in 1 % BSA in PBS, and then developed using TMB substrate. The reaction was stopped with H_2SO_4, and the optical density measured at 450/595 nm using a spectrophotometer. The standard curve produced using this procedure is shown in Fig 7.3.
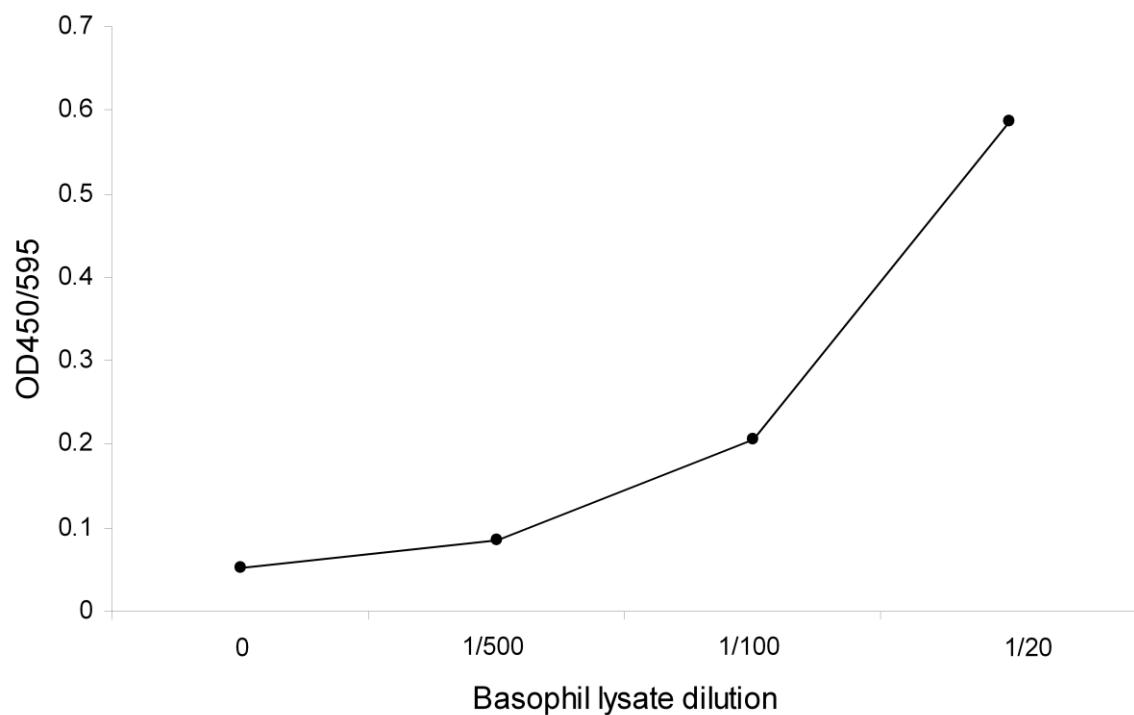
Fig 7.3 Standard curve in basogranulin ELISA. BB5 was used for capture and BB1 for detection, followed by a secondary antibody against murine IgG. A lysate of human basophils was used as the standard.

7.3 Discussion

We have successfully produced new monoclonal antibodies against human tryptase and developed for the first time a prototype ELISA for measurement of basogranulin. Time did not permit further studies in this area. However, there is potential for development of a more sensitive and specific tryptase ELISA and for an assay that can indicate basophil activation.

New monoclonal antibodies against human tryptase

We have raised new monoclonal antibodies that bind to α and β (total) tryptase, and also antibodies that bind to either α or β tryptase specifically. These have been purified, and biotinylated antibodies prepared. Furthermore, we have shown some of the antibodies to work effectively within sandwich ELISA. The properties of the new antibodies require further characterization, but they are likely to be useful in the development of more specific tryptase assays.

The ELISA for tryptase used in most of the present studies has employed polyclonal antibody for capture, and a monoclonal antibody for detection. For optimal sensitivity and specificity, ideally two monoclonal antibodies that bind to different epitopes of the protease should be used. This could be achieved by pairing the currently used antibody, AA5, with a new monoclonal antibody; or alternatively by using a pair of the new antibodies. A technique with greater sensitivity may allow for more accurate measurement of tryptase in other human biological fluids such as saliva.

The commercially available tryptase assay currently used in clinical diagnosis, and also our in-house tryptase ELISA, measures total circulating tryptase (α and β) levels. However, a test for β tryptase may provide a better indication of mast cell activation. In addition, measurement of α tryptase levels may help predict susceptibility to severe reactions, as alleles for this form of the protease compete with some alleles for β tryptase at one site on the human tryptase locus (Soto *et al.*, 2002). Expression of genes for α tryptase, and thus higher levels of this form of the protease, may be indicative of lower risk of anaphylaxis due to occupation of a site that that would otherwise contain genes expressing

the β form. Approximately one third of the population was found to be genetically deficient of α tryptase (Soto *et al.*, 2002). It would therefore be interesting to examine differences in allergic history and severity of allergic reactions in subjects expressing and deficient in α tryptase.

To date there has been no means for distinguishing between circulatory levels of subtypes of tryptase. Previously, an antibody was raised that was thought to be specific for β tryptase (G5); and an ELISA for measurement of this subtype was developed (Schwartz et al., 1995). Alpha tryptase levels were assessed by subtracting levels of β tryptase from total concentrations. However, the G5 antibody was subsequently found to bind specifically to the active mature forms of tryptase (both α and β) rather than to β tryptase alone. Our new antibodies that are specific for α and β tryptase may be used to develop novel assays for measurement of levels of the two subtypes within human biological fluids. This should shed further light on the theory that β tryptase is the most relevant form involved in allergic reactions in humans, and that expression of α tryptase may indicate reduced risk of anaphylaxis.

Novel assay for basogranulin

We have developed a novel ELISA for measurement of basogranulin. This is the first assay with the potential to measure basophil activation in allergic disease. At this stage the ELISA developed should be regarded simply as a prototype technique; and it requires further development and validation for use with human biological fluids. This includes methods described in Chapters 3 and 4, including determination of limits of detection and also sample dilution and spiking studies. Following this, measures of intra- and inter-assay variability will need to be assessed, as will *in vivo* and *in vitro* stability of the protease within biological fluids.

This work ideally requires basogranulin to be purified, and its concentration to be determined. This has not yet been achieved. In the absence of purified basogranulin, lysed basophils could be used. However, in order to maintain consistency throughout the development process and in use of the new assay, a large stock of the lysate must first be established. This has not been possible

within the current study due to the large numbers of volunteers and volumes of blood required to achieve a suitable yield.

Basophils are derived from a common progenitor to mast cells (Li *et al.*, 1998), and have been shown to contain similar granular constituents such as histamine and tryptase (Foster *et al.*, 2002; Li *et al.*, 1998). We detected a bimodal pattern of histamine release into the nasal lavage of patients with seasonal allergic rhinitis following allergen challenge with grass pollen (Chapter 4). The first histamine peak may be a result of mast cell degranulation, and the second may be from basophils which have migrated into the tissues. Measurement of basogranulin levels, alongside histamine and other markers of mast cell activation, in human biological fluids may be useful in examining this concept further. Release of basogranulin at approximately the same time as the second peak of histamine would suggest that it is indeed basophil derived.

The role of IgE, mast cells and histamine in allergic reactions is well described, and the concept of IgE-dependent mast cell and basophil driven responses is widely accepted. However, there has been evidence presented from studies in mice of an independent IgG-mediated systemic anaphylaxis, involving basophil activation and PAF release (Tsujimura *et al.*, 2008). Basophils of mice sensitized with penicillin V-specific IgG1 and then challenged with penicillin V-conjugated BSA were found to capture the IgG1-allergen complex via FcγRII-III. Resulting anaphylaxis was blocked by PAF (but not histamine) inhibition. Depletion of basophils prevented IgG-mediated anaphylaxis, and also rescued mast cell deficient mice (previously sensitized with penicillin V-OVA) from anaphylactic death.

IgG-mediated anaphylaxis has not been described in humans. However, peripheral blood basophils are reported to be more numerous in patients with asthma, allergy or drug reactivity than in healthy control subjects (Li *et al.*, 1998). Release of PAF (alongside histamine) from human basophils upon stimulation with anti-IgE antibody *in vitro* has been reported (Lewis *et al.*, 1995; Camussi *et al.*, 1981); and circulating PAF concentrations were found to be higher in patients with acute allergic reactions compared to healthy control subjects (Vadas *et al.*, 2008). Thus there is some evidence for a role of

basophils and PAF *in vivo*. Measurement of basogranulin levels (in comparison to those of mast cell markers) within the circulation following acute allergic reactions may shed further light on the possibility of an alternative pathway of anaphylaxis in humans.

Summary

Our production of new monoclonal antibodies against α and β tryptase and a novel prototype ELISA for basogranulin may allow development of two new tests for use in diagnosis of allergic reactions. Furthermore, these assays may have the capacity to distinguish between reactions that are mediated by mast cells and/or basophils. The potential for development of more sensitive assays may also allow for production of diagnostic tests that utilize saliva rather than blood.

As well as being used as diagnostic tools, new ELISAs for tryptase and basogranulin may have the capacity to help predict susceptibility of an individual to severe allergic reactions. In addition, the assays may contribute to increased understanding of allergic mechanisms, including contributions of mast cells and basophils in different types of reactions.

CHAPTER 8: GENERAL DISCUSSION

In the current study we have found that mast cell proteases, including tryptase, chymase, carboxypeptidase and DPPI, should be of value as confirmatory markers in cases of suspected allergic reactions. Furthermore, we have shown for the first time that baseline levels of these proteases may be predictive of types and severity of reactions to foods and drugs. Here we discuss the context and implications of these findings.

Diagnostic markers

We have demonstrated that serum, and perhaps saliva, measurement of mast cell proteases may be useful in confirming diagnosis of allergic reactions to foods, drugs, and possibly other allergens. Previous studies revealed that serum/plasma tryptase levels increased during cases of severe allergic reactions to insect venom and drugs (Schwartz *et al.*, 1989; Yunginger *et al.*, 1991), and this is the basis for the recommendations of the resuscitation that concentrations are measured in cases of suspected anaphylaxis. In our study, serum tryptase concentrations were not raised in reactions to foods, but salivary levels increased in children with isolated oral allergy. Thus perhaps release of tryptase is more localised in food allergy. In support of this, previous studies have reported a lack of high tryptase levels in serum following exposure to foods in allergic individuals (Santos *et al.*, 1999; Vila *et al.*, 2001); but the protease was detected locally within the jejunum following intra-luminal allergen provocation (Santos *et al.*, 1999). We did not detect increased serum or saliva tryptase levels following positive drug challenge. However, this likely reflects the mild nature of the reactions.

Serum concentrations of chymase did not increase during allergic reactions to food, but high levels were detected after diagnostic drug challenge in patients who responded to skin testing. There is only one published study of *in vivo* chymase release in anaphylaxis. In this study chymase was detected in all of eight cases of fatal anaphylaxis, but in less than 2 % of deaths due to other causes (Nishio *et al.*, 2005). In support of our findings, most of the allergic reactions occurred in response to drugs.

Previously, levels of carboxypeptidase in serum were found to be higher in patients with anaphylaxis compared to healthy blood donors (Xiaoying Zhou *et al.*, 2006). The blood donors employed as controls are likely to have included both atopic and non-atopic phenotype, and it is possible that some may have previously experienced significant allergic reactions. For this reason, the higher levels of carboxypeptidase seen in patients following anaphylaxis were assumed to be due to an increase during the allergic event, rather than simply a difference between allergic and non-allergic individuals.

In the current study, serum levels of carboxypeptidase were higher in children who reacted to food challenge both at baseline (discussed below) and post challenge. Levels were not significantly raised after reactions compared to at baseline, but concentrations were associated with those of DPPI, which did increase. Serum carboxypeptidase levels were not increased in drug challenge, again likely reflecting the mild nature of responses. However, concentrations in saliva were increased after challenge in those who responded to skin testing. This is interesting given that, when subjects experienced symptoms in addition to positive skin test, these were often confined to the oral mucosa (such as oral pruritus and angioedema).

Serum DPPI levels were high in adults presenting at the Accident and Emergency Department with anaphylaxis to foods, drugs or insect venom; and concentrations were increased after food challenge in children with moderate to severe objective symptoms. Furthermore, post food challenge levels of serum DPPI were associated with the severity of reactions experienced. In addition, saliva levels of DPPI increased after drug challenge in adults who presented symptoms; and serum concentrations were associated with those of chymase (which were high after positive skin testing).

Overall, our findings from patients undergoing food and drug challenges suggest that measurement of a panel of markers may be most effective in confirming diagnosis of allergic reactions and anaphylaxis. This could perhaps be performed using a multiplex assay. Results of this and previous studies support the measurement of tryptase, chymase, carboxypeptidase and DPPI.

There are many potential implications of a new reliable and effective test for confirming diagnosis of allergic reactions. A key example of this is in cases of suspected anaphylaxis in the Accident and Emergency Department. If a patient has experienced an allergic reaction it is important that they are accurately diagnosed to ensure appropriate treatment is given (such as adrenaline). In addition, the allergen must be identified, appropriate rescue medication prescribed, and relevant advice given. Conversely, if it is incorrectly thought that a patient has experienced a reaction to an allergen, this may unnecessarily lead to lifestyle restrictions.

In order for a diagnostic test to influence medication given at the time of a suspected anaphylactic reaction, it would have to yield accurate results very quickly. Thus, development of an assay that can measure levels of a range of mediators rapidly would be optimum. Ideally this could take the form of a bedside test, similar to the pregnancy or blood glucose tests. A test that utilizes saliva rather than blood may be of particular use due to the ease of sampling as compared with obtaining venous access.

As described previously, allergic reactions and anaphylaxis may be underreported due to the difficulties in diagnosis. Easier diagnosis, due to availability of a better test, may allow for increased accuracy in recorded events. This is important in order for sufficient specialized medical staff to be trained, including doctors, nurses and dieticians. Furthermore, more accurate recording of incidence of anaphylaxis may be useful in studies that have the potential to influence policy on care of allergic individuals.

Predictive markers

We have shown for the first time that baseline levels of the mast cell proteases chymase, carboxypeptidase and DPPI may be useful in predicting susceptibility to severe allergic reactions. We have found this to be true for reactions to food in children, and also reactions to drugs in adults. It would be reasonable to suppose that baseline protease measurement may be useful in cases of reactions to any allergen in either age group. However, this would require confirmation.

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There are very few previous studies that have investigated baseline levels of mast cell proteases with respect to severity of allergic reactions. Studies of this nature that have been published have shown associations between baseline levels of serum tryptase and severity of reactions to insect venom (Ludolph Hauser *et al.*, 2001; Potier *et al.*, 2009; Ruëff *et al.*, 2010). No associations have been reported for other allergen types; and in the current study baseline serum or saliva levels of tryptase were not predictive of severity of reactions to foods or drugs.

Results from the present study revealed carboxypeptidase to be the most valuable mast cell protease in predicting reaction severity. Baseline concentrations correlated with severity of historical reactions to both foods and drugs, and also reactions experienced in food challenge. Furthermore, high concentrations were predictive of experiencing angioedema and perhaps wheeze in reactions to food in children, and cardiovascular compromise in reactions to drugs in adults.

Serum carboxypeptidase levels were consistently associated with those of DPPI. Baseline serum concentrations of this protease correlated with severity scores of food challenge reactions, and were higher in children who experienced throat tightness. Levels of carboxypeptidase and DPPI were also associated with those of chymase, and high baseline levels of chymase were found to be predictive of experiencing cyanosis during historical reactions to food. There were some cases in which levels of only one of carboxypeptidase, chymase or DPPI were raised in the absence of other markers. These findings support the measurement of a panel of markers, perhaps in the form of a multiplex assay, in predicting susceptibility to severe reactions.

Assessing susceptibility to severe reactions by way of biomarker measurement, considered alongside other known risk factors, could potentially have important implications in advising patients with allergies. A key risk factor for severe or fatal reactions is late administration of adrenaline (Bock *et al.*, 2001; Bock *et al.*, 2007). In one study, almost half of school children did not carry an adrenaline auto-injector with them (Ben-Shoshan *et al.*, 2008). In most of these cases the device was kept in a nurse's office (or another school office); but a full time

nurse was available in less than 20 %. This suggests that there is likely to be a delay in access to required medication in the event of an allergic reaction in many of these children. Anecdotal observations from our own study suggested that some subjects delayed using their adrenaline auto-injector to see if symptoms worsened, and because they 'didn't want to use it up'. Perhaps knowledge that one is at risk of anaphylaxis, as determined by baseline measurement of biomarkers considered alongside other risk factors, may encourage compliance with carrying medication (or ensuring it is accessible) at all times and immediate action in the event of a reaction.

Availability of a test for predicting susceptibility to severe reactions may affect consumer choices of patients with food allergy. Although some packaged foods are clearly labelled with allergens that they do or do not contain, others are ambiguous, stating that they 'may contain' or 'may contain traces of' allergens. This creates a dilemma for individuals with food allergy in deciding what is safe to eat. A recent study of choices made by teenagers and adults with nut allergies revealed that most sufferers felt that avoidance of all foods that 'may contain' allergens would severely impact on their quality of life (Barnett *et al.*, 2011; Leftwich *et al.*, 2011). Participants also reported that they were more likely to eat foods that 'may contain' allergens if they considered that any resulting reaction was likely to be mild. However, inevitably severe reactions do occur in some cases. A test that can help to establish susceptibility to anaphylaxis may aid patients in making these decisions. Risk-taking behaviour may be reduced in those more susceptible, and anxiety of potential consequences of accidental allergen exposure may be reduced in those at low risk.

The benefits of new tests for predicting susceptibility to anaphylaxis described above are perhaps less relevant for patients allergic to drugs. Once diagnosis of an allergy to a therapeutic agent has been established, alternatives would be sought upon future requirement of medication (if available). Accidental allergen exposure is less likely. However, there are other implications.

Drug allergy is more prevalent in adults than in children and adolescents (Wu *et al.*, 2010); and anaphylaxis is commonly considered to be associated with

myocardial ischaemia (Mueller *et al.*, 2007; Triggiani *et al.*, 2008), a condition which is increasingly prevalent in the aging population. Coronary artery disease is associated with increased severity of allergic reactions (Mueller *et al.*, 2007; Triggiani *et al.*, 2008), and anaphylaxis to insect venom or drugs has been shown to trigger myocardial ischaemia (Yildiz *et al.*, 2006; Tigen *et al.*, 2007; Del Furia *et al.*, 2007; López-Abad *et al.*, 2004). In the current and previous studies, cardiovascular symptoms were a key feature of anaphylaxis to drugs (Cianferoni *et al.*, 2001; Gomes *et al.*, 2004). Thus perhaps tests that predict susceptibility to severe reactions, particularly cardiovascular symptoms, should be considered alongside factors such as age and co-existing disease when prescribing medication that is more frequently associated with allergy. Furthermore, this practice could be applied in considerations towards potential surgical procedures involving drugs such as neuromuscular blocking agents, which can directly activate cardiac mast cells (Genovese *et al.* 2010).This may be particularly relevant in atopic patients, or those with a family history of drug allergy.

Confounding factors

We found that reactions occurring in response to foods in children varied in symptomatology from those occurring in response to drugs in adults. Whereas respiratory involvement was a key feature of paediatric anaphylaxis, severe reactions in adults more often involved cardiovascular compromise (data not shown). These observations were mainly concerning historical reactions, in which information was reported by patients and obtained from medical notes. However, differences were also apparent during the more serious reactions occurring in allergen challenge.

Similarly to our results, in an Italian study of patients admitted to hospital with anaphylaxis, respiratory symptoms were most commonly provoked by foods (Cianferoni *et al.*, 2001). Reactions to drugs occurred most commonly in adults, and frequently involved the cardiovascular system. This suggests that there are differences in the allergenic potential of, and immunological responses to, different allergens. However, the findings may also indicate that changes in the immune system and allergic mechanisms occur with age.

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Instances whereby age appears to play a key role in allergic disease are frequently reported. Some food allergies persist from childhood into adulthood; whereas others are outgrown by, or conversely developed during, adulthood (Yun and Katelaris, 2009). For example, allergy to milk and egg is typically a childhood phenomenon, and is often outgrown before adulthood (Bishop *et al.*, 1990; Savage *et al.*, 2007). On the other hand, allergy to shellfish appears to be more prevalent in adults (Osterballe *et al.*, 2005). Also, most cases of fatal reactions to foods are in adolescents and young adults (Bock *et al.*, 2001; Bock *et al.*, 2007); whereas the severity of venom-induced reactions has been shown to increase with age (Guenova *et al.*, 2010; Kucharewicz *et al.*, 2007). Perhaps there are fundamental changes to mast cells during development from childhood to adulthood that alter their reactive potential to different types of allergens.

Previously, baseline serum tryptase levels have been shown to correlate with age in populations of patients allergic to insect venom (Guenova *et al.*, 2010; Kucharewicz *et al.*, 2007). In our study, baseline concentrations of tryptase in serum were low in all children with food allergy. All subjects had levels below the cut off for normal levels (12 ng/ml), and only one had levels above 5 ng/ml. However, concentrations were also low in our adult population. In support of this, a study by Bonadonna *et al.* (2009) revealed that only nine of 137 subjects (aged five to 79 years) with food or drug allergies, had elevated tryptase levels. The authors of this study did not report an association between tryptase levels and age.

Despite low tryptase levels, concentrations of carboxypeptidase were generally high in children with food allergy compared to levels previously measured in blood donors (Xiaoying Zhou *et al.*, unpublished observation). Furthermore, we found that levels of carboxypeptidase and chymase were higher in children than in adults.

Mast cell protease expression has been reported to be dependent upon cell maturation, which in turn is affected by the cytokine environment. Murine bone marrow-derived mast cells developed with IL-3 contained higher levels of mouse mast cell protease (MMCP) -5 (chymase) and carboxypeptidase mRNA

than MMCP-6 (tryptase) mRNA (Gurish *et al.*, 1992). These cells stained negative with safranin, indicating that they were not granular, and they did not contain mRNA for MMCP-2 or -4 (genes that are expressed late in the differentiation of progenitor cells). Conversely, bone marrow-derived mast cells developed with c-kit ligand were granular and contained MMCP-4 and -6 mRNA, but histamine and carboxypeptidase activity were low.

In later studies, knockout of carboxypeptidase in mice resulted in mast cells with histochemical and biochemical similarities to immature cells, thus suggesting that this protease also plays a role in mast cell maturation (Feyerabend *et al.*, 2005). Ultra-structural analysis revealed that all mast cells in new newborn foreskin were immature; whereas approximately 90 % in adult lung, foreskin and bowel mucosa/submucosa appeared mature (Craig *et al.*, 1989).

The results of these studies suggest that the proportion of mature compared to immature mast cells increases with age; and the level of maturation affects (and is affected by) intracellular protease composition. Chymase and carboxypeptidase appear to be present at high concentrations in immature cells, and carboxypeptidase may play a key role in the maturation process. Although these findings are from investigations in mice, they are supportive of our results showing higher levels of these proteases in children than in adults. In the mouse studies, tryptase concentration was higher in mature mast cells than in immature cells, supporting previous findings of associations between circulating tryptase levels and age (Guenova *et al.*, 2010; Kucharewicz *et al.*, 2007). The potential for differences in reactive potential of mast cells at various stages of maturation requires investigation.

Despite the observed differences in levels of certain mast cell proteases between children and adults in the current and previous studies, our findings support their measurement in confirming diagnosis of reactions and predicting susceptibility to anaphylaxis in both children and adults. Although serum carboxypeptidase levels were higher in children than adults, measurement of the protease was equally associated with severity of reactions in both populations. Thus, it is not anticipated that potential changes in levels with

aging should confound the use of our assays. However, normal levels should be established for different age groups and clinical results interpreted accordingly.

Study strengths and limitations

In this study we have investigated levels of mast cell proteases in allergic reactions occurring in allergen challenge and historically. There were many strengths but also some limitations to the methods employed. Some of these have been discussed in Chapters 5 and 6. Where limitations presented, measures were taken to reduce their effects.

One of the key strengths of investigating allergic reactions occurring in allergen challenge was the capacity for accurate and detailed clinical phenotyping. Presenting symptoms could be observed (or directly described by the patient), and signs such as changes in temperature or blood pressure could be measured. In addition, quantities of allergen required to elicit a response could be precisely recorded.

This level of detail and accuracy was more difficult to achieve when investigating historical reactions, as recording of information was largely reliant on descriptions provided by the patient and/or their family. Patients may not have fully remembered the sequence of events that occurred, or may have forgotten about minor symptoms if more severe symptoms presented. This effect would have been most pronounced in cases where reactions occurred many years prior to recruitment. For a significant proportion of the children recruited, reactions occurred in infancy. In these cases symptoms were described by parents/guardians. However, this would have affected reporting of any subjective symptoms that the child was not able to communicate. In addition, measurements of clinical signs such as changes in blood pressure were not available for most historical reactions.

To reduce effects of limitations relating to recall of events, subjects and their families were asked in detail about historical reactions using a standardized questionnaire. They were asked if they had experienced specific symptoms listed in the questionnaire in order to aid recall and prevent exclusion of minor

symptoms. Most subjects had received medical attention during or soon after the allergic event, and information was obtained from medical notes where possible. All scoring of paediatric reactions to food was carried out by one team member (myself) in order to maintain consistency in interpretation of data.

The main strength of examining reactions occurring historically was that symptoms were likely to have progressed and manifested further than in allergen challenge. Within challenges, patients were exposed to the allergen gradually, and were treated immediately upon onset of symptoms. Thus some symptoms, particularly those of a severe nature, are more likely to have occurred historically than in allergen challenge. Furthermore, anxiety of allergen exposure in open allergen challenges can occasionally trigger symptoms that may be mistaken as an allergic response, leading to a false positive outcome. Ideally double-blind placebo-controlled allergen challenges would have been carried out. However, this was beyond the scope of the current study. False positive reactions are less likely to have occurred within the community as subjects were probably unaware of contact with a potential allergen.

The study of reactions occurring in food challenge was especially beneficial for investigating confirmatory markers. Levels measured after allergen exposure could be compared with those at baseline in order to examine whether concentrations had changed; and timing of sampling could be controlled. However, a limitation within our study was that, in most children undergoing food challenge, only one blood sample was collected following allergen challenge as cannulation was not routinely performed. This may have resulted in sampling at times different to those at which levels of potential markers of reactions increased within the circulation. Although there is no data published regarding times at which increases in chymase, carboxypeptidase and DPPI may be observed, tryptase is reported to be released by thirty minutes and peak between one and two hours (Schwartz *et al.*, 1989). Within the current study post challenge blood sampling occurred between 90 minutes and two hours after initial allergen exposure. Saliva samples, which were easier to obtain, were taken at regular time intervals.

In order to maximize strengths and reduce limitations when investigating potential markers for predicting susceptibility to anaphylaxis, baseline levels were studied with respect to allergic responses occurring both in food challenge and historically. As described above, reactions occurring in food challenge may not have been completely representative of those occurring within the community. However, this method allowed for baseline blood sampling to occur on the day of the reaction. There may have been a large gap in time between obtaining blood samples and historical reactions, and it is possible that levels of the markers may have changed during this time. In addition, a limitation with basing susceptibility to future anaphylaxis on data from historical reactions is that some patients may be at risk without previously having experienced a severe reaction. This may have occurred, for example, if the patient was only previously exposed to very small quantities of the allergen. However, drawing upon results from both challenge and historical reactions strengthens the validity of our findings.

Future directions

Many of our study participants had very low serum and saliva levels of tryptase, some of which were below the levels of detection of the assay. We did find evidence of increased salivary tryptase levels during food challenge in children with isolated oral allergy; but to confirm these findings, and to investigate changes in salivary concentrations further, an assay with greater sensitivity is required. Furthermore, assays that are specific for α and β tryptase may be useful.

We have produced new tryptase-specific murine monoclonal antibodies. There is now the potential for pairs of monoclonal antibodies to be used in development of an ELISA with greater sensitivity than current commercial assays and in-house ELISAs. This may be particularly useful for further research into, and diagnostic potential of, salivary levels. Furthermore, some of the antibodies appear to have differing affinities for α and β tryptase, and thus levels of the two subtypes could potentially be determined. This could have major implications for the specificity and reliability of tryptase measurement in future diagnosis of allergic reactions.

The mediators that we have investigated in this study are predominantly markers of mast cell activation. Tryptase has previously been shown to be present in basophils, but at lower quantities than in mast cells (Xia *et al*., 1995). DPPI is expressed in numerous cell types, including cytotoxic T lymphocytes (Brown *et al*., 1993). However, due to the key role of mast cells in the immediate allergic response, the release seen in our study is likely to be from these cells.

As described in Chapter 1, basophils have similarities to mast cells (Kirshenbaum *et al.,* 1991; Hempstead *et al.,* 1979) and are also thought to play a role in the immediate IgE-mediated allergic response. Their involvement has been indicated using *in vitro* basophil activation tests (Eberlein-König *et al*., 2006). However, the extent of basophil contribution to the pathogenesis of the *in vivo* allergic response in humans is not known. Due to the lack of an increase in serum tryptase levels in some cases of anaphylaxis, there has been speculation about basophil-driven anaphylaxis.

Two murine monoclonal antibodies were previously raised against the basophil-specific protease, basogranulin (McEuen *et al*., 1999; McEuen *et al*., 2001; Fawzy *et al*., 2011). Using the two antibodies, and lysate of basophils purified from human blood, we successfully developed a prototype assay for measurement of the protease. This assay requires further development and validation for use with human biological fluids, such as plasma and saliva. However, it is the first assay that has the potential to examine *in vivo* basophil involvement in allergic reactions, allowing us to test the hypothesis that there are distinct forms of reactions that involve either mast cells or basophils. Furthermore, basogranulin measurement may be useful in confirming diagnosis of reactions. This new and novel assay may have major implications, both clinically and in research.

Summary

We have found for the first time that serum (and perhaps saliva) measurement of other mast cell proteases in addition to tryptase may be useful in confirming diagnosis and predicting severity of allergic reactions to foods, drugs, and

possibly other allergens. Our results, taken together with those from previous studies, suggest that the most effective test may incorporate simultaneous measurement of tryptase, chymase, carboxypeptidase and DPPI levels. Results could be interpreted with respect to clinical signs and symptoms of a patient presenting with a suspected allergic reaction to aid diagnosis; and baseline levels could be considered in addition to history, SPT, IgE measurement and presence of co-existing conditions in assessing potential risk of anaphylaxis. Statistical modelling, including multi-variate analysis, is required to further investigate the complex relationships between the proteases and other clinical indicators, and to establish their usefulness in combination in diagnosis of allergic reactions and predicting susceptibility to anaphylaxis.

In addition to the potential for clinical applications, our novel assays may also be useful in future studies aimed at increasing our understanding of allergic mechanisms. These may include underlying cellular processes that incur susceptibility to severe reactions, and how the mast cell and its mediators contribute to the allergic response. Ultimately this could lead to development of intervention therapies.

We now have new monoclonal antibodies against human tryptase that can be utilized for future development of a more sensitive ELISA, and possibly even assays that can measure levels of the α and β subtypes specifically. These new techniques have the potential to improve upon the reliability of the current test for tryptase. In addition, we have produced a prototype assay for measurement of the basophil-specific protein basogranulin. Further development and validation of this novel ELISA would allow *in vivo* basophil involvement in the allergic response to be investigated for the first time.

Development of novel ELISAs within our laboratory for measurement of chymase, carboxypeptidase and DPPI, and the results generated within this study using these techniques, have huge potential clinical implications for patients who suffer from allergic diseases and the medical staff who care for them. Development of new assays for tryptase and basogranulin may increase this potential further still due to specific measurement of mast cell and basophil activation.

APPENDIX A: Assessment of methods for saliva collection

Assessment of methods for collection of saliva

Several methods for obtaining saliva samples were explored, and the results compared. Saliva was collected from four healthy adult volunteers by salivette, saliva swab or expectoration. Subjects were asked to rinse their mouths with water before each method of collection. Protein content of samples was measured by the Coomassie blue binding assay (BioRad), using bovine serum albumin (BSA) as standards.

No significant differences were seen in protein concentrations of saliva collected by salivette or expectoration (Fig A1). Concentrations were higher in saliva collected by swab. However, after centrifugation of swab samples, cellular deposits formed at the bottom of the tubes, probably accounting for the high protein levels. The salivette yielded the highest volumes of saliva, and was the preferred method by the subjects.
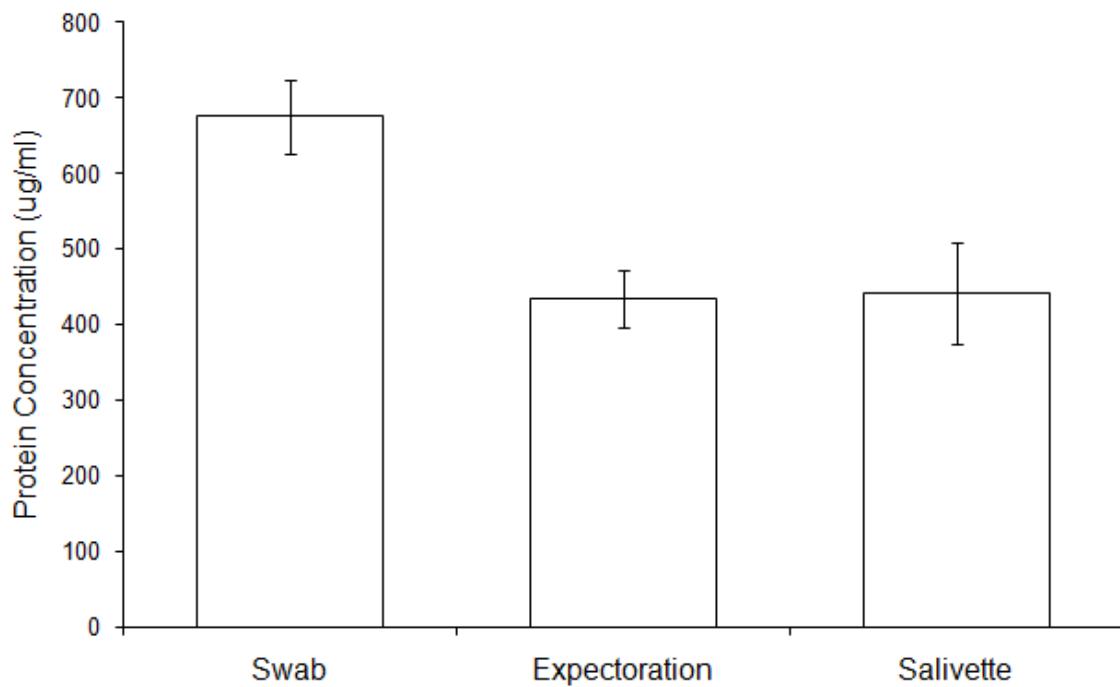
Fig A1.1 Protein concentration of saliva collected by swab, salivette and expectoration. Protein levels were higher in saliva collected by swab, but similar in samples collected by salivette and expectoration. Bars represent mean ± standard error (n = 4).

Protein concentrations were also compared in saliva samples collected by salivette with no prior mouth wash, and with one, two, or three mouth washes with water. Protein levels were higher in samples taken with no prior mouth wash compared to one wash, but did not significantly differ with consecutive washes (Fig A2). The high levels with no wash are probably due to protein from residual food/drink; once this was washed out it appeared that there was no need for further washes. Therefore, prior to saliva collection, study subjects were asked to perform one mouth wash with fresh water. In addition, to ensure that saliva collected was representative of that from all areas of the mouth, patients were asked to chew gently and to move the salivette around the mouth whilst the sample was being collected.
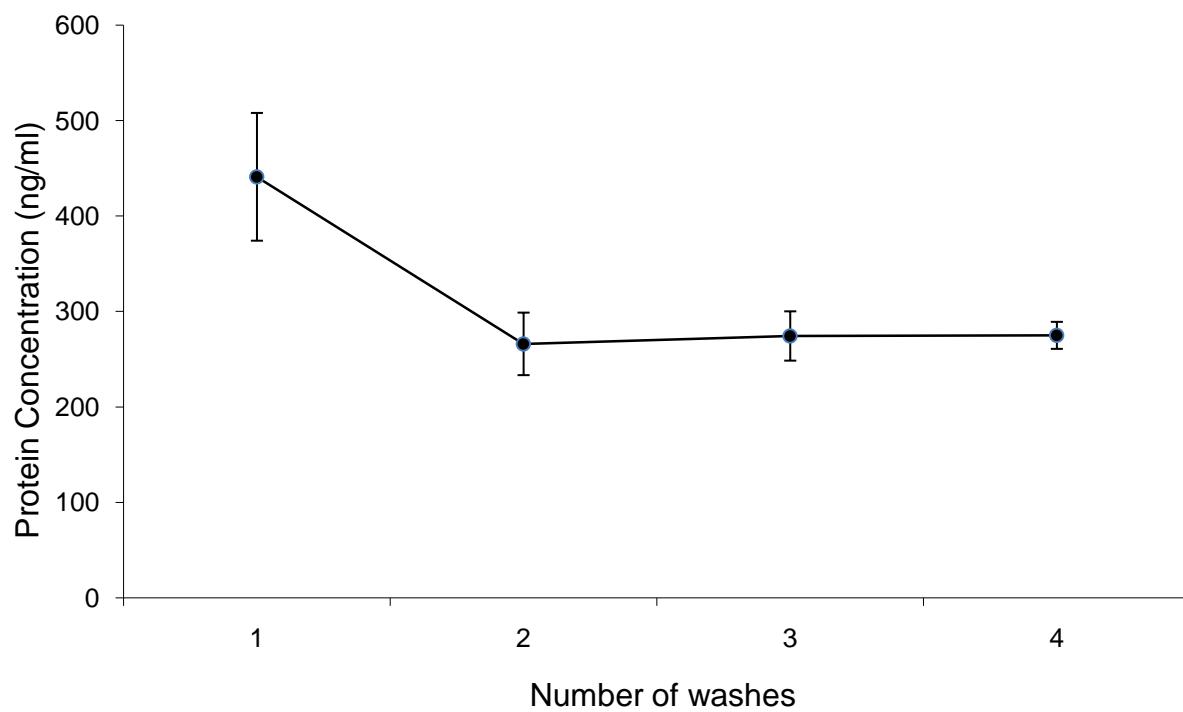
Fig A1.2 Protein concentration of saliva samples collected by salivette with no washes, and with one, two or three washes. After one initial wash, protein levels remained stable. The graph shows mean values ± standard error (n = 4).

APPENDIX B: New biomarkers of anaphylaxis food challenge record

New biomarkers of anaphylaxis: food challenge record

Study number: 08/H0501/17   **Date: ....**

Subject number: …………   **Age: ……yrs……mths**   **Gender: M / F**

1. Food being challenged to: _____

2. Allergic reaction during challenge? Y / N

Symptoms experienced

Generalised hives		Generalised swelling	
Persistent crampy abdominal pain		Cough	
Stridor (upper airway swelling)		Persistent vomiting	
Throat tightness		Croup (upper airway cough)	
Chest tightness		Wheeze	
Hypoxia		Impending doom	
Hypotension		Collapse	
Cough			

Other

_____

Anaphylaxis? Y / N

3. Observations

	Time	Temp	BP	HR	SATS	PEFR
	(24 hr)	° C	mmHg	BPM	%	L/min
1						
2						
3						
4						
5						

4. Challenge timing:

	Time	Cumulative hrs/mins
Contact with potential allergen		
Onset of first symptoms		
Onset of severe symptoms*		
First treatment		
IM/IV adrenaline		
Blood sample 1 (serum/plasma?)		
Blood sample 2 (serum/plasma?)		
Blood sample 3 (serum/plasma?)		
Urine sample 1		
Urine sample 2		
Urine sample 3		
Saliva sample 1		
Saliva sample 2		
Saliva sample 3		

*cough, stridor, croup, throat tightness, chest tightness, wheeze, breathing difficulties, feeling of impending doom, collapse

5. Previous reactions to food being challenged to: Y / N

If yes…

Symptoms experienced

Generalised hives		Generalised swelling	
Persistent crampy abdominal pain		Cough	
Stridor (upper airway swelling)		Persistent vomiting	
Throat tightness		Croup (upper airway cough)	
Chest tightness		Wheeze	
Hypoxia		Impending doom	
Hypotension		Collapse	

Other

_____

6. Previous reactions to other foods/medications/insects: Y / N

If yes…

Cause(s) of reaction(s)

_____

Worst symptoms experienced

Generalised hives		Generalised swelling	
Persistent crampy abdominal pain		Cough	
Stridor (upper airway swelling)		Persistent vomiting	
Throat tightness		Croup (upper airway cough)	
Chest tightness		Wheeze	
Hypoxia		Impending doom	
Hypotension		Collapse	

7. Ever had asthma? Y / N

If yes…

Physician diagnosed? Y / N

Currently has blue reliever (e.g. salbutamol, ventolin, bricanyl)? Y / N

If yes…

Frequency of use _____

Effective? Y / N

8. Ever had hay fever? Y / N

9. Ever had eczema? Y / N

10. Previous allergen testing:

Skin prick testing

Allergen	Date	Wheal size (mm)
+'ve		

Food challenge

Allergen	Date	Result

Serum specific IgE

Allergen	Date	Result (kU/L)

Serum total IgE _____

Reaction Scoring

Community Reaction:

	No reaction	Localised urticaria/ Pruritus	Generalised urticaria	Abdominal pain/ Vomiting/ Angioedema	Laryngeal oedema/ Respiratory problems	Anaphylaxis /Systemic symptoms
Inhalation /Skin contact	0	6	12	18	20	25
Mucosal touch – no ingestion	0	4	10	16	19	24
Fragment swallowed	0	3	8	13	17	23
Small quantity ingested	0	2	7	11	15	22
Large quantity ingested	0	1	5	9	14	21

Food Challenge Reaction:

	No reaction	Localised urticaria/ Pruritus	Generalised urticaria	Abdominal pain/ Vomiting/ Angioedema	Laryngeal oedema/ Respiratory problems	Anaphylaxis /Systemic symptoms
Inhalation /Skin contact	0	6	12	18	20	25
Mucosal touch – no ingestion	0	4	10	16	19	24
Fragment swallowed	0	3	8	13	17	23
Small quantity ingested	0	2	7	11	15	22
Large quantity ingested	0	1	5	9	14	21

APPENDIX C: New biomarkers of anaphylaxis drug challenge record

New biomarkers of anaphylaxis drug challenge record

Subject Details

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

Observations

	° 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	

Time recorded	1		3		5	
	2		4			

Challenges

Challenge drugs	1		3	
	2		4	

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	Dose	1/	1/	1/	1/	1/	
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No	☐-ve control
	Time						

Drug 2

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

Drug 3

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

Drug 4

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

Sampling time points

	Time sampled		Time sampled
Blood 1		Saliva 1	
Blood 2		Saliva 2	
Blood 3		Saliva 3	

Result

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[*]	

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)	☐

Reaction observations	

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			

Symptoms

	Allergen 1 2 3 4 5		Allergen 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	☐ ☐ ☐ ☐ ☐

Existing allergy notes	
Atopy	☐ Hay fever ☐ Asthma ☐ Eczema

Anaesthetic History

Previous anaesthesia before index reaction	☐ Yes ☐ No
Drugs administered previously	

Index anaesthetic reaction signs

	Present
Rash	☐
Generalised angioedema	☐
Hypotension	☐
Bronchospasm	☐
Tachycardia	☐
Bradycardia	☐
Vomiting	☐

Reaction observations	

Medical history

Drug history	
Family history of allergy	
Additional notes	

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