Investigating the immune mechanisms of anaphylaxis

Abbie Francis
BSc (Hons) BForSc

This thesis is presented for the degree of Doctor of Philosophy
School of Medicine
Discipline of Emergency Medicine

2017
Statement of candidate contribution

All work presented in this thesis was performed by the author unless otherwise acknowledged in the text.

Abbie Francis

(PhD candidate)

Erika Bosio

(Coordinating supervisor)
Thesis declaration

I, Abbie Francis, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

This thesis does not contain material that has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

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The research involving human data reported in this thesis was assessed and approved by The University of Western Australia Human Research Ethics Committee. Approval #: RA/4/1/6565

Written patient consent has been received and archived for the research involving patient data reported in this thesis.

The following approvals were obtained prior to commencing the relevant work described in this thesis: Site specific HREC approvals for each participating hospital as follows: Royal Perth Hospital, Fremantle Hospital, and Armadale-Kelmscott Memorial Hospital: EC 2009/080; South West Health Campus: 2012:31; Austin Hospital: H2012/04477

This thesis contains published work and work submitted for publication, some of which has been co-authored.

Signature:  

Date: 15/09/2017
Authorship declaration: co-authored publications

This thesis contains work that has been submitted for publication.

<table>
<thead>
<tr>
<th>Details of the work:</th>
<th>Serum mast cell tryptase measurements: sensitivity and specificity for a diagnosis of anaphylaxis in patients with shock or hypoxemia</th>
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<tr>
<td>Location in thesis:</td>
<td>Sections 2.2 – 2.8 in chapter 2</td>
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<td>Student contribution to work:</td>
<td>The student performed all experiments, analysed the data, and prepared the manuscript. Co-authors recruited patients for the study, collected samples, and reviewed the manuscript.</td>
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<table>
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<th>Details of the work:</th>
<th>Neutrophil activation during acute human anaphylaxis: analysis of MPO and sCD62L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location in thesis:</td>
<td>Sections 5.2 – 5.8 in chapter 5</td>
</tr>
<tr>
<td>Student contribution to work:</td>
<td>The student performed all experiments, analysed the data, and prepared the manuscript. Co-authors recruited patients for the study, collected samples, and reviewed the manuscript.</td>
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<td>15/09/2017</td>
</tr>
</tbody>
</table>

Student signature: Erika Bosio
Date: 15/09/2017

I, Erika Bosio, certify that the student statements regarding their contribution to each of the works listed above are correct.

Coordinating supervisor signature: 15/09/2017
Summary

Anaphylaxis is a potentially life-threatening condition involving multiple organ systems that affects an increasing number of individuals. IgE-mediated mast cell activation is an important trigger of these reactions. However, not all reactions involve IgE and, regardless, the subsequent immune mechanisms that rapidly amplify a localised allergic response into systemic anaphylaxis are poorly understood. This thesis aimed to improve our understanding of anaphylaxis by firstly questioning the established dogma, before investigating other facets of the immune system during acute reactions.

The preferred clinical biomarker for anaphylaxis is mast cell tryptase (MCT), which is released from activated mast cells. Whilst healthy individuals have stable and low MCT, concentrations are transiently elevated within the first few hours of symptom onset during anaphylaxis. However, the validity of MCT as a diagnostic marker to differentiate anaphylaxis from other causes of systemic inflammation had not been assessed. Failure to accurately diagnose anaphylaxis can result in avoidable and potentially fatal secondary reactions. We measured MCT at least three times over the first 24 hours of admission in a large cohort of patients with anaphylaxis and non-anaphylactic critical illnesses (e.g. sepsis and trauma). Both peak and delta-MCT (difference from the highest to lowest concentrations) were higher in patients with anaphylaxis compared to non-anaphylactic critical illnesses. However, the sensitivity and specificity of MCT in isolation was insufficient for distinguishing anaphylaxis from other causes of shock. This highlighted the insufficiency of the IgE-mediated/mast cell pathway to define the anaphylactic process. Clearly, anaphylaxis is far more
complicated and likely involves an array of cell types at different stages from triggering,
to amplification, and resolution of anaphylactic inflammation.

To improve our understanding of the complexity of the immune response, we
undertook two microarray studies investigating differential gene regulation in peripheral
blood leukocytes during anaphylaxis. The first focussed on changes that occur over
time, with samples analysed from the first three hours following emergency department
(ED) admission. It analysed patients with moderate anaphylaxis and was undertaken
prior to the commencement of this thesis. Pathway and upstream regulator analyses
suggested that innate immunity and neutrophil activation played critical roles during
anaphylaxis. Building on these results, we performed validation studies investigating
the mRNA and protein levels of “hub genes” and upstream regulators in a larger and
more diverse cohort as part of this thesis. We observed elevated concentrations of key
markers involved in innate immunity, inflammation, apoptosis, and neutrophil
activation (IL-6, IL-10, S100A9, MMP9, FasL, and TREM1) in anaphylaxis compared
with controls. Alongside this, we undertook a second microarray that included both
moderate and severe anaphylaxis patients and focussed on early changes at ED arrival.
We also compared the anaphylaxis cohort to patients with other inflammatory
conditions (sepsis and head trauma), to identify changes specific to anaphylaxis. This
microarray analysis identified upregulation of natural killer (NK) cell signalling, Type 1
and Type 2 T helper cell (Th1 and Th2) activation pathways, and a snoRNA network.
The putative drivers of these responses included the cytokines IL-2, IL-15, IL-21, and
IFN-α.
After evaluating our results, neutrophils stood out as an important cell type in the anaphylactic response. Studies by other groups in mice had established a critical role for neutrophils in the development of anaphylaxis. We had demonstrated elevated S100A8, S100A9, TREM1 and MMP9 in humans during anaphylaxis, all primarily derived from neutrophils. Next, we directly investigated neutrophil activation by measuring myeloperoxidase (MPO), an enzyme released only from activated neutrophils, and soluble CD62L, a surface protein shed during transendothelial migration. We found MPO concentrations were consistently elevated and soluble CD62L concentrations were consistently reduced over the first three hours since ED arrival. These findings specifically confirm, for the first time, that neutrophils are activated during acute human anaphylaxis. Circulating neutrophils are highly abundant and, once activated, they are able to recruit and activate additional immune cells. Therefore, this could represent one mechanism driving the rapid amplification of the inflammatory response seen in anaphylaxis. Understanding the mechanism underlying the anaphylactic response will be critical in improving available treatments and prevention of allergies and anaphylaxis.
Papers and Presentations

The work presented in chapter 5.2-5.8 of this thesis has been published as follows:


Note: In 2016 I changed my name from Abbie Creamer to Abbie Francis.


Creamer, A. (2014, June) Downregulation of MMP9 and TREM1 mRNA is observed with acute human anaphylaxis. Paper presented at: ASMR Medical Research Week, Perth.


Francis, A. (2016, October) Anaphylaxis: Diagnosis and immune mechanisms. Paper presented at: School of Primary, Aboriginal and Rural Health Care Seminar Series, Perth.


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Acknowledgements

Firstly I would like to extend my sincerest gratitude towards Shelley Stone and Simon Brown whose interest in venoms first caught my attention. Thank you both for offering me this amazing project and cultivating my love of anaphylaxis and all things research.

Many thanks to the other members of my superstar supervisor team Erika Bosio, Daniel Fatovich, and Matthew Linden for providing me with stimulating discussion, valuable insight, and genuinely helpful feedback.

Huge thanks to all the research nurses past and present (special mention to Ellen) for identifying cases and collecting precious samples for me. Also, thank you to all the patients (and controls) who have contributed to this project. Without you, quite simply this thesis would not have been possible.

Lisa, Claire, Moira, and Sally thank you all for your support and encouragement over the years, and thanks for the morning teas that made Mondays that much brighter!

Thank you to all of my friends who have been there every step of the way, helping me to de-stress and unwind.

To the best in-laws ever, thanks John and Julie for doing so very much for us. Thank you for opening your home to me and for helping us to build a home of our own. You have both done so much for us and we are forever grateful.
To my wonderful family, thank you all for everything. Mum and Dad, I would not have gotten this far without your unconditional love and support. Thank you for being so proud of me, it means so much.

Although many people advised me against it, getting a puppy during my PhD was honestly the best idea. I’m sure everybody says this but Ghost is the best dog ever. She has been such a joy to have around. She’s kept me company in my thesis-writing cave and she always makes me smile.

To my partner Jylan Francis, this thesis is dedicated to you. You’ve been right there with me, cheering me on since the start. You’ve seen me through all the ups and downs. Your faith in me has never waivered, and you were right, I got there in the end. You’re my lobster.

This PhD research was supported by an Australian Postgraduate Award, a University of Western Australia Top-Up Scholarship, a Centre for Clinical Research in Emergency Medicine Ad Hoc Top-Up Scholarship, a UWA Emergency Medicine Ad Hoc Scholarship, and an Australian Government Research Training Program (RTP) Scholarship. Project-specific funding sources are acknowledged in each chapter.
Abbreviations

%CC  % correctly classified
ΔMCT  Change in MCT concentrations
AH  Austin Hospital
AKMH  Armadale Kelmscott Memorial Hospital
AUC  Area under the (ROC) curve
bp  Base pairs
CAMERA  Correlation-adjusted mean rank
CBA  Cytometric bead array
CD  Cluster of differentiation
cDNA  Complimentary DNA
CI  Confidence interval
CISS  Critical Illness and Shock Study
CNRQ  Calibrated normalised relative quantity
COPD  Chronic obstructive pulmonary disease
DAMP  Danger-associated molecular pattern
DC  Dendritic cell
DNA  Deoxyribonucleic acid
ED  Emergency department
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme-linked immunosorbsent assay
FAHF-2  Food Allergy Herbal Formula-2
FasL  Fas ligand
FC  Fold change
FDR  False discovery rate
FH  Fremantle Hospital
fMLP  N-formyl-methionyl-leucyl-phenylalanine
G-CSF  Granulocyte colony-stimulating factor
GCS  Glasgow Coma Score
GI   Gastrointestinal
GM-CSF  Granulocyte macrophage colony-stimulating factor
GTP  Guanosine-5'-triphosphate
HR   Heart rate
HGNC HUGO Gene Nomenclature Committee
ICAM Intercellular adhesion molecule
IFN  Interferon
Ig   Immunoglobulin
IgER IgE receptor
IL   Interleukin
INR  International Normalised Ratio
IQR  Interquartile range
IV   Intravenous
LOC  Loss of consciousness
LPS  Lipopolysaccharide
LTP  Lipid transfer proteins
MAP  Mean arterial pressure
MBA  Motorbike accident
MCT  Mast cell tryptase
MLE  Maximum Likelihood Estimation
MMP  Matrix metalloproteinase
MPO  Myeloperoxidase
mRNA Messenger RNA
MyD88 Myeloid differentiation primary response 88
MVA  Motor vehicle accident
NA   Not available
NE   Neutrophil elastase
NET  Neutrophil extracellular trap
<table>
<thead>
<tr>
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<th>Full Form</th>
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<tr>
<td>NIAID/ FAAN</td>
<td>National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network</td>
</tr>
<tr>
<td>NISS</td>
<td>New Injury Severity Score</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerisation domain</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drug</td>
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<tr>
<td>OSCAR</td>
<td>Osteoclast-associated immunoglobulin-like receptor</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAF-AH</td>
<td>PAF-acetylhydrolase</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood leukocyte</td>
</tr>
<tr>
<td>PCA</td>
<td>Passive cutaneous anaphylaxis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEF</td>
<td>Peak expiratory flow</td>
</tr>
<tr>
<td>PSA</td>
<td>Passive systemic anaphylaxis</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPH</td>
<td>Royal Perth Hospital</td>
</tr>
<tr>
<td>S100A8/A9</td>
<td>S100 calcium-binding protein A8/A9</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>sCD62L</td>
<td>Soluble L-selectin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>sIgE</td>
<td>Allergen-specific IgE</td>
</tr>
<tr>
<td>sIgG</td>
<td>Allergen-specific IgG</td>
</tr>
<tr>
<td>SnoRNA</td>
<td>Small nucleolar RNA</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SOFA</td>
<td>Sequential Organ Failure Assessment</td>
</tr>
<tr>
<td>SpO₂</td>
<td>Blood oxygen saturations measured by pulse oximetry</td>
</tr>
<tr>
<td>sTNFR1</td>
<td>Soluble tumour necrosis factor receptor 1</td>
</tr>
<tr>
<td>SWHC</td>
<td>South West Health Campus</td>
</tr>
<tr>
<td>TGM2</td>
<td>Transglutaminase 2</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>TREM-1</td>
<td>Triggering receptor expressed on myeloid cells-1</td>
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Chapter 1     Literature Review

1.1 Introduction

Anaphylaxis is an immediate-type, generalized hypersensitivity reaction affecting multiple organ systems, characterized at its most severe by bronchospasm, laryngeal angioedema, hypoxemia, hypotension, and collapse. It is defined simply as “a serious allergic reaction that is rapid in onset and may cause death” [1]. Anaphylaxis was first recognised over 100 years ago by Portier and Richet, who described attempts to immunise dogs against jellyfish venom, unexpectedly resulting in lethal reactions upon repeated exposure [2]. Richet coined the term anaphylaxis to describe the phenomenon and was later awarded a Nobel Prize [3].

Despite remarkable progress in our understanding of immunology, the pathways of signal amplification that rapidly progress a localised allergic response into systemic life-threatening anaphylaxis remain elusive. Whilst several immune processes have been shown in mouse models of anaphylaxis, few have been evidenced in humans. The most well understood immune mechanism leading to anaphylaxis is the IgE-mediated pathway, which primarily involves B cells, mast cells, and basophils [4]. However, some reactions occur without IgE involvement, instead involving other processes that may include an IgG-mediated pathway, complement generation, and immune complex formation [5]. Regardless of the triggering mechanism, it remains unclear how the initial immunogenic activation at the site of allergen exposure becomes rapidly amplified.
1.2 Pathogenesis of anaphylaxis

1.2.1 Triggers

Anaphylaxis can occur in response to a variety of triggers including foods, venoms, and drugs, which account for roughly equal proportions of reactions [6, 7]. Patients may react to certain triggers but not others, even within the same class of trigger. For example, a patient may react to peanuts but not tree nuts. Alternatively, a patient may be sensitized to multiple, seemingly unrelated triggers. The reasons behind this are unknown. There is even significant variability in the immune responses of a single person to the same trigger at different exposure events [6].

1.2.1.1 Foods

One of the primary triggers of anaphylaxis are foods, which account for approximately one third of all reactions [5]. Hospital admissions due to food-induced anaphylaxis have reportedly increased, particularly in children [8]. Approximately 1 to 2% of the general population and up to 10% of young children suffers from food sensitivities [9, 10]. These reactions usually take effect within seconds of ingestion, and can become life threatening within a few minutes, although delayed reactions have been reported [11]. Peanuts and tree nuts are the most common trigger of severe food-induced reactions. Other common food triggers include fish/shellfish, milk, eggs, soy, wheat, and sesame, although any food is potentially allergenic [6, 9, 12-16]. Patients with suspected food-induced anaphylaxis will typically undergo skin-prick testing to identify the allergen/s they are sensitized against. During skin-prick testing, a drop of allergen is applied to the skin, which is then pricked with a fine needle, allowing the
allergen to penetrate. The size of the swelling and redness (wheal and flare respectively) is an indication of whether the person is sensitised, and is predictive of anaphylaxis risk [17]. A test is considered positive when the allergen elicits a wheal 3 mm larger than that caused by the negative control (e.g. saline) [18].

Treatments may not always be readily available to anaphylaxis suffers, and even when administered they are not always effective [14]. Therefore, of greater importance is the issue of prevention. In cases of food anaphylaxis where the trigger is identified, prevention of future episodes is most effective through strict elimination and avoidance of the offending substances [14]. With widespread education, this strategy has enormous potential for reducing anaphylaxis cases in the community. Indeed, a large number of schools and workplaces are now nut-free to protect anaphylaxis-prone individuals. Despite such preventative strategies, up to 20% of those presenting with anaphylaxis are experiencing a second episode, while for 5% it is a third event. The median time between presentations is 395 days [19]. Avoidance of food allergens is made particularly difficult through inappropriate or inadequate labelling of processed/packaged foods. However, over 30% of secondary food reactions are due to deliberately ignoring or failing to read labels [20, 21].

1.2.1.2 Drugs

Anaphylactic reactions to drugs are also very common, accounting for approximately one third of reported anaphylaxis cases [5]. The incidence of drug-induced anaphylaxis by all drug classes is rising. Unlike food-induced anaphylaxis, reactions to drugs are most commonly observed in middle-aged or older adults [11, 14,
Regardless of admission diagnosis, approximately 3% of all hospitalized patients experience adverse drug reactions during their admission, with 1 in every 2700 suffering severe drug-induced anaphylaxis [11, 23, 24]. Although any drug has the potential to illicit an adverse reaction, the most common triggers include β-lactam antibiotics (including penicillin), radiocontrast media, intravenous anaesthetic drugs, nonsteroidal anti-inflammatory drugs (NSAIDs), and opioid analgesics [11, 14]. In addition, whilst not allergenic of themselves, certain drugs increase the risk of adverse reactions to other drugs. For example, ACE inhibitors and β-blocking agents increase the risk of anaphylactic reactions to other triggers [25-29]. Biological agents that can trigger anaphylaxis include allergens used in immunotherapy (e.g. bee venom extracts) and monoclonal antibodies such as cetuximab (an epidermal growth factor receptor inhibitor used to treat some metastatic cancers), infliximab (a TNFα blocker used to treat autoimmune diseases), and omalizumab, designed to bind IgE to reduce allergic asthma and urticaria [14, 30]. Although rare, anaphylactic reactions to vaccines have also been reported. However, in such events the causative agent is most likely a secondary component of the vaccine such as gelatine [11, 31, 32].

1.2.1.3 Venom

Stinging insects belonging to the Hymenoptera order include honeybees, bumblebees, yellow jackets, hornets, wasps, and ants [33]. Anaphylaxis from Hymenoptera stings is estimated to occur in 0.5 to 5% of the general population, and is more common in adults than in children [34]. The reported incidence of death from insect sting anaphylaxis is between 2 and 3 per year in Australia, and between 40 and 100 per year in the United States, but these numbers are likely to be severely
underestimated [11, 22]. Anaphylaxis to other types of venoms, including that from snakes, does occur and is often associated with concurrent sensitivity to Hymenoptera venom [35]. Unsurprisingly, venom-induced anaphylaxis occurs with greatest frequency during the summer months and in individuals who are highly exposed (i.e. spend large amounts of time outdoors) [11, 12, 22].

1.2.1.4 Other Triggers

Other less common triggers include latex, cleaning agents, and environmental allergens [19]. Latex-triggered anaphylaxis may initially manifest as dermatitis, with subsequent progression to anaphylaxis if exposure is repeated or continued [36, 37]. The incidence of latex sensitivity is reported to be 6.5% in patients who have undergone multiple surgeries [38], 8 to 17% in health care workers [37, 39, 40], and 1 to 6% in the general population [11, 36]. Non-immunologic triggers include exercise (particularly following ingestion of certain foods, i.e. summative trigger), exposure to extremes of temperature, and opioids [6, 41]. Particularly with delayed-onset reactions, specific triggers can be difficult to identify. Reactions are deemed idiopathic if there was no likely trigger identified and no sensitivity observed during follow-up testing [42]. Idiopathic anaphylaxis accounts for approximately 3% of reactions [42], and is of particular concern as no avoidance strategies can be put into place to prevent subsequent reactions.

1.2.2 IgE-Mediated Anaphylaxis

Regardless of the triggering agent, anaphylaxis typically occurs through an IgE-mediated pathway. This is a multistep process involving an initial sensitisation process
(Figure 1.1A), with subsequent exposure triggering rapid immune activation and a mediator storm (Figure 1.1B).

Sensitisation is a process whereby the initial exposure of an allergen to the immune system results in the production of allergen-specific antibodies. The presence of allergen causes naïve CD4+ T helper cells (Th0) to differentiate into Th2 cells, which secrete cytokines including IL-4, IL-5, and IL-13 [4, 43]. In the presence of these cytokines, B cells undergo class-switching and produce allergen-specific IgE antibodies (sIgE) [44]. High and low affinity IgE receptors, FcεRI and FcεRII (CD23) respectively, are present on an array of cell types including mast cells, basophils, neutrophils, and monocytes. The sIgE antibodies bind to these receptors on mast cells and basophils, essentially priming them to respond to that allergen [45, 46]. There is limited evidence of sIgE binding to other cell types expressing IgE receptors.

The second step in IgE-mediated anaphylaxis occurs on a subsequent exposure to either the same allergen or one sufficiently similar so as to bind to the sIgE (for example, bee-sensitised individuals may also react to stings from wasps or other types of insects without having prior sensitisation). The binding of even minute quantities of allergen causes sIgE to cross-link and the IgE receptors, which do not co-localise in the absence of allergen, then aggregate on the surface of mast cells and basophils [4, 5]. This activates the cells, leading to degranulation and the release of an array of biochemical mediators including mast cell tryptase (MCT), histamine, prostaglandins, leukotrienes, and numerous cytokines and chemokines [4]. These mediators can activate the surrounding cells, recruit additional immune cells, and act directly on smooth
muscle and blood vessels to contribute to symptoms such as bronchoconstriction, vasodilation, and increased vascular permeability [47]. Contrary to expectations, sensitised individuals with high levels of serum sIgE and strongly positive skin-prick tests may exhibit no clinical reactivity even when exposed to large amounts of allergen (i.e. during deliberate sting, food or drug challenge) [4, 48]. Conversely, patients with undetectable or very low sIgE and minimally positive skin-prick tests can experience life-threatening reactions [4, 48]. In addition, patients may present with reactions that vary significantly on different exposure events, from mild local reactions to life-threatening severe anaphylaxis [1, 49].

Anaphylaxis can also occur following initial exposure to an allergen, particularly in response to drug and venom triggers. This occurs without sensitisation or the involvement of IgE through lesser-understood non-IgE mediated pathways [50]. It is likely that different individuals develop anaphylaxis through different immune processes and perhaps even within the same patient upon different exposure events, which may contribute to the variation in clinical features. Since very small quantities of allergen can induce severe anaphylactic reactions, it is unlikely that IgE-mediated mast cell activation at the site of allergen exposure is solely responsible for the clinical symptoms of anaphylaxis. The amplification of effector cell activation probably occurs through systemic release of immune mediators from multiple cell sources.
Figure 1.1A: IgE-mediated anaphylaxis: sensitisation to allergen.

The presence of allergen stimulates naïve CD4+ T helper cells (Th0) to differentiate into CD4+ Th2 cells, which produce the cytokines interleukin-4 (IL-4), IL-5, and IL-13. These cytokines prompt B cells to produce allergen-specific IgE antibodies (sIgE) that bind to IgE receptors (IgER) on the surface of effector cells (mast cells and basophils).
Figure 1.1B: IgE-mediated anaphylaxis: the response of sensitised cells to allergen.

On a subsequent exposure, the allergen binds to sIgE antibodies on the surface of mast cells and basophils. This causes crosslinking of the sIgE, which activates the cell and initiates degranulation. Mediators including mast cell tryptase (MCT) and histamine are released. These mediators can activate surrounding cells, recruit additional immune cells, and contribute directly to the physical symptoms of anaphylaxis such as swelling and hypotension by increasing vascular permeability and dilating blood vessels.
1.2.2.1 Mediators of Anaphylaxis

Investigation into the IgE-mediated anaphylaxis pathway has identified a large number of mediators contained within mast cells and basophils that can contribute to symptoms. These mediators have traditionally been placed into three classes: (1) preformed mediators stored in the cytoplasmic granules of effector cells that are released by degranulation; (2) newly synthesised pro-inflammatory lipid mediators generated over minutes; and (3) newly synthesised growth factors, cytokines and chemokines generated over hours (Table 1.1) [47]. There is supporting evidence that several of these mediators are elevated during acute human anaphylaxis including MCT, histamine, interleukin (IL)-6, IL-10, C-reactive protein, leukotrienes, platelet activating factor (PAF) (with corresponding low levels of PAF-acetylhydrolase (PAF-AH) activity), prostaglandins, and soluble tumour necrosis factor receptor 1 (sTNFR1) [51-57]. Furthermore, concentrations of a number of these mediators correlate with reaction severity in humans, suggesting a role in symptom development [55, 58]. Variation in target organ sensitivities and levels of regulatory mediators between individuals further complicates the analysis of mediator levels.

1.2.2.1.1 Histamine

One of the most well-characterised mediators of anaphylaxis is histamine. Histamine is a hydrophilic vasoactive amine formed from the decarboxylation of histidine. It is normally confined within vesicles of mast cells and basophils, and is released into the bloodstream following activation of the cell by certain stimuli, including allergen exposure [14]. Histamine has a number of effects including promoting vasodilation, increasing vascular permeability, decreasing blood pressure,
stimulating smooth muscle contractions, and increasing heart rate and the strength of cardiac contractions [59]. Histamine was first identified as a potential mediator of anaphylaxis in 1929 [60], and antihistamines have been used to treat mild allergies and hay fever for over 60 years [61]. Mast cells were first shown to degranulate and release histamine in response to allergens in the 1960s [62].

Once in the extracellular environment, histamine is rapidly metabolised by histamine methyl transferase [61]. It has a half-life in the bloodstream of 30 minutes, which greatly limits its utility as a marker of mast cell and basophil activation [58]. Plasma histamine levels have been shown to correlate with reaction severity in samples drawn within 60 minutes of anaphylaxis symptom onset, and a concentration greater than 1.2 ng/mL is considered positive (based on the 99th percentile of healthy controls) [14, 55]. However, patients often have difficulty in arriving at a hospital or medical centre within this time frame. A less time-sensitive alternative is to measure urinary histamine metabolites (e.g. methylhistamine), which may be elevated for up to 24 hours following the onset of symptoms [52]. However, this is not common practice for anaphylaxis emergency department admissions. The specificity and sensitivity of urinary histamine metabolites for a diagnosis of anaphylaxis has not been defined.
Table 1.1: Mediators of anaphylaxis.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Known or possible effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preformed for immediate release</strong></td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>Vasodilation and oedema, bronchoconstriction, mucous secretion, and nerve stimulation.</td>
</tr>
<tr>
<td>Heparin</td>
<td>Mediates capillary leakage and oedema by initiating the formation of bradykinin, a vasoactive and pro-inflammatory peptide hormone.</td>
</tr>
<tr>
<td>Tryptase</td>
<td>Leukocyte migration and activation, bronchoconstriction, vasodilation and oedema, tissue degradation and cell proliferation. Potentially plays a role in the amplification of an allergic response (positive feedback on effector cells).</td>
</tr>
<tr>
<td>Chymase</td>
<td>Vasodilation and oedema, mucous secretion, leukocyte activation, and tissue degradation.</td>
</tr>
<tr>
<td>TNFα</td>
<td>Bronchoconstriction, leukocyte adhesion, migration and activation, and may play a role in delayed phase reactions.</td>
</tr>
<tr>
<td><strong>Newly generated over minutes</strong></td>
<td></td>
</tr>
<tr>
<td>Cyclooxygenase products (e.g. PGD₂)</td>
<td>Vasodilation and oedema, mucous secretion, bronchoconstriction, and nerve stimulation.</td>
</tr>
<tr>
<td>Lipoxygenase products (LTB4, LTC4, LTD4, LTE4)</td>
<td>Vasodilation and oedema, mucous secretion, bronchoconstriction, and leukocyte recruitment.</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activation, leukocyte migration and activation, histamine release, and reduced myocardial contractility.</td>
</tr>
<tr>
<td><strong>Newly generated over hours</strong></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Leukocyte adhesion, migration, and activation.</td>
</tr>
<tr>
<td>IL-4, IL-5, IL-13</td>
<td>Allergen-specific IgE production, and upregulation of FceRI expression.</td>
</tr>
<tr>
<td>IL-10</td>
<td>Anti-inflammatory cytokine that reduces activation and degranulation of mast cells, and induces increased numbers of</td>
</tr>
</tbody>
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regulatory T cells.

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<table>
<thead>
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</tr>
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<tbody>
<tr>
<td>IL-6</td>
<td>Pro-inflammatory cytokine that correlates with hypotension and the extent of erythema, upregulates FcεRI expression and intracellular histamine, and prevents mast cell apoptosis.</td>
</tr>
<tr>
<td>PAF-AH</td>
<td>Enzyme that inactivates PAF, and reduced levels of PAF-AH has been reported in fatal anaphylaxis.</td>
</tr>
<tr>
<td>Anaphylatoxins (C3a, C4a, C5a)</td>
<td>Complement activation products that stimulate mast cell and neutrophil degranulation, and cause smooth muscle contraction and increased vascular permeability.</td>
</tr>
<tr>
<td>Chemokines (RANTES, IL-8, MCP-1)</td>
<td>Chemotactic recruitment and activation of additional immune cells, and histamine and serotonin release from mast cells.</td>
</tr>
<tr>
<td>Sulphidoleukotrienes</td>
<td>Bronchoconstriction and smooth muscle contraction.</td>
</tr>
</tbody>
</table>

1.2.2.1.2 Tryptase

Mast cell tryptase (MCT) is a 134 kDa tetrameric serine esterase stored primarily in mast cell secretory granules, although small amounts of tryptase are present in basophils [47, 63, 64]. When released, MCT is secreted as a large ~200kDa active proteoglycan complex, with its size limiting diffusion away from mast cell activation sites [47]. Therefore, large increases in circulating tryptase levels are generally only observed after the extensive mast cell activation seen in anaphylaxis [64]. Serum tryptase can activate surrounding mast cells, and therefore may assist in spreading the degranulation signal from mast cell to mast cell [47]. Serum tryptase peaks 60 to 90 minutes after the onset of anaphylaxis and, unlike histamine, remains elevated for up to 6 hours, making it a more useful laboratory marker for anaphylaxis [65]. A positive result for serum tryptase is defined as a total tryptase concentration greater than 11.4
ng/mL, the 95\textsuperscript{th} percentile of healthy controls as determined by the ImmunoCAP\textsuperscript{®} tryptase test manufacturer (Phadia (now Thermo Fisher Scientific)) [14, 51, 52]. The change in total tryptase over time (delta-MCT) is also useful in diagnosing anaphylaxis, with a change greater than 2 ng/mL considered outside the normal variation observed in healthy controls [51, 55].

There are several tryptase subtypes, primarily $\alpha$ and $\beta$, which share approximately 90\% sequence homology despite vast differences in their activity and function [64]. The $\alpha$-tryptases are released constitutively from mast cells and, due to a defect in the catalytic domain, they are largely inactive [64, 66]. Elevated $\alpha$-tryptase levels, and a consistently elevated total MCT, may indicate clonal mast cell disorders such as mastocytosis, characterized by an overabundance of mast cells [57]. This highlights the importance of collecting serial blood samples. The $\beta$-tryptases are stored in mast cell granules and are released during degranulation. Serum $\beta$-tryptase is typically only elevated to detectable levels after anaphylaxis [57, 67]. However, the sensitivity of tryptase assays is limited as anaphylaxis can occur in the absence of mast cell activation. In addition, even where mast cell activation occurs, the true peak of MCT may be missed or fall within the normal range of the assay [47].

1.2.2.1.3 Cytokines and Chemokines

Cytokines represent an integral component of the signalling networks among various cells, essential for the development and regulation of innate and adaptive immune processes [68]. They are a large family of small proteins produced by immune and non-immune cells that act locally on neighbouring cells to direct important
biological processes including inflammation, immunity, repair, and angiogenesis [69]. Several studies have identified the potential involvement of a number of cytokines during anaphylaxis (including TNFα, IL-6, and IL-10).

TNFα is the major inflammatory cytokine and is produced following activation of an array of immune cell types including mast cells and is released following cell activation [70]. Elevated TNFα production has been observed during severe IgE-mediated anaphylaxis [55]. It activates neutrophils, increases monocyte chemotaxis, and enhances the production of other cytokines by T cells [70]. As such, TNFα is likely to be involved in spreading the immune activation signal from the site of allergen exposure [47].

Another cytokine reported to play a role in anaphylaxis is IL-6, which is released by macrophages, T cells, and mast cells upon their activation [71]. IL-6 has been shown to have both pro- and anti-inflammatory effects depending upon the stimulus and surrounding environment. However, it is proposed to be pro-inflammatory in the context of anaphylaxis [72]. Indeed, elevated levels of IL-6 correlated with the occurrence of hypotension (a severe symptom) in human patients with anaphylaxis [53, 55].

The anti-inflammatory cytokine IL-10 has also been implicated in anaphylaxis. IL-10 may contribute to the resolution of anaphylaxis by reducing activation and degranulation of mast cells and/or basophils and dampening the effect of pro-inflammatory mediators [73]. IL-10 was found to have a protective effect against
anaphylaxis when administered to mice prior to allergen challenge [74]. In addition, elevated levels of serum IL-10 have been identified during moderate and severe human anaphylaxis. This occurred approximately one hour after the observed peak in inflammatory mediators such as histamine and MCT [55]. IL-10 has been shown to upregulate regulatory T cells (Tregs), which suppress anaphylaxis [75, 76]. This presents a potential mechanism whereby IL-10 production may offer protection from future reactions [55]. Although other cytokines are also likely to be involved in anaphylaxis, there is limited evidence to support that at this stage, particularly in humans.

1.2.2.2 Mast Cells

The immune cell type with the most widely evidenced role in anaphylaxis is the mast cell, which releases all of the aforementioned mediators. Mast cells are leukocytes derived from haematopoietic progenitor cells that circulate in the blood in an immature form [64]. They migrate to vascularised tissues and undergo final differentiation and maturation as guided by cytokines secreted from endothelial cells and fibroblasts [77]. Mature mast cells are large, terminally differentiated cells that play an important role in the immune response to pathogens [64, 66, 78]. They are found in most tissues in the body, with the largest numbers present at junctions to the external environment such as the skin, airways, and gastrointestinal tract [77].

1.2.2.2.1 Structure

Mast cells have a large round or ovoid shaped nucleus and numerous surface receptors and cytoplasmic granules [79]. In the 1970s, a receptor with a uniquely high-
affinity for IgE was first identified on mast cells, named FcεRI [80]. Mast cells also express receptors for IgG, which can induce anaphylaxis in mouse models (chapter 1.2.3), including the high affinity IgG receptor FcγRI (CD64) [81]. Mast cells have been shown to express a variety of complement receptors, such as C5a receptor, allowing them to also respond to complement activation signals [77]. Pattern-recognition receptors, including toll-like receptors (TLRs), such as TLR4, are also expressed and can act as activating or inhibitory receptors [82]. The mast cell populations of different tissues express different surface markers depending on their location within the body and the functions required [83]. The effects of certain mediators may be restricted to specific tissues due to the differential expression of their receptors on different populations of mast cells [84]. Therefore, the severity and symptoms of anaphylaxis may be partially dependent on the route of allergen exposure (e.g. ingested or injected) and the specific mast cell population activated.

Mast cells contain a large number of cytoplasmic granules that encapsulate an array of different enzymes and proteins. Following activation, mast cells can degranulate, which releases these mediators into the extracellular environment, allowing mast cells to exert their effects on the offending pathogen [4, 78]. As with the surface receptors, the expression of particular mast cell mediators differ between mast cells of different tissue locations [85]. Two major mast cell components, histamine and tryptase, can be expressed in vastly different proportions depending upon the tissue microenvironment in which the mast cells mature [85]. The histamine content of skin, heart, and lung mast cells is comparable, whilst the tryptase content is much higher in skin mast cells than in either heart or lung mast cells [79, 85]. In addition to the
mediators released upon degranulation, mast cells also modify transcription to upregulate production of proteins as required depending upon the stimulus [79]. Therefore, mast cells are highly effective response cells, critical in defence against foreign pathogens.

1.2.2.2 Activation

Mast cell activation can occur through several pathways including via IgE binding to FcεRI (in the context of anaphylaxis) and via pattern recognition receptors (e.g. TLRs) directly interacting with pathogens [77]. Depending on the triggering stimulus, mast cell activation can result in each of the following responses, alone or in combination: cytokine release; degranulation (i.e. the rapid release of pre-formed mediators from cytoplasmic granules, including primarily histamine, tryptase, and chymase) [78]; the production of lipid-derived eicosanoids (e.g. prostaglandin D2 and leukotriene C4) within minutes [77]; and, over the course of hours, the upregulation of cytokines and chemokines (e.g. IL-4 and additional TNFα) [77, 86].

Despite their fundamental role in IgE-mediated anaphylaxis, mast cell activation/degranulation is not critical for the development of anaphylaxis. Mast cell deficient, IgE deficient, and FcεRI (IgE receptor) knockout mice are able to mount anaphylactic responses to allergens [87-89]. In addition, many cases of human anaphylaxis occur without evidence of mast cell activation. One or more peripheral blood leukocytes, including neutrophils, DCs, monocytes, and T cells, are likely to be involved in the initiation and/or amplification of systemic anaphylaxis.
1.2.2.3 Biological Functions

Even though mast cells have the potential to trigger severe anaphylactic responses, they are of great importance in several biological processes, particularly host defence. Mast cells contribute to host defence in a variety of ways through both direct and indirect mechanisms. They can directly kill invading organisms through phagocytosis and the production of reactive oxygen species (ROS) and antimicrobial peptides [90, 91]. Mast cells can also produce extracellular traps that encompass and kill organisms through exposure to high concentrations of antimicrobials [92]. However, due to the relatively small number of mast cells in tissues, it is unlikely that the direct killing of pathogens by mast cells plays a large role in overall host defence. The indirect effects of mast cells in coordinating the immune response are likely to be much more important.

The indirect immunoregulatory actions of mast cells involve both the innate and adaptive immune systems. Degranulation causes the release of a large array of mediators and chemokines that are involved in innate immunity. The release of vasoactive mediators (e.g. histamine) increases vascular permeability and local blood flow, enhances epithelial cell mucus production, and can act on smooth muscle to immobilise pathogens and increase the expulsion of mucosal parasites [77, 83, 93]. Mast cell-mediated production of chemotactic factors recruits inflammatory cells including neutrophils, eosinophils, and natural killer (NK) cells [94]. Mast cell products enhance maturation of immature dendritic cells (DCs), upregulating antigen presentation and expression of co-stimulatory molecules [77, 78]. In addition, mast cell-derived cytokines and chemokines enhance the migration of DCs and effector T cells to
the site of infection and draining lymph nodes [83]. Mast cells also function directly as antigen presenting cells, presenting foreign antigens to T cells, particularly CD8+ T cells [95]. Whilst mast cell responses beneficially increase host defence locally at the site of infection, mast cell-mediated enhancement of inflammation can also induce damage to host tissues and worsen outcome during some infections [77, 96].

1.2.3 **IgG-Mediated Anaphylaxis**

There is a significant degree of similarity between IgG- and IgE-mediated anaphylaxis with similar sensitization and reaction steps. Initial exposure to allergen causes differentiation of Th0 cells into Th2 cells and stimulates the production of cytokines, which cause B cells to produce allergen-specific IgG (sIgG) [89]. These antibodies then bind to IgG receptors, including high (FcγRI) and low (FcγRII and FcγRIII) affinity receptors, on the surface of effector cells [97]. Mast cells, basophils, neutrophils, eosinophils, monocytes, macrophages, Langerhans’ cells, DCs, and platelets all express IgG receptors [81, 98]. On subsequent exposure, the binding of allergen/sIgG complexes causes the IgG receptors to cross-link, which activates the effector cell [81]. Unlike IgE-mediated anaphylaxis where histamine is considered the major mediator, IgG-mediated anaphylaxis is primarily associated with the release of platelet activating factor (PAF) [97]. Binding of PAF to its receptor on the surface of mast cells can activate them, inducing histamine release in the absence of direct allergen binding [49, 99]. However, the evidence for IgG-mediated anaphylaxis is currently limited to animal models and *in vitro* cultures. The mechanisms for IgG-mediated anaphylaxis in humans are not well understood.
The variability of the symptoms and severity of anaphylaxis, both between and within susceptible individuals, may be attributed to whether the allergen bound to sIgE or sIgG on each event and in what proportions. Despite the two processes being similar, eliciting IgG-mediated anaphylaxis typically requires considerably more antibody than IgE-mediated reactions [89, 100]. Therefore, sIgG may be protective against IgE-mediated anaphylaxis by binding the allergen preferentially, thereby preventing allergen from binding to sIgE.

1.2.4 Other Mechanisms

A number of other mechanisms of anaphylaxis have also been proposed. Immunological (i.e. allergic) mechanisms include complement generation, immune complex formation, coagulation system activation, neuropeptide release, cytotoxicity, and leukocyte activation. Non-immunological (i.e. non-allergic) pathways include immune cell activation through physical stress (e.g. exercise or temperature) [83, 101, 102].

Both IgE- and IgG-mediated anaphylaxis require sensitization through prior exposure. However, up to half of patients with drug-induced anaphylaxis claim no prior contact with the triggering drug [103], suggesting a non IgE-/IgG-mediated reaction. These reactions may be caused by cross-reactivity between drug compounds and other allergens, or the drugs may be bound up in a drug-protein complex recognised as a danger signal by the innate immune system [23, 104]. The latter could cause upregulation of co-stimulatory molecules and elevated cytokine production, culminating in an anaphylactic reaction.
Exposure to certain substances, such as tryptase, results in rapid activation of complement, producing large amounts of the anaphylatoxins C3a, C4a, and C5a [105]. High levels of plasma C3a and C5a have been observed during human anaphylaxis, which correlate with reaction severity [106]. The anaphylatoxins can act directly causing smooth muscle contraction and enhanced vascular permeability [107]. They can also act indirectly by stimulating macrophages, basophils, and mast cells to secrete PAF and histamine [108]. Immune complexes generated by complement activation and anaphylatoxins may lead to recruitment and activation of inflammatory cells such as neutrophils and monocytes, contributing to the immune amplification seen in anaphylaxis. Although the field of allergy and immunology is rapidly advancing, much remains unknown about the mechanisms of anaphylaxis that occur in humans.

1.2.5 Mouse Models of Anaphylaxis

Mice are the predominant laboratory animals used for research into many diseases. They are favoured for their small size, short breeding cycles, manageable housekeeping, and the relative ease of genetic manipulation, compared with larger models [109, 110]. However, mouse models are not ideal surrogates for human anaphylaxis for a number of reasons. Firstly, there is limited evidence to suggest that anaphylaxis naturally occurs in mice unlike dogs and cats, for example [111]. As a result, anaphylaxis is difficult to diagnose in mice, and does not present with features akin to those seen in human anaphylaxis. Additionally, there are differences between the properties and binding affinities of mouse and human IgE/IgG receptors, perhaps contributing to differences in pathways of anaphylaxis [112]. Therefore, it is likely that
the mediators and cellular environment required for anaphylaxis, as it occurs in humans, is not complete in mice.

There are two different mouse models widely used to study anaphylactic responses: passive cutaneous anaphylaxis (PCA); and passive systemic anaphylaxis (PSA). The PCA model induces anaphylaxis in mice by the direct injection of antigen-specific IgE. The extent of the response is modelled by measuring vascular extravasation through tracking the release of Evans blue dye from the injection site [4]. The PSA model is a process more similar to the IgE-mediated anaphylaxis pathway seen in humans. Mice undergo sensitization through the repetitive exposure to allergen, followed by an allergen challenge via systemic injection. In these mice, anaphylaxis is diagnosed by: measuring the levels of circulating histamine; noting decreases in activity; and measuring a reduction in core temperature, a symptom not typically observed in human anaphylaxis [4, 97]. Although these models do not encapsulate the spectrum of responses associated with human anaphylaxis, they have provided considerable information about the signalling molecules that regulate mast cell activation. These studies have also supported comprehensive in vitro studies conducted in bone marrow-derived mast cells from knockout and transgenic mice [4].
1.3 Physiology of anaphylaxis

1.3.1 Diagnosis

The diagnosis of anaphylaxis is based on the presence of certain features as detailed in the current National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network (NIAID/FAAN) criteria (Table 1.2) [1].

Table 1.2: NIAID/FAAN criteria for the diagnosis of anaphylaxis.

<table>
<thead>
<tr>
<th>1. Acute onset illness (minutes to hours) with involvement of skin and/or mucosa (generalized hives, pruritus or flushing, swollen lips &amp;/or tongue) <strong>and at least one of the following:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Respiratory compromise (dyspnoea, wheeze, bronchospasm, stridor, hypoxemia (SpO₂ ≤ 92% and/or cyanosis))</td>
</tr>
<tr>
<td>b. Hypotension (systolic blood pressure (SBP) &lt; 90 mm Hg) or associated symptoms of end organ dysfunction (loss of consciousness (LOC), collapse, syncope, incontinence)</td>
</tr>
<tr>
<td>2. Two or more of the following within minutes to several hours of exposure to a likely allergen for that patient:</td>
</tr>
<tr>
<td>a. Skin or mucosal involvement as above</td>
</tr>
<tr>
<td>b. Airway compromise as above</td>
</tr>
<tr>
<td>c. Hypotension or associated symptoms as above</td>
</tr>
<tr>
<td>d. Persistent gastrointestinal symptoms (e.g. cramping abdominal pains, vomiting)</td>
</tr>
<tr>
<td>3. Hypotension within minutes to hours following exposure to a known allergen for that patient</td>
</tr>
</tbody>
</table>

The differential diagnosis of anaphylaxis also overlaps with other common conditions, such as acute generalized hives, acute asthma, syncope, panic attack, aspiration of a foreign body, and cardiovascular or neurologic events [6, 14, 113]. A diagnosis of anaphylaxis is supported by blood tests showing increased histamine or MCT, which are commonly present in anaphylaxis, as mentioned previously (chapter
1.2.2.1). However, these features are not specific to anaphylaxis and not seen in all patients [14, 49, 93]. Due to the apparent involvement of basophils in the anaphylactic response, a basophil activation test using CD63 and CD203 expression as markers of anaphylaxis has been developed and used as a diagnostic tool, with a sensitivity of 85% – 100% and specificity of 83% – 100% [114, 115].

In cases of sudden death due to suspected but un witnessed exposure to allergen, post-mortem diagnosis of fatal anaphylaxis is difficult and requires careful investigation. Post-mortem measurement of mediator levels, such as MCT, may assist with diagnosis. However, moderately elevated MCT levels are common in post mortem sera following non-anaphylactic deaths. In these scenarios, much higher cut-points are needed to define a positive result, and values above 45 ng/mL may support the diagnosis of fatal anaphylaxis [51, 57, 116, 117].

1.3.2 Symptoms

The symptoms of anaphylaxis include urticaria (hives/rash), erythema (flushing), angioedema (swelling), nausea, vomiting, bronchospasm, hypoxemia/cyanosis, and collapse (unconsciousness) due to arterial hypotension [1]. All these changes result primarily from the release of mediators that cause: blood vessel dilation; extravasation of fluid from the circulation into tissues; smooth muscle constriction; and myocardial dysfunction [58]. Involvement of particular organ systems varies amongst patients, and even within the same patient from one episode to another. However, some general patterns have been observed. Urticaria and angioedema are the most common clinical symptoms, seen in over 80% of cases, followed by shortness of breath/wheeze in 70%,
gastrointestinal tract involvement in 45%, and hypotension in 35% of reactions [14, 118]. Patients may present with any combination of these symptoms. Importantly, the less severe symptoms, such as urticaria, do not always precede more severe symptoms as a warning or indicator of an oncoming reaction [11].

1.3.2.1 Severity Grading

The severity of a reaction is commonly defined as either mild (grade 1), moderate (grade 2), or severe (grade 3) based on the symptoms experienced by the patient (Table 1.3). This grading system was developed in 2004 and is the current standard for anaphylaxis classification [119]. The severity of a reaction correlates with systemic concentrations of anaphylaxis mediators, such as histamine and MCT, which are commonly measured to confirm a diagnosis of anaphylaxis (chapter 1.2.2.1) [41].
Table 1.3: Severity grading system for hypersensitivity reactions.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Defined by:</th>
<th>Grade</th>
<th>Defined by:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mild (skin and subcutaneous tissues only)</td>
<td>2</td>
<td>Moderate (features suggesting respiratory, cardiovascular, or gastrointestinal involvement)</td>
</tr>
<tr>
<td></td>
<td>Generalized erythema, urticaria, or angioedema</td>
<td></td>
<td>Dyspnoea, stridor, wheeze, nausea, vomiting, dizziness (pre-syncope), diaphoresis, chest or throat tightness, or abdominal pain</td>
</tr>
<tr>
<td>3</td>
<td>Severe (hypoxemia, hypotension, or neurological compromise)</td>
<td></td>
<td>Cyanosis or SpO₂ ≤ 92%, hypotension (SBP &lt; 90 mm Hg in adults), confusion, LOC, collapse, or incontinence at any stage</td>
</tr>
</tbody>
</table>

1.3.3 Biphasic Reactions

Biphasic anaphylaxis is defined as a second reaction occurring 1 – 72 hours after initial recovery. Symptoms of the second phase reaction can be similar, milder, or more severe than the initial reaction [120]. Hypotension and airway obstruction during the initial reaction are a common feature of biphasic anaphylaxis [121]. These types of anaphylactic reactions have been reported in up to 11% of children and up to 20% of adults presenting to emergency departments with anaphylaxis [120, 122]. Biphasic reactions are a significant problem and account for 25% of cases of fatal or near-fatal food reactions and 23% of drug reactions. However, biphasic reactions are uncommon in anaphylaxis of other causes [121].

1.3.4 Risk Factors

Risk factors for anaphylaxis are difficult to define and although certain predispositions have been identified, they are often not easily recognizable or testable. The greatest risk factor for anaphylaxis is having had a prior reaction to a particular allergen. Therefore, the most effective and reliable determination of anaphylaxis risk is
through a documented history of previous allergic/anaphylactic episodes [11, 123]. This method is clearly not ideal since some individuals die from their first anaphylactic reaction [11, 22]. Other factors reported to increase the risk of anaphylaxis include high pollen counts, foreign travel or other disruption of routine, fever, acute infection (such as an upper respiratory tract infection), emotional stress, menses (premenstrual and ovulatory phases), and ingestion of ethanol [14].

Patients may be at risk of increased reaction severity and fatality due to advanced age, concomitant disease, and concurrent medications [14]. For women who have a reaction during pregnancy, anaphylaxis places the mother and especially the baby at high risk of fatality or permanent central nervous system damage [124]. Pre-existing conditions, such as asthma and other chronic respiratory diseases, cardiovascular disease, mastocytosis or clonal mast cell disorders, allergic rhinitis, and eczema are all associated with increased risk of life-threatening or fatal anaphylaxis [14, 16, 125]. Concurrent medications, such as β-blockers and angiotensin-converting enzyme inhibitors increase the severity of anaphylaxis, and make reactions more difficult to treat [14].

1.3.5 Treatments

Due to the lack of understanding of the complexity of the immune response during anaphylaxis, the symptoms are primarily treated through physiological antagonism. Epinephrine is the primary intervention for anaphylaxis and acts as an effective cardiac stimulant and bronchodilator, and also reduces fluid extravasation from capillaries [93, 126, 127]. In the event of accidental ingestion or contact with a
known trigger, prompt treatment with epinephrine is effective in minimizing reaction severity [11]. For this reason, patients with a history of anaphylaxis are advised to carry devices to self-administer epinephrine (e.g. EpiPen®) in the event of a reaction [126, 128]. Anaphylaxis fatalities are often attributed to delays in epinephrine administration, although even those patients who receive prompt treatment do not always survive [127-129]. The current Australasian Society of Clinical Immunology and Allergy Guidelines also recommend a number of supplementary treatments as follows: patients with hypotension may be given intravenous fluid therapy to restore blood volume in vessels; steroids may be given to treat persistent wheeze, although the benefits are unproven; high flow oxygen and bronchodilators may be given to patients with respiratory symptoms; and gastrointestinal symptoms may be treated with anti-nausea medication or anti-emetics [130]. Despite evidence of elevated plasma histamine during anaphylaxis, antihistamines have little to no benefit as a treatment or to prevent progression of symptoms [130].

Venom immunotherapy (desensitisation) immediately after a sting reaction is extremely effective at preventing future anaphylactic reactions and is recommended for up to 3 to 5 years after the initial incident [14]. In adults with a history of anaphylaxis to Hymenoptera sting, those who do not receive immunotherapy have a risk of anaphylaxis from future stings of approximately 50%. In patients who do receive immunotherapy, the risk is less than 3%, and venom immunotherapy is generally very well tolerated [131]. Only approximately 6% of patients develop allergic adverse reactions to the immunotherapeutic injections [34]. Sting avoidance is advised through the use of appropriate apparel, insect repellent, and caution. For sensitised individuals, in the event
of a sting, prompt treatment with epinephrine is suggested and sufferers are encouraged to carry an epinephrine auto-injector at all times [11].

Desensitisation for food sensitivities remains experimental and is not yet used in routine clinical practice. There are currently no known guidelines describing the optimal candidates for food desensitisation, or the safest and most effective dosing schedule. Food allergen immunotherapy through the oral or sublingual routes may reduce the risk of adverse events when compared with subcutaneous desensitisation [6, 132]. One potential treatment, Food Allergy Herbal Formula-2 (FAHF-2), has been found to block peanut-induced anaphylaxis in a mouse model [133]. This protection was due to a FAHF-2-induced reduction in basophil and mast cell numbers, and suppression of IgE-mediated mast cell activation, including reduced IgE receptor (FcεRI) expression and release of histamine after allergen challenge [134]. In an acute Phase I study of patients with food sensitivities, 6 months of three times daily doses of FAHF-2 were shown to be safe and well tolerated. This dosage schedule resulted in a significant reduction in basophil CD63 expression (a basophil activation marker) in response to ex vivo stimulation, suggesting a reduction in sensitivity [135].

1.4 Summary and Aims

Allergy and anaphylaxis are an increasing health concern worldwide. Despite large advances in scientific techniques and our understanding of immunology, much remains unknown about the mechanisms of anaphylaxis. In some cases, the immune system is sensitized through repeated exposure to allergen and ultimately the presence
of allergen causes effector cells, including mast cells, to degranulate. This causes an array of mediators to be released into the peripheral blood. It is unclear precisely how a localised mast cell response becomes rapidly amplified into a systemic, potentially life-threatening response. A number of different cell types including neutrophils, basophils, DCs, and T cells are likely involved. These cell types may contribute to reactions that occur on a first exposure, without mast cell sensitisation and involvement. Improving our understanding of anaphylaxis at an immunological level is critical for identifying at-risk individuals, improving therapies, and preventing future reactions.

The overarching aim of this thesis is to investigate the immune mechanisms of anaphylaxis, particularly those involved in the rapid amplification of the allergenic signal. The overall hypothesis is that key immune pathways are activated during the acute period of anaphylaxis that amplify, rather than resolve, the localised response to allergen. Neutrophils, in particular, were hypothesized to be involved in this process due to their abundance in peripheral blood and capacity for rapid signalling.

The thesis is divided into four smaller aims, which have been formatted (submitted and/or published) as journal articles. First, the usefulness of the serum mast cell tryptase (MCT) assay (which measures the degree of systemic mast cell activation) in the diagnosis of anaphylaxis in the emergency department was investigated. Specifically, we aimed to explore the ability of MCT measurements to differentiate between anaphylaxis and other forms of critical illness, which can often present similarly. Second, we aimed to validate the key findings of a previous microarray performed in our laboratory. This microarray highlighted the importance of the innate
immune system in anaphylaxis. We investigated this further by analysing mRNA and protein levels of neutrophil activation markers, danger signals, and regulators of apoptosis. Third, we undertook a second microarray study of a larger patient cohort that included both anaphylaxis reaction severities, in addition to sepsis and head trauma patients for comparison. The aim of this study was to identify key immune pathways activated during the acute phase of anaphylaxis and investigate whether these differed based on reaction severity. We also aimed to identify pathways that may be specific to anaphylaxis, compared to other inflammatory conditions. Differential expression of key hub genes and suspected molecular drivers of the response were validated using qPCR and protein assays. Fourth, as the involvement of neutrophils during anaphylaxis was becoming more evident, we then aimed to specifically investigate neutrophil activation during anaphylaxis. The neutrophil-specific activation marker myeloperoxidase (MPO) was measured in stored plasma samples from anaphylaxis patients.

Taken together, the work presented in this thesis aimed to improve our understanding of the immune mechanisms of anaphylaxis by initially taking a broad look at the systems and pathways activated during a reaction. It then focuses more closely on the involvement of one particular standout candidate cell type, neutrophils.
Chapter 2  Serum mast cell tryptase measurements: sensitivity and specificity for a diagnosis of anaphylaxis in patients with shock or hypoxemia

2.1 Background

The initial direction of this thesis was to investigate mast cell activation in anaphylaxis patients that presented to Australian emergency departments. The IgE-mediated/mast cell pathway described in Chapter 1.2.2 is currently the only mechanism well evidenced in humans. Mast cell tryptase (MCT) is released from mast cells during this process. MCT is the preferred biomarker for anaphylaxis, as it remains elevated in the serum for several hours after the onset of symptoms. Previous studies, in our laboratory and others, have shown that MCT is frequently elevated in patients with anaphylaxis, and rarely in healthy volunteers. However, not all cases of anaphylaxis are associated with mast cell degranulation, and other types of shock can also activate mast cells. The following study addressed this concern by investigating the usefulness of MCT to differentiate anaphylaxis from other types of shock frequently seen in emergency departments. As such, our aim was to determine the sensitivity and specificity of MCT as an independent biomarker to separate anaphylactic from non-anaphylactic shock in critical patients. Characterisation of the variability in MCT response as outlined in this chapter also underpins investigation of the role of mast cell degranulation in activation of immune pathways in anaphylaxis, as outlined in subsequent chapters. The experimental work for this chapter has been submitted for publication in the European Journal of Immunology, as follows.
Title: Serum mast cell tryptase measurements: sensitivity and specificity for a diagnosis of anaphylaxis in patients with shock or hypoxemia

Short title: Serum mast cell tryptase to diagnose anaphylaxis

Abbie Francis¹,², Daniel M Fatovich¹,²,³, Glenn Arendts¹,²,³,⁴, Stephen PJ Macdonald¹,²,³,⁵, Erika Bosio¹,², Yusuf Nagree¹,⁴,⁶, Hugh Mitenko¹,⁷, and Simon GA Brown¹,²,³,⁸

1. Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Perth, WA, Australia
2. Discipline of Emergency Medicine, School of Medicine, Faculty of Health and Medical Sciences, University of Western Australia, Crawley, WA, Australia
3. Emergency Department, Royal Perth Hospital, Perth, WA, Australia
4. Emergency Department, Fiona Stanley Hospital, Perth, WA, Australia
5. Emergency Department, Armadale Kelmscott Memorial Hospital, Mount Nasura, WA, Australia
6. Emergency Department, Fremantle Hospital and Health Service, Fremantle, WA, Australia
7. Emergency Department, South West Health Campus, Bunbury, WA, Australia
8. Emergency Department, Royal Hobart Hospital, Hobart, TAS, Australia; and School of Medicine, University of Tasmania, Hobart, TAS, Australia

Key words: allergic shock, anaphylaxis, clinical immunology, delta-MCT, immunologic tests, mast cell tryptase, mast cells, MCT

Corresponding author: Abbie Francis, Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Level 6 MRF Building, Rear 50 Murray Street, Perth, Western Australia 6000.
Abbreviations

%CC, % correctly classified; AUC, area under the (ROC) curve; CI, confidence interval; CISS, Critical Illness and Shock Study; ED, emergency department; HR, heart rate; IQR, interquartile range; MAP, mean arterial pressure; MCT, mast cell tryptase; NIAID/FAAN, National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network; NSAID, non-steroidal anti-inflammatory drug; ROC, Receiver Operating Characteristic; SBP, systolic blood pressure; SD, standard deviation; SpO\textsubscript{2}, oxygen saturations measured by pulse oximetry.

2.2 Abstract

Background

Laboratory confirmation of anaphylaxis would be clinically useful, particularly for patients with atypical symptoms. We investigated mast cell tryptase (MCT) measurement, transiently elevated in most anaphylaxis patients, for differentiating anaphylaxis from other causes of critical illness.

Methods

MCT was measured in patients with anaphylaxis and non-anaphylactic critical illness (controls) at emergency department arrival, and after 1-2, 3-4, and 12-24 hours. Differences between groups were investigated using linear regression models, and diagnostic ability was analysed using Receiver Operating Characteristic curves.
Results

Peak MCT was 4.0-fold (95%CI: 2.9, 5.5) higher in anaphylaxis patients (n=67) than controls (n=120) (p<0.001). Delta-MCT was 5.1-fold (95%CI: 2.9, 8.9) higher in anaphylaxis than controls (p<0.001). Optimal test characteristics (sensitivity of 72% (95%CI: 59, 82) and specificity of 72% (95%CI: 63, 80)) were observed when MCT concentrations were greater than 11.4 ng/mL and/or delta-MCT ≥2.0 ng/mL. For hypotensive patients, peak MCT >11.4 ng/mL had an improved sensitivity of 85% (95%CI: 65, 96) and specificity of 92% (95%CI: 85, 97); delta-MCT reduced test specificity.

Conclusion

A dichotomous result for MCT was not sufficiently accurate to distinguish anaphylaxis from other critical illnesses. When considered in combination with clinical features, particularly hypotension, MCT is a useful diagnostic marker of anaphylaxis.

2.3 Introduction

Anaphylaxis is a potentially life-threatening systemic allergic reaction that progresses rapidly following exposure to an allergen [1, 119]. It is distinguished from mild allergic reactions by the involvement of multiple organ systems and/or potentially lethal cardiorespiratory compromise [1, 136]. Diagnosis is straightforward in cases that present with typical skin features (i.e. erythema, urticaria, and/or angioedema) together with objective evidence of additional organ system involvement, such as hypotension or hypoxemia [41, 137]. However, some cases present with subtle skin changes that are easily missed or only appear after resuscitation from cardiovascular collapse [1, 118, 138]. Furthermore, multisystem involvement may only be evident from subjective
clinical features such as itch, dyspnoea, nausea, and dizziness, and potentially effort-dependent or psychogenic features such as wheeze and stridor [1]. These features often resolve prior to first medical assessment.

In cases where the diagnosis is uncertain, laboratory confirmation of anaphylaxis can be clinically useful for subsequent risk assessment and management. However, with the array of different mediators, multiple inflammatory pathways, and various cell types involved, no “gold standard” biomarker has been identified [139, 140]. The only widely available and well-standardised biomarker assay is a mast cell tryptase (MCT) test, which measures total tryptase in serum (ImmunoCAP®, Thermo Fisher Scientific, Uppsala, Sweden). Total tryptase is a mixture of mature β-tryptases released by mast cell degranulation, a process that occurs following allergen exposure in most cases of anaphylaxis, plus baseline or “constitutive” pro-tryptases (α and β) that are present in the absence of anaphylaxis [64, 116]. Typically serum MCT levels begin to rise within 30 minutes of symptom onset, reaching a peak after approximately 3 hours, before returning again to baseline [64, 141]. Importantly, a single MCT measurement may miss the peak of mature β-tryptase, or alternatively, the peak may occur within the normal range for the assay, rendering the assay insensitive for anaphylaxis. Furthermore, a single measurement may also lack specificity as it cannot always differentiate anaphylaxis from conditions such as mastocytosis and other mast cell disorders that cause persistently high MCT values [57, 116].

Serial measurement of MCT, by identifying maximum MCT concentrations and investigating changes in concentration (delta-MCT), may overcome the lack of sensitivity and specificity of single MCT measurements, and identify patients with
anaphylaxis whose peak MCT falls within the normal range. A previous study in our laboratory showed that an *absolute* delta-MCT ≥ 2 ng/mL has a diagnostic sensitivity in the order of 80% and is highly specific for a diagnosis of anaphylaxis, when compared to non-reacting, healthy controls [51]. Another study investigating patients with a history of hypersensitivity to venom, found that a *relative* delta-MCT, measuring the difference in the peak relative to baseline, with a cut-point of 135% was positive for 17 of 20 (85%) patients who reacted upon re-exposure to venom and none of the 15 (0%) non-reactors [142]. However, these approaches have not been assessed for their ability to differentiate anaphylaxis from shock and/or hypoxemia due to other causes, the most likely clinical scenario where an MCT assay would be measured.

The aim of this study was to investigate this question by measuring MCT concentrations and changes in patients with shock and/or respiratory compromise from a range of causes, and evaluating the diagnostic abilities of different methods for determining a positive result. Our hypothesis was that MCT release during non-anaphylactic shock would limit the diagnostic utility of MCT for differentiating anaphylactic from non-anaphylactic shock.

### 2.4 Materials and methods

#### 2.4.1 Patient recruitment

Patients (age ≥ 16 years) meeting the inclusion criteria listed in Box 1 were enrolled in the Critical Illness and Shock Study (CISS)[143] in the emergency departments (ED)s of three Australian metropolitan hospitals (Royal Perth Hospital, Perth WA; Armadale Kelmscott Memorial Hospital, Mount Nasura WA; and Austin Hospital, Heidelberg VIC) between March 2010 and January 2014. Clinical details were
recorded onto structured datasheets and samples were collected as soon as practicable after enrolment criteria were met in the ED, and 1 – 2, 3 – 6, and 12 – 24 hours post enrolment, where possible. Blood was collected into serum separating tubes (Vacutainer® SST™ tubes, Becton Dickinson & Co., New Jersey, USA), and processed, then stored immediately at -80°C until analysis. The CISS enrolment criteria exclude mild (skin-only) allergic reactions. A sample size was not pre-specified and we aimed to recruit as many cases of anaphylaxis as possible over the 4-year period of funding.

2.4.2 Ethics approval and consent

Ethics approval was obtained from the Human Research Ethics Committees at each site (Royal Perth Hospital and Armadale Kelmscott Memorial Hospital: EC 2009/080; Austin Hospital: H2012/04477). As emergency care took priority, waiver of initial formal consent was approved under the provision of paragraph 2.3.6 of the National Health and Medical Research Council Ethical Conduct guidelines (2007). Patients enrolled under this waiver could subsequently choose to provide delayed informed consent or withdraw from the study.

2.4.3 Case selection

Suspected anaphylaxis was identified on enrolment based on the National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network (NIAID/FAAN) consensus definition (Box 2) [1]. Atypical cases (e.g. two affected organ systems without skin reaction, or hypotension or wheeze where anaphylaxis was considered as a possible diagnosis) had no requirement for allergen exposure, as this may have been uncertain at the time of presentation. Patients were excluded if features
had resolved prior to ED arrival. Three clinical investigators (SB, DF, and GA) subsequently and independently applied the NIAID/FAAN criteria, based on the available clinical information for each case of suspected anaphylaxis. Investigators were blinded to MCT results. The cases were then classified as either definite (i.e. strict adherence with NIAID-FAAN criteria) or possible anaphylaxis. Sequentially enrolled non-anaphylaxis cases with hypotension (systolic blood pressure (SBP) < 90 mm Hg) and/or hypoxemic respiratory failure (SpO₂ ≤ 92% and/or cyanosis) served as a control group.

2.4.4 Mast cell tryptase measurement

The concentration of MCT in neat patient sera was determined using a clinical laboratory ImmunoCAP® Tryptase system (Phadia (now Thermo Fisher Scientific), Uppsala, Sweden). In accordance with the product protocol, concentrations greater than 11.4 ng/mL (the 95th percentile of healthy control values determined by the manufacturer) were considered positive. The lower limit of detection for serum MCT was 0.707 ng/mL.

2.4.5 Statistical analysis

For each patient and control, an absolute delta-MCT was calculated as the difference in concentration between highest and lowest MCT values observed in each set of serial samples. In addition, a relative delta-MCT was calculated as the change (as a percentage) from the highest to the lowest MCT values. Both peak MCT and absolute delta-MCT were log transformed to normalise the distribution of results. Linear regression models were used to test for differences between groups, adjusted for age and
sex. To investigate the diagnostic utility of each method and investigate alternative cut-points, ROC curve analysis was used. The sensitivity, specificity, area under the ROC curve (AUC), and % correctly classified (CC), with corresponding 95% confidence intervals (CIs) as appropriate, were calculated. Finally, we performed a descriptive analysis of confirmed anaphylaxis cases with a negative MCT, and non-anaphylaxis cases with a positive MCT (defined using an 11.4 ng/mL peak MCT cut-point and/or an absolute delta-MCT cut-point of 2 ng/mL) by comparing their characteristics (main clinical features) with those correctly classified, using linear regression models as appropriate. All statistical analyses were performed with Stata version 12.1 (StataCorp, College Station, Texas).

2.5 Results

2.5.1 Patient characteristics

Of the 83 patients enrolled with suspected anaphylaxis, 67 were classified as definite anaphylaxis (including all 36 severe cases), 15 as possible anaphylaxis, and 1 patient was given a diagnosis of chronic urticaria and excluded. The clinical characteristics of the definite and possible anaphylaxis cases are detailed in Table 2.1. Of the definite cases, the majority of moderate reactions were triggered by foods (19/31 (61%)) and most severe reactions were drug-triggered (20/36 (56%)). Reaction characteristics of the possible anaphylaxis patients are described in detail in Table S2.1. The major clinical characteristics of the critically ill non-anaphylaxis controls are described in Table S2.2; they differed from anaphylaxis patients in several ways. Hypotension was observed in 26 (32%) definite anaphylaxis patients, no possible anaphylaxis patients, and 103 (86%) non-anaphylaxis controls. There were no deaths among the anaphylaxis cohort, whilst 23 (19%) controls died. Control patients stayed in
hospital 10.6 days (95% CI: 7.8, 13.5) longer than definite anaphylaxis patients (p < 0.001), who in turn stayed for 7.0 hours (95% CI: 0.45, 13.6) longer than those with possible anaphylaxis (p = 0.036). Patients with possible anaphylaxis took 59 minutes (95% CI: 10, 109) longer from symptom onset to first blood sampling than definite anaphylaxis (p = 0.020).

### 2.5.2 Mast cell tryptase concentrations

Peak MCT concentrations, absolute delta-MCT concentrations, and relative delta-MCT percentages for definite anaphylaxis, possible anaphylaxis, and non-anaphylaxis controls are summarised in Figure 2.1. Definite anaphylaxis patients had peak MCT concentrations ranging from 1.2 – 163.1 ng/mL (median (interquartile range (IQR)): 13.3 (25.4)). Possible anaphylaxis patients had peak MCT concentrations ranging from 3.6 – 45.7 ng/mL (median (IQR): 12.5 (14.3)). Non-anaphylaxis controls had peak MCT concentrations ranging from 0.2 – 44.3 ng/mL (median (IQR): 4.2 (4.4)). Peak concentrations were 4.0-fold higher (95% CI: 2.9, 5.5) in definite anaphylaxis patients than controls (p < 0.001), and 2.6-fold higher (95% CI: 1.7, 3.9) in possible anaphylaxis than controls (p < 0.001). There was no difference between the peak MCT concentrations of definite and possible anaphylaxis (p = 0.108).

The absolute delta-MCT of definite anaphylaxis patients ranged from 0.1 – 114.1 ng/mL (median (IQR): 4.0 (14.3) ng/mL). Possible anaphylaxis patients had absolute delta-MCT ranging from 0.1 – 14.2 ng/mL (median (IQR): 2.9 (7.6)). Non-anaphylaxis controls had absolute delta-MCT ranging from 0 – 22.3 ng/mL (median (IQR): 1.0 (1.6)). The absolute delta-MCT of definite anaphylaxis patients was 5.1-fold (95% CI: 2.9, 8.9) higher than non-anaphylaxis controls (p < 0.001), and possible
anaphylaxis had *absolute* delta-MCT concentrations 2.7-fold (95% CI: 1.1, 6.6) higher than controls (p = 0.025). There was no difference between definite and possible anaphylaxis (p = 0.123).

Lastly, the *relative* delta-MCT of definite anaphylaxis ranged from 101 – 690% (median (IQR): 149 (109)). The *relative* delta-MCT of possible anaphylaxis patients ranged from 103 – 302% (median (IQR): 137 (70)). Non-anaphylaxis controls ranged from 100 – 650% (median (IQR): 131 (47)). The *relative* delta-MCT of definite anaphylaxis patients was 51% higher (95% CI: 14, 89) than that of controls (p = 0.007). There were no differences between the other groups (p ≥ 0.145).

### 2.5.3 Validation of current diagnostic limits

Using ROC curve analysis, the validity of currently recommended cut-points for peak MCT and *absolute* and *relative* delta-MCT [51, 142] (both alone and in combination) to correctly diagnose anaphylaxis was investigated (Table 2.1, Figure 2.2). The combination of sensitivity, specificity, and %CC for peak or delta-MCT (*absolute*/*relative*) was not improved by selecting different cut-points. Major cut-points for peak MCT and *absolute* delta-MCT are listed in Tables S2.3 and S2.4 respectively, and the sensitivity, specificity, and %CC for each are detailed. Although peak MCT > 11.4 ng/mL had the highest specificity and %CC, the sensitivity was low. The combined approach where a positive result was defined by a peak MCT > 11.4 ng/mL and/or *absolute* delta-MCT ≥ 2 ng/mL optimized both the sensitivity and specificity, and correctly identified 72% (95% CI: 65, 78) of patients. *Relative* delta-MCT alone was the poorest performer with a low sensitivity, specificity, AUC, and %CC. Interestingly, when considering only patients (and controls) with hypotension the diagnostic ability of

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MCT using all methods was improved (Table 2.3, Figure 2.3). In patients with hypotension, optimum sensitivity, specificity, AUC, and %CC was achieved when a positive result was defined by peak MCT alone.

### 2.5.4 False positive and false negative MCT results

The method describing a positive result as peak MCT > 11.4 ng/mL and/or absolute delta-MCT ≥ 2 ng/mL was investigated further to identify common features of outliers. Firstly, we considered the definite anaphylaxis cases with a negative MCT result (false negatives). These 19 patients spanned a large age range (18 – 69 years old), were primarily female (12 (63%)), and stayed in hospital for 2 – 23 hours, all features of which were not significantly different from the correctly classified patients (p ≥ 0.070). The majority of these false negative patients had moderate reactions (15 (79%)) (p = 0.001). The major triggers were foods (12 (63%)) and drugs (4 (21%)). These patients displayed a variety of symptoms, and almost all were administered epinephrine (18 (95%)), and 7 (39%) of these received multiple doses. There were no common or unique features that defined this group. The peak MCT ranged from 1.2 – 10.8 ng/mL, and the absolute delta-MCT ranged from 0.1 – 1.9 ng/mL.

Lastly, we considered the non-anaphylaxis controls with a positive peak MCT and/or absolute delta-MCT result (false positives). Thirty-four such patients were identified, being between 22 and 86 years, primarily male (25 (74%)), and stayed in hospital for 0 – 50 days, all features that were not significantly different from controls with negative MCT results (p ≥ 0.094). All of these patients had hypotension, and the predominant conditions were sepsis (12 (35%)), cardiac conditions (8 (24%)), and trauma (7 (21%)). The peak MCT for false positives ranged from 2.8 – 44.3 ng/mL, and the absolute delta-MCT ranged from 1.9 – 22.3 ng/mL.
2.6 Discussion

Laboratory confirmation of a diagnosis of anaphylaxis is important to facilitate risk minimization strategies (trigger avoidance, desensitization therapy, and access to epinephrine auto-injectors (e.g. EpiPen®)) for susceptible patients. Peak MCT and delta-MCT measurements are moderately effective at differentiating anaphylactic patients from healthy/non-reacting individuals [51, 142]. This study is the first to investigate MCT as a diagnostic marker, to differentiate anaphylaxis from other types of shock in the emergency setting.

Both peak and absolute delta-MCT concentrations were higher in patients with anaphylaxis compared to critically ill non-anaphylactic controls. However, not all anaphylactic patients had disturbances in MCT and, conversely, several non-anaphylactic controls had abnormal MCT. Indeed, other studies have also shown MCT is not elevated in a considerable proportion of anaphylaxis cases [51, 142, 144]. The best test characteristics we found used a combined approach whereby a positive result is defined by a peak MCT > 11.4 ng/mL and/or an absolute delta-MCT ≥ 2.0 ng/mL. This provided better sensitivity, but specificity was still poor. Whilst using peak MCT alone is not sufficiently sensitive for diagnosing anaphylaxis, the high specificity, particularly at higher cut-points, suggests a positive result would provide useful diagnostic support.

When the analysis focused on hypotensive patients, the sensitivity of peak MCT in particular was greatly improved, without compromising specificity. We have previously shown that MCT concentrations correlated with hypotension in anaphylaxis patients [55], so the increased sensitivity was predictable. However, the finding that peak MCT retained a high specificity for anaphylaxis amongst other critical illnesses
with associated hypotension, is novel. Hypotension was frequently observed in non-anaphylactic controls without detectable changes in MCT, and it is therefore unlikely that hypotension alone triggers sufficient mast cell activation to alter systemic MCT concentrations. When considered in combination with clinical features, particularly hypotension, serum MCT concentrations provide useful additional information to assist with the diagnosis of anaphylaxis.

Disturbances in MCT concentrations are not solely attributed to allergy and anaphylaxis. In addition to mastocytosis and other clonal mast cell disorders, which are associated with elevated baseline MCT levels due to abnormally high numbers of mast cells [57, 116, 145], elevated MCT has previously been observed in non-anaphylactic deaths and trauma patients due to cell lysis [146-148]. In this study we also observed elevated MCT levels in trauma patients. This is most likely the result of physical stress following trauma causing mast cells to lyse and release mediators including MCT. Furthermore, it is also possible that delta-MCT measurements may not accurately reflect mast cell activation in patients who receive large volumes of fluid therapy; significant haemodilution may cause a positive delta-MCT (decrease from pre- to post-treatment) despite a lack of substantial mast cell involvement.

Not all cases of anaphylaxis involve mast cell activation. There are a number of other mechanisms of anaphylaxis that have been proposed, including non-immunological pathways, with varying degrees of evidence in humans [5, 89]. A lack of mast cell involvement would account for a normal and stable serum MCT concentration in some patients. Due to the large degree of variation in symptoms, severity, triggers, routes of allergen exposure, and immune mechanisms, it seems unlikely that any single
biomarker test will have better sensitivity and specificity than MCT in helping to establish a diagnosis of anaphylaxis in hypoxic or hypotensive patients, particularly in cases where the physiological shock may be attributable to another cause.

We acknowledge that the delta-MCT values obtained in this study may not fully represent the change due to illness, as the design of the CISS protocol sampled patients only until discharge. This is in contrast to other studies that measured baseline MCT weeks after the event. However, the purpose of our study was focused on identifying events measurable during ED presentation and it was important to determine whether the delta-MCT concentration measurable during this period could be useful diagnostically. The ROC analysis was made difficult, as there is no existing ‘gold standard’ test for anaphylaxis to compare MCT with. We used the current, widely accepted NIAID/FAAN criteria for diagnosing true cases, with the diagnosis made by experienced emergency physicians blinded from the MCT results. As these criteria are still open to interpretation, we minimized potential bias by having multiple clinicians independently apply the criteria to each case.

In summary, both peak and delta-MCT concentrations are higher in anaphylaxis than other forms of critical illness. MCT values may help in establishing a diagnosis of anaphylaxis when all clinical features are considered. However, setting a cut-point for either peak MCT or delta-MCT to determine a dichotomous positive/negative result cannot be used in isolation to distinguish anaphylaxis from other causes of shock and/or respiratory compromise.
Acknowledgements

The authors appreciate and acknowledge the research nursing staff at Royal Perth, Armadale, and Austin hospitals, including Dr. Mani Rajee (Austin Hospital lead investigator) and Ellen MacDonald (Clinical Nurse Manager), for identifying patients for the study and collecting and processing blood samples. The authors also thank Dr. Chris Bundell for her assistance with the ImmunoCAP® technique and Ms. Sally Burrows for her expert statistical knowledge and assistance in data analysis. Prof. Simon Brown is supported by a NHMRC Career Development Fellowship and laboratory work was supported by the RPH Medical Research Foundation. Funding bodies played no role in study design, data collection, or analysis.

Author contributions

All authors assisted with data interpretation, drafting and editing of the manuscript, and approved the final version. AF contributed to the study concept and design, performed the laboratory experiments, contributed to data analysis, and drafted the manuscript. SM, YN, HM, DF, GA, and SB identified and recruited patients, and DF, GA, and SB also critically reviewed clinical information. EB assisted with data analysis. SB contributed to study concept and design, and obtaining funding support.

Conflicts of interest

The authors declare that they have no conflicts of interest.
Box 1: Critical Illness and Shock Study (CISS) inclusion criteria.

1. Shock – ANY cause (e.g. septic, traumatic, haemorrhagic, cardiogenic, anaphylactic), with any of:
   a. systolic blood pressure (SBP) ≤ 90 mm Hg, or
   b. mean arterial pressure (MAP) ≤ 65 mm Hg, or
   c. heart rate (HR) ≥ SBP (i.e. shock index HR/SBP ≥ 1), or
   d. lactate ≥ 4 mmol/L

2. Hypoxemic respiratory failure – ANY cause (e.g. infective, traumatic, cardiogenic, non-cardiogenic), with either:
   a. keeping SpO₂ > 90% requiring > 6 L/min O₂ by face mask, or
   b. PaO₂ (mm Hg)/FiO₂ < 200 (Ventilated, BiPAP, Venturi mask)

3. Post-cardiac arrest, i.e. any spontaneous circulation in ED post-arrest

4. Severe sepsis, defined as: sepsis (likely infection +2 or more of; temp > 38°C or < 36°C, HR > 90, respiratory rate > 20, white cell count > 12 or < 4) plus either:
   a. shock or hypoxemic respiratory failure as defined above, or
   b. organ dysfunction (oliguria/acute renal failure or acute altered mental state)

5. Acute anaphylaxis defined as a reaction, which at the time of enrolment involves two or more organ systems:
   a. Skin: generalized erythema or itch, urticaria, angioedema
   b. Gastrointestinal tract: nausea, vomiting, abdominal or pelvic pain, incontinence
   c. Respiratory system: dyspnoea, wheeze, stridor, chest tightness, hypoxemia (SpO₂ ≤ 92% and/or cyanosis)
   d. Cardiovascular system: diaphoresis, dizziness/pre-syncope, collapse, altered mental state, hypotension (SBP < 90/MAP < 65, or relative BP drop of > 30% from normal for that individual)

OR:
Any acute onset (minutes-hours) of hypotension or bronchospasm where anaphylaxis is considered possible, even if the typical skin features listed above are not present.
Box 2: NIAID/FAAN clinical criteria for diagnosing anaphylaxis [1].

| Anaphylaxis is highly likely when any one of the following 3 criteria are fulfilled: |
| 1. Acute onset of an illness (minutes to several hours) with involvement of the skin, mucosal tissue, or both (e.g., generalized hives, pruritus or flushing, swollen lips-tongue-uvula) AND AT LEAST ONE OF THE FOLLOWING: |
| a. Respiratory compromise (e.g., dyspnoea, wheeze-bronchospasm, stridor, reduced PEF, hypoxemia) |
| b. Reduced BP or associated symptoms of end-organ dysfunction (e.g., hypotonia [collapse], syncope, incontinence) |
| 2. Two or more of the following that occur rapidly after exposure to a likely allergen* for that patient (minutes to several hours): |
| a. Involvement of the skin-mucosal tissue (e.g., generalized hives, itch-flush, swollen lips-tongue-uvula) |
| b. Respiratory compromise (e.g., dyspnoea, wheeze-bronchospasm, stridor, reduced PEF, hypoxemia) |
| c. Reduced BP or associated symptoms (e.g., hypotonia [collapse], syncope, incontinence) |
| d. Persistent gastrointestinal symptoms (e.g., cramping abdominal pain, vomiting) |
| 3. Reduced BP after exposure to known allergen* for that patient (minutes to several hours): |
| a. Infants and children: low systolic BP (age specific) or greater than 30% decrease in systolic BP† |
| b. Adults: systolic BP of less than 90 mm Hg or greater than 30% decrease from that person’s baseline |

PEF, peak expiratory flow; BP, blood pressure.

* For the purposes of Definitions 2 and 3 we considered that the conditions for allergen exposure were met if the patient had been exposed to either (i) an infrequently encountered precipitant just prior to onset, such that both exposure and the occurrence of anaphylaxis were unlikely to be coincidental or (ii) a commonly encountered substance (e.g. food) if it was a previously known allergen for that patient or subsequently identified through allergen-specific IgE testing and/or challenge testing to be the likely cause. †Not applicable in this study as all participants were aged 16 years or older.
# Chapter 2

## 2.7 Tables

Table 2.1: Clinical reaction features of possible and definite anaphylaxis presentations.

<table>
<thead>
<tr>
<th></th>
<th>Possible anaphylaxis</th>
<th>Definite anaphylaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>67</td>
</tr>
<tr>
<td>Age (years), mean (SD)</td>
<td>45 (18)</td>
<td>40 (16)</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>8 (53)</td>
<td>30 (45)</td>
</tr>
<tr>
<td><strong>Suspected cause</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food, n (%)</td>
<td>6 (40)</td>
<td>22 (33)</td>
</tr>
<tr>
<td>Summative (food + exercise), n (%)</td>
<td>–</td>
<td>3 (4)</td>
</tr>
<tr>
<td>Injected diagnostic contrast, n (%)</td>
<td>–</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Injected medicine, n (%)</td>
<td>–</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Oral medicine, n (%)</td>
<td>3 (20)</td>
<td>23 (34)</td>
</tr>
<tr>
<td>Venoms, n (%)</td>
<td>3 (20)</td>
<td>5 (7)</td>
</tr>
<tr>
<td>Unknown or other, n (%)</td>
<td>3 (20)</td>
<td>10 (15)</td>
</tr>
<tr>
<td><strong>Onset to enrolment (mins), median (IQR)</strong></td>
<td>125 (140)</td>
<td>75 (50)</td>
</tr>
<tr>
<td>Any skin feature, n (%)</td>
<td>14 (93)</td>
<td>65 (97)</td>
</tr>
<tr>
<td>Urticaria, n (%)</td>
<td>7 (47)</td>
<td>40 (60)</td>
</tr>
<tr>
<td>Erythema, n (%)</td>
<td>12 (80)</td>
<td>47 (70)</td>
</tr>
<tr>
<td>Oedema, n (%)</td>
<td>4 (27)</td>
<td>26 (39)</td>
</tr>
<tr>
<td>Periorbital oedema, n (%)</td>
<td>2 (13)</td>
<td>18 (27)</td>
</tr>
<tr>
<td>Any gastrointestinal feature, n (%)</td>
<td>10 (67)</td>
<td>29 (43)</td>
</tr>
<tr>
<td>Nausea</td>
<td>9 (60)</td>
<td>27 (40)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>3 (20)</td>
<td>10 (15)</td>
</tr>
<tr>
<td>Abdominal/pelvic pain</td>
<td>–</td>
<td>8 (12)</td>
</tr>
<tr>
<td>Any respiratory feature, n (%)</td>
<td>12 (80)</td>
<td>59 (88)</td>
</tr>
<tr>
<td>Chest/throat tightness</td>
<td>12 (80)</td>
<td>41 (61)</td>
</tr>
<tr>
<td>Dyspnoea, n (%)</td>
<td>2 (13)</td>
<td>39 (58)</td>
</tr>
<tr>
<td>Stridor, n (%)</td>
<td>1 (7)</td>
<td>6 (9)</td>
</tr>
<tr>
<td>Wheeze, n (%)</td>
<td>2 (13)</td>
<td>25 (37)</td>
</tr>
<tr>
<td>Hypoxemia, n (%)</td>
<td>–</td>
<td>9 (13)</td>
</tr>
<tr>
<td>Any cardiovascular feature, n (%)</td>
<td>7 (47)</td>
<td>42 (63)</td>
</tr>
<tr>
<td>Dizziness</td>
<td>6 (40)</td>
<td>19 (28)</td>
</tr>
<tr>
<td>Diaphoresis</td>
<td>2 (13)</td>
<td>20 (30)</td>
</tr>
<tr>
<td>Hypotension (SBP &lt; 90 mm Hg), n (%)</td>
<td>–</td>
<td>26 (39)</td>
</tr>
<tr>
<td>Incontinence</td>
<td>–</td>
<td>3 (4)</td>
</tr>
<tr>
<td>Confusion</td>
<td>–</td>
<td>8 (12)</td>
</tr>
<tr>
<td>Collapse</td>
<td>–</td>
<td>10 (15)</td>
</tr>
<tr>
<td>Loss of consciousness</td>
<td>–</td>
<td>6 (9)</td>
</tr>
<tr>
<td>Treated with epinephrine, n (%)</td>
<td>13 (87)</td>
<td>63 (94)</td>
</tr>
<tr>
<td>Multiple doses, n (%)</td>
<td>2 (13)</td>
<td>28 (42)</td>
</tr>
<tr>
<td>Length of stay (hrs), median (IQR)</td>
<td>5 (3)</td>
<td>7 (11)</td>
</tr>
</tbody>
</table>
SD, standard deviation; IQR, interquartile range; SBP, systolic blood pressure.

*The most common medications responsible for anaphylactic episodes were antibiotics (14 (47%)), followed by non-steroidal anti-inflammatory drugs (10 (33%)), intravenous contrast (2 (7%)), and vaccines (2 (7%)). The most common foods implicated were nuts and legumes (15 (48%)), followed by seafood (3 (10%)), fruits and vegetables (3 (10%)), and meat, egg and dairy (1 (3%)). The most common venom trigger was honeybee (7 (88%)), followed by paper wasp (1 (12%)).
Table 2.2: Sensitivity, specificity, AUC, and %CC for the diagnosis of anaphylaxis using different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>AUC</th>
<th>%CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak MCT &gt;11.4ng/mL</td>
<td>58.2 (45.5, 70.2)</td>
<td>93.3 (87.3, 97.1)</td>
<td>0.76 (0.69, 0.82)</td>
<td>81</td>
</tr>
<tr>
<td>Absolute delta-MCT ≥2ng/mL</td>
<td>67.2 (54.6, 78.2)</td>
<td>72.5 (63.6, 80.3)</td>
<td>0.70 (0.63, 0.77)</td>
<td>71</td>
</tr>
<tr>
<td>Peak MCT &gt;11.4ng/mL &amp;/or delta-MCT ≥2ng/mL</td>
<td>71.6 (59.3, 82.0)</td>
<td>71.7 (62.7, 79.5)</td>
<td>0.72 (0.65, 0.78)</td>
<td>72</td>
</tr>
<tr>
<td>Peak MCT &gt;11.4ng/mL &amp;/or delta-MCT ≥7ng/mL</td>
<td>58.2 (45.5, 70.2)</td>
<td>91.7 (85.2, 95.9)</td>
<td>0.75 (0.68, 0.81)</td>
<td>80</td>
</tr>
<tr>
<td>Relative delta-MCT ≥135%</td>
<td>59.7 (47.0, 71.5)</td>
<td>52.5 (43.2, 61.7)</td>
<td>0.56 (0.49, 0.64)</td>
<td>55</td>
</tr>
<tr>
<td>Peak MCT &gt;11.4ng/mL and/or delta-MCT ≥135%</td>
<td>74.6 (62.5, 84.5)</td>
<td>50.0 (40.7, 59.3)</td>
<td>0.62 (0.55, 0.69)</td>
<td>59</td>
</tr>
</tbody>
</table>

AUC, area under the (ROC) curve; CC, correctly classified; MCT, mast cell tryptase.

Values are displayed as the estimate (95% CI), or % only for CC.

Note: determined using ROC analysis of definite anaphylaxis cases (n = 67) and non-anaphylaxis controls (n = 120).
Table 2.3: Sensitivity, specificity, AUC, and %CC for the diagnosis of anaphylaxis in patients with hypotension (SBP < 90 mm Hg) using different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>AUC</th>
<th>%CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak MCT &gt; 11.4 ng/mL</td>
<td>84.6 (65.1, 95.6)</td>
<td>92.2 (85.3, 96.6)</td>
<td>0.88 (0.81, 0.96)</td>
<td>91</td>
</tr>
<tr>
<td>Absolute delta-MCT ≥ 2 ng/mL</td>
<td>88.5 (69.8, 97.6)</td>
<td>69.9 (60.1, 78.5)</td>
<td>0.79 (0.71, 0.87)</td>
<td>74</td>
</tr>
<tr>
<td>Peak MCT &gt; 11.4 ng/mL &amp;/or delta-MCT ≥ 2 ng/mL</td>
<td>96.2 (80.4, 99.9)</td>
<td>68.9 (59.1, 77.7)</td>
<td>0.83 (0.77, 0.88)</td>
<td>74</td>
</tr>
<tr>
<td>Peak MCT &gt; 11.4 ng/mL &amp;/or delta-MCT ≥ 7 ng/mL</td>
<td>84.6 (65.1, 95.6)</td>
<td>91.3 (84.1, 95.9)</td>
<td>0.88 (0.80, 0.96)</td>
<td>90</td>
</tr>
<tr>
<td>Relative delta-MCT ≥ 135%</td>
<td>65.4 (44.3, 82.8)</td>
<td>51.5 (41.4, 61.4)</td>
<td>0.58 (0.48, 0.69)</td>
<td>56</td>
</tr>
<tr>
<td>Peak MCT &gt; 11.4 ng/mL and/or delta-MCT ≥ 135%</td>
<td>92.3 (74.9, 99.1)</td>
<td>48.5 (38.6, 58.6)</td>
<td>0.70 (0.63, 0.78)</td>
<td>57</td>
</tr>
</tbody>
</table>

AUC, area under the (ROC) curve; CC, correctly classified; MCT, mast cell tryptase; SBP, systolic blood pressure.

Values are displayed as the estimate (95% CI), or % only for CC.

Note: determined using ROC analysis of definite anaphylaxis cases with hypotension (n = 26) and non-anaphylaxis controls with hypotension (n = 103).
### 2.8 Figures

**Figure 2.1: Summary of the mast cell tryptase (MCT) concentrations.**

Samples were collected from critically ill non-anaphylaxis controls (e.g. sepsis, trauma) \((n = 120)\), possible \((n = 15)\) and definite \((n = 67)\) anaphylaxis patients over the first 24 hours since emergency department admission. Definite anaphylaxis patients strictly adhered to the NIAID/FAAN criteria for diagnosis, whilst possible anaphylaxis patients did not meet criteria but their most likely diagnosis was anaphylaxis. MCT was measured in singlicate in neat sera using diagnostic, validated ImmunoCAP® methodology. Boxplots identify the median, upper and lower quartiles, and the range.

- **A)** Peak MCT concentrations. Dashed line indicates a peak MCT of 11.4 ng/mL, the 95th percentile of healthy controls.
- **B)** Absolute delta-MCT concentrations (change in...
concentration between the highest and lowest values). Dashed line indicates an absolute delta-MCT of 2.0 ng/mL. C) Relative delta-MCT percentages (percentage change between the highest and lowest values). Dashed line indicates a relative delta-MCT of 135%.

Note: p-values are derived using linear regression models testing the ratio of the two means following log transformation, adjusted for age and sex.
Figure 2.2: Receiver Operating Characteristic (ROC) curves showing sensitivity and specificity for a diagnosis of anaphylaxis.

Curves show results for A) peak mast cell tryptase (MCT); B) absolute delta-MCT; and C) relative delta-MCT when used as diagnostic markers for a sample of 67 anaphylaxis cases (defined using NIAID/FAAN criteria) and 120 non-anaphylactic critically ill controls, with ng/mL (A and B) or % (C) cut-offs indicated. MCT was measured in singlicate using the diagnostic, validated ImmunoCAP® protocol in neat serum samples collected from patients over the first 24 hours since their enrolment to the emergency department.
Figure 2.3: Receiver Operating Characteristic (ROC) curves showing sensitivity and specificity for a diagnosis of anaphylaxis in hypotensive patients.

Curves show results for A) peak mast cell tryptase (MCT); B) absolute delta-MCT; and C) relative delta-MCT when used as diagnostic markers for a sample of 26 anaphylaxis cases (defined using NIAID/FAAN criteria) with hypotension (systolic blood pressure < 90 mm Hg) and 103 non-anaphylaxis critically ill controls with hypotension, with ng/mL (A and B) or % (C) cut-offs indicated. MCT was measured in singlicate using the diagnostic, validated ImmunoCAP® protocol in neat serum samples collected from patients over the first 24 hours since their enrolment to the emergency department.
Table S2.1: Clinical reaction features of patients with possible anaphylaxis (not strictly adhering to NIAID/FAAN diagnostic criteria).

<table>
<thead>
<tr>
<th>Time (mins)*</th>
<th>Age/sex</th>
<th>Cause</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin features + subjective features only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>51M</td>
<td>Insect (honeybee)</td>
<td>Erythema, urticaria, angioedema, chest/throat tightness, nausea</td>
</tr>
<tr>
<td>54</td>
<td>64F</td>
<td>Drug (herbal)</td>
<td>Erythema, nausea</td>
</tr>
<tr>
<td>55</td>
<td>16F</td>
<td>Food (unknown)</td>
<td>Erythema, chest/throat tightness, stridor, nausea, vomiting</td>
</tr>
<tr>
<td>65</td>
<td>31M</td>
<td>Insect (honeybee)</td>
<td>Erythema, urticaria, chest/throat tightness</td>
</tr>
<tr>
<td>110</td>
<td>25M</td>
<td>Drug (oral antibiotic)</td>
<td>Erythema, puffy eyes, chest/throat tightness, vomiting</td>
</tr>
<tr>
<td>125</td>
<td>45M</td>
<td>Insect (honeybee)</td>
<td>Erythema, chest/throat tightness, nausea, dizziness</td>
</tr>
<tr>
<td>134</td>
<td>32M</td>
<td>Food (nuts/legumes)</td>
<td>Erythema, chest/throat tightness, nausea</td>
</tr>
<tr>
<td>140</td>
<td>46F</td>
<td>Unknown</td>
<td>Erythema, urticaria, angioedema, nausea</td>
</tr>
<tr>
<td>146</td>
<td>38F</td>
<td>Food (nuts/legumes)</td>
<td>Erythema, chest/throat tightness, nausea, dizziness</td>
</tr>
<tr>
<td>205</td>
<td>49F</td>
<td>Unknown</td>
<td>Erythema, urticaria, angioedema, chest/throat tightness</td>
</tr>
<tr>
<td>270</td>
<td>80F</td>
<td>Unknown</td>
<td>Erythema, urticaria, chest/throat tightness, dizziness</td>
</tr>
<tr>
<td>561</td>
<td>56M</td>
<td>Food (nuts/legumes)</td>
<td>Urticaria, angioedema, chest/throat tightness</td>
</tr>
<tr>
<td>Multisystem involvement but not typical skin+respiratory or skin+cardiovascular, and no clearly identified trigger</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>73F</td>
<td>Food (possibly nuts)</td>
<td>Nausea, dyspnoea, chest/throat tightness, dizziness (no skin features)</td>
</tr>
<tr>
<td>245</td>
<td>29M</td>
<td>Food (unknown)</td>
<td>Erythema, urticaria, nausea, vomiting, dizziness, diaphoresis</td>
</tr>
<tr>
<td>Respiratory and/or subjective features only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>38M</td>
<td>Drug (oral NSAID)</td>
<td>Chest/throat tightness, dyspnoea, wheeze, dizziness, puffy eyes</td>
</tr>
</tbody>
</table>

NSAID, non-steroidal anti-inflammatory drug.

*time between the onset of symptoms and first blood sample
## Table S2.2: Clinical features of non-anaphylaxis controls.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>120</td>
</tr>
<tr>
<td>Age (years), mean (SD)</td>
<td>61 (18)</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>74 (62)</td>
</tr>
<tr>
<td>Major pathologies</td>
<td></td>
</tr>
<tr>
<td>Sepsis, n (%)</td>
<td>47 (39)</td>
</tr>
<tr>
<td>Pneumonia, n (%)</td>
<td>22 (47)</td>
</tr>
<tr>
<td>Cellulitis, n (%)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Urosepsis, n (%)</td>
<td>7 (15)</td>
</tr>
<tr>
<td>Other, n (%)</td>
<td>17 (36)</td>
</tr>
<tr>
<td>Cardiac conditions, n (%)</td>
<td>24 (20)</td>
</tr>
<tr>
<td>Myocardial infarction, n (%)</td>
<td>6 (25)</td>
</tr>
<tr>
<td>Atrial arrhythmia, n (%)</td>
<td>6 (25)</td>
</tr>
<tr>
<td>Ventricular arrhythmia, n (%)</td>
<td>5 (21)</td>
</tr>
<tr>
<td>Cardiac arrest, n (%)</td>
<td>7 (29)</td>
</tr>
<tr>
<td>Respiratory conditions, n (%)</td>
<td>32 (27)</td>
</tr>
<tr>
<td>Asthma, n (%)</td>
<td>6 (19)</td>
</tr>
<tr>
<td>COPD, n (%)</td>
<td>18 (56)</td>
</tr>
<tr>
<td>Other, n (%)</td>
<td>9 (28)</td>
</tr>
<tr>
<td>Stroke, n (%)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Trauma, n (%)</td>
<td>12 (10)</td>
</tr>
<tr>
<td>Renal failure, n (%)</td>
<td>12 (10)</td>
</tr>
<tr>
<td>Liver failure, n (%)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Toxicology, n (%)</td>
<td>6 (5)</td>
</tr>
<tr>
<td>Haemorrhage, n (%)</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Other, n (%)</td>
<td>23 (19)</td>
</tr>
<tr>
<td>Hypotension (SBP&lt;90), n (%)</td>
<td>103 (86)</td>
</tr>
<tr>
<td>Length of stay (days), median (IQR)</td>
<td>6 (13)</td>
</tr>
<tr>
<td>Death, n (%)</td>
<td>23 (19)</td>
</tr>
</tbody>
</table>

SD, standard deviation; COPD, chronic obstructive pulmonary disease; SBP, systolic blood pressure; IQR, interquartile range.
Table S2.3: ROC table showing different cut-points for peak MCT based on analysis of definite anaphylaxis cases (n=67) and non-anaphylaxis controls (n=120).

<table>
<thead>
<tr>
<th>Peak MCT cut-point (ng/mL)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>%CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>97</td>
<td>20</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>87</td>
<td>58</td>
<td>68</td>
</tr>
<tr>
<td>7.5</td>
<td>75</td>
<td>80</td>
<td>78</td>
</tr>
<tr>
<td>10</td>
<td>61</td>
<td>86</td>
<td>77</td>
</tr>
<tr>
<td>12.5</td>
<td>54</td>
<td>95</td>
<td>80</td>
</tr>
<tr>
<td>15</td>
<td>45</td>
<td>96</td>
<td>78</td>
</tr>
<tr>
<td>20</td>
<td>36</td>
<td>98</td>
<td>75</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
<td>99</td>
<td>73</td>
</tr>
<tr>
<td>45</td>
<td>19</td>
<td>100</td>
<td>71</td>
</tr>
</tbody>
</table>

Table S2.4: ROC table showing different cut-points for absolute delta-MCT based on analysis of definite anaphylaxis cases (n=67) and non-anaphylaxis controls (n=120).

<table>
<thead>
<tr>
<th>Absolute delta-MCT cut-point (ng/mL)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>%CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>93</td>
<td>24</td>
<td>49</td>
</tr>
<tr>
<td>1.0</td>
<td>84</td>
<td>48</td>
<td>61</td>
</tr>
<tr>
<td>1.5</td>
<td>75</td>
<td>62</td>
<td>66</td>
</tr>
<tr>
<td>2.0</td>
<td>67</td>
<td>72</td>
<td>71</td>
</tr>
<tr>
<td>2.5</td>
<td>61</td>
<td>78</td>
<td>72</td>
</tr>
<tr>
<td>3.0</td>
<td>58</td>
<td>82</td>
<td>74</td>
</tr>
<tr>
<td>4.0</td>
<td>51</td>
<td>87</td>
<td>74</td>
</tr>
<tr>
<td>5.0</td>
<td>45</td>
<td>90</td>
<td>74</td>
</tr>
<tr>
<td>7.0</td>
<td>40</td>
<td>96</td>
<td>76</td>
</tr>
<tr>
<td>10.0</td>
<td>33</td>
<td>98</td>
<td>74</td>
</tr>
<tr>
<td>15.0</td>
<td>27</td>
<td>99</td>
<td>73</td>
</tr>
<tr>
<td>22.5</td>
<td>16</td>
<td>100</td>
<td>70</td>
</tr>
</tbody>
</table>
Chapter 3  Validation of innate immune pathways and neutrophil activation markers identified by microarray

3.1  Background

The results of the previous chapter have demonstrated that mast cell tryptase (MCT) measurements are not adequately sensitive or specific for anaphylaxis diagnosis in the absence of hypotension. Therefore, the activation of mast cells alone clearly does not represent the breadth of immune activation occurring during anaphylaxis. We still do not sufficiently understand the immune processes and reaction pathways activated during spontaneous human reactions. In an effort to better understand these immune processes, our laboratory has previously performed a microarray study that compared six moderate anaphylaxis patients with six healthy controls [149]. A large number of genes were differentially expressed including those involved in innate immunity, particularly at one and three hours after emergency department arrival. The current chapter aims to validate the findings of this microarray by investigating several hub genes and upstream regulators identified. Both mRNA and protein levels were analysed in a larger cohort of patients with both moderate and severe reactions, sampled over the course of their emergency department presentation. The experimental work for this chapter has been prepared as an article for submission, as follows.
Title: Markers involved in innate immunity and neutrophil activation are elevated during anaphylaxis: validation of a microarray study

Short title: Innate immunity and neutrophils in anaphylaxis

Abbie Francis¹, ², Erika Bosio¹, ², Shelley F Stone¹, ², Daniel M Fatovich¹, ², ³, Glenn Arendts¹, ², ³, ⁴, Sally Burrows⁵, and Simon GA Brown¹, ², ³, ⁶

1. Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Perth, WA, Australia
2. Discipline of Emergency Medicine, School of Medicine, Faculty of Health and Medical Sciences, University of Western Australia, Perth, WA, Australia
3. Emergency Department, Royal Perth Hospital, Perth, WA, Australia
4. Emergency Department, Fiona Stanley Hospital, Murdoch, WA, Australia
5. School of Medicine & Pharmacology, University of Western Australia, Perth, WA, Australia
6. Emergency Department, Royal Hobart Hospital, Hobart, TAS, Australia

Key words: anaphylaxis, basic mechanisms, clinical immunology, innate immunity, neutrophils

Corresponding Author: Abbie Francis, Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Level 6 MRF Building, Rear 50 Murray Street, Perth, Western Australia 6000.

Phone: +61 8 9224 0356 Email: Abbie.Francis@uwa.edu.au
Abbreviations

CD64, cluster of differentiation 64; cDNA, complimentary DNA; CI, confidence interval; CISS, Critical Illness and Shock Study; DAMP, danger-associated molecular pattern; ED, emergency department; FasL, fas ligand; IQR, interquartile range; MCT, mast cell tryptase; MMP9, matrix metalloproteinase 9; MyD88, myeloid differentiation primary response 88; NIAID/FAAN, National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network; OSM, oncostatin M; PBL, peripheral blood leukocyte; qPCR, quantitative PCR; RIN, RNA integrity number; S100A8, S100 calcium-binding protein A8; S100A9, S100 calcium-binding protein A9; SD, standard deviation; TLR4, toll-like receptor 4; TREM-1, triggering receptor expressed on myeloid cells-1.

3.2 Abstract

Background

Anaphylaxis describes an uncontrolled inflammatory response following exposure to allergens. The mechanisms that amplify the localised allergic signal are poorly understood. We have previously identified upregulation of the innate immune response, neutrophil activation, and apoptosis using a microarray approach.

Objective

To validate differential gene expression and investigate protein concentrations of “hub genes” and upstream regulators during anaphylaxis.
Methods

Samples were collected from patients with anaphylaxis upon arrival to the emergency department, and again after 1 and 3 hours. Messenger RNA (mRNA) levels of 11 genes (IL6, IL10, OSM, S100A8, S100A9, MMP9, FASL, TLR4, MYD88, TREM1, and CD64) were measured in peripheral blood leucocytes using quantitative PCR. Protein concentrations were measured in serum/plasma by ELISA or cytometric bead array for seven of these candidates. Results were compared with healthy control levels and between severities using parametric and non-parametric methods, as appropriate.

Results

Of 69 anaphylaxis patients enrolled, 36 (52%) had severe reactions, and 38 (55%) were female. Increases in both mRNA and protein of IL-10, S100A9, MMP9, and TREM1 were observed during anaphylaxis. OSM, S100A8, TLR4, and CD64 were upregulated during anaphylaxis; protein concentrations were not measured. IL-6 protein concentrations were higher in patients than controls, whilst the corresponding mRNA expression was similar for all groups. MYD88 expression was elevated in patients, with no significant differences in protein concentrations. Finally, both mRNA and protein levels of FasL decreased during anaphylaxis.

Conclusion

These results validate the previous microarray findings and provide evidence for the involvement of innate immune pathways and myeloid cells during human anaphylaxis. Neutrophils are a standout candidate with several results from this study such as elevated S100A8, S100A9, TLR4, MYD88, and TREM1 strongly suggesting that they are activated during acute anaphylaxis.
3.3 Introduction

Allergies are a global health concern, with increases in the numbers of adverse reactions to foods in children and drugs in adults [150, 151]. The rapid progression from the onset of allergic symptoms through to a potentially life-threatening reaction affecting multiple organ systems is a hallmark of anaphylaxis [1]. The immune mechanisms driving human anaphylaxis are poorly understood. However, an IgE-mediated pathway, involving the activation of mast cells, has been the predominant focus of clinical investigation [14]. During this process, initial exposure to an allergen stimulates the production of allergen-specific IgE, which bind to IgE receptors on the surface of mast cells or basophils. On a subsequent exposure, allergens bind to these antibodies, causing IgE receptor cross-linking, which initiates degranulation [4, 16]. This releases an array of mediators including histamine and mast cell tryptase (MCT) that contribute to the physiological symptoms of anaphylaxis, such as vasodilation and bronchoconstriction [47, 51, 52, 55]. Much still remains unknown about how the localized mast cell signal becomes rapidly amplified into a potentially lethal degree of immune activation.

A previous microarray study performed in our laboratory aimed to investigate differential gene expression during the course of anaphylaxis. This study investigated six patients with moderate anaphylaxis who were sampled over the first 3 hours after arrival at the emergency department (ED). An increasing number of genes were differentially expressed over this time, culminating in a storm of genetic activation. Several key pathways including inflammation, innate immunity, apoptosis, neutrophil activation, and chemotaxis were upregulated suggesting these pathways likely play central roles in the anaphylactic response [149]. The present study aimed to investigate
some of these processes more closely during anaphylaxis by analysing the mRNA expression of several important genes in peripheral blood leucocytes (PBLs) and their corresponding protein levels in serum/plasma, where possible.

The selected targets were either “hub genes” or upstream regulators for the pathways of interest including: interleukin (IL)-6; IL-10; oncostatin M (OSM); S100 calcium-binding proteins A8 (S100A8) and A9 (S100A9); matrix metalloproteinase 9 (MMP9); fas ligand (FasL); toll-like receptor 4 (TLR4); myeloid differentiation primary response 88 (MyD88); triggering receptor expressed on myeloid cells 1 (TREM1); and cluster of differentiation 64 (CD64). IL-6, IL-10, and OSM are cytokines that play important roles in innate immunity by performing either pro- or anti-inflammatory functions [152-154]. S100A8 and S100A9 are proteins contained within phagocytes that are released following cellular activation or damage [155]. They are known as danger-associated molecular patterns (DAMPs) because the binding of extracellular S100A8/S100A9 to certain activating surface receptors including TREM1 and TLR4 (which associates with the intracellular adaptor protein MyD88) amplifies inflammation [156]. MMP9 is released primarily from neutrophils, in addition to other myeloid cells, following their activation/degranulation [157, 158]. FasL is a surface protein that stimulates apoptosis of FasL-expressing cells following binding to fas. However, once cleaved, soluble FasL competes with surface FasL for binding and does not trigger apoptosis. Therefore, whilst surface FasL is pro-inflammatory, high concentrations of soluble FasL promote cell survival [159]. Finally, CD64 is the high-affinity IgG receptor present on the surface of macrophages, monocytes, and neutrophils. Anaphylaxis via an IgG-mediated pathway has been demonstrated in mouse models but is not well evidenced in humans [160].
protein concentrations of this panel of targets would provide insight into the cell types and pathways activated during anaphylaxis.

3.4 Methods

3.4.1 Patient recruitment

Study participants and healthy controls were enrolled in our prospective, observational Critical Illness and Shock Study (CISS) between September 2010 and January 2014 in four Australian EDs. The methodology for CISS has been previously described [143]. Briefly, patients enrolled in the study meet a case definition of critical illness, which excludes mild (skin-only) allergic reactions, and then undergo serial blood sampling and clinical data collection at protocolled time points. The time points for this study were: as soon as practicable after enrolment criteria were identified (T0); one hour later (T1); and three hours after enrolment (T3). At each time point, 2 x 4 mL EDTA plasma tubes, 1 x 3.5 mL serum separating tube and 2 x 2.5 mL Blood RNA PAXgene™ tubes (PreAnalytiX GmbH, Switzerland) were collected. Serum and EDTA plasma were collected, aliquoted, and stored immediately at -80°C until analysis. RNA PAXgene™ tubes were collected and placed immediately at 4°C, then transferred to -20°C within 72 hours, before final storage at -80°C until analysis.

3.4.2 Ethics approval and consent

Ethics approval was obtained from the Human Research Ethics Committees at each hospital (Royal Perth Hospital, Fremantle Hospital, Armadale-Kelmscott Memorial Hospital: EC 2009/080; Austin Hospital: H2012/04477). Since the need for emergency care took priority, waiver of initial consent was approved under the
provision of paragraph 2.3.6 of the National Health and Medical Research Council Ethical Conduct guidelines (2007). Once the study could be explained to them, fully informed written consent was obtained. Patients were given the option of declining further involvement and having samples collected up to that point destroyed.

### 3.4.3 Patient cohort

We selected cases that satisfied a clinical definition of anaphylaxis based on National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network (NIAID/FAAN) criteria [1]. To test for differences associated with severity, cases were reviewed by three physician investigators (SB, DF, and GA) and classified into two severity groups, moderate or severe anaphylaxis, based on clinical features according to established criteria [119]. Both moderate and severe anaphylaxis involves multiple organ systems, whilst the presence of hypoxemia (SpO$_2$ ≤ 92% or cyanosis), hypotension (systolic blood pressure < 90 mm Hg), and/or neurological compromise (confusion, collapse, loss of consciousness, or incontinence) classifies a reaction as severe. This classification was undertaken separately and blinded to the laboratory analyses. Healthy controls for this study were selected to match the age and sex distributions of the patient cohort.

### 3.4.4 RNA extraction

RNA was extracted using PAXgene™ Blood RNA Extraction Kits (PreAnalytiX GmbH, Switzerland) by automation with a QIAcube instrument (Qiagen, USA). The purity and integrity of the RNA was assessed on a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and a 2100 Bioanalyzer (Agilent, USA). Samples with RNA integrity number (RIN) values < 7 and total RNA < 1 µg were excluded. As a
result, the quality of included samples was very high (median (interquartile range, IQR) 260/280 absorbance ratio: 2.04 (0.09); and median (IQR) RIN: 8.7 (0.9).

3.4.5 **Quantitative PCR**

Complimentary DNA (cDNA) was synthesized using SuperScript™ III reverse transcriptase according to the manufacturer’s protocol (Thermo Fisher Scientific, USA). cDNA samples were stored immediately at -20°C until utilized for qPCR. Quantitative PCRs (qPCRs) were performed in a total volume of 10 µL, comprising 37.5 ng of each primer (Table S3.1), 0.5 µL of LightCycler® 480 ResoLight Dye (Roche Diagnostics, USA), and 1 µL of 10X PCR buffer, 5 mM MgCl₂, 0.2 mM dNTPs, 0.33 Units Platinum® *Tag* DNA Polymerase (all from Thermo Fisher Scientific, USA) and 2 µL of 1/10 cDNA. A Rotor-Gene™ 6000 (Corbett, Australia) was used to optimise annealing temperatures and magnesium concentrations for each set of primers. Triplicate reactions were set up in 384 well plates and a ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA) was used with the following cycling conditions: 50°C for 2 min; 95°C for 10 min; and followed by 40 cycles of 95°C for 15 sec, annealing temperature (Table S3.1) for 15 sec, and 72°C for 15 sec. Single product amplification was confirmed using dissociation curves established by increasing the temperature of the samples from 60°C to 95°C stepwise at 0.05°C per second.

3.4.5.1 **Template cloning for standard curve preparation**

RNA extracted from PBLs stimulated overnight with phorbol myristate acetate (Sigma-Aldrich, Australia) was used to prepare cDNA. Targets of interest were amplified using the same primers used for qPCR and products were ligated into
pGEM®-T Easy vectors (Promega, Australia). JM109 competent cells (Promega, Australia) were transformed with the construct. Ampicillin-resistant colonies were grown in liquid culture and plasmid DNA was prepared using the QIAprep Spin Miniprep Kit (Qiagen, Australia). Cloned sequences were verified on both strands using Sanger sequencing at the Australian Genome Research Facility. Plasmids were linearized with AatII (New England Biolabs, USA) and standard curves were prepared.

3.4.5.2 Analysis using qBase plus

Viia™ software (Thermo Fisher Scientific, USA) determined Cq values using the Baseline Threshold algorithm. Three reference genes, RPS18, HPRT, and YWHAZ, were determined as appropriate to normalize Cq data using qBase+ software, v. 2.6 (Biogazelle, Belgium). Replicates that varied by greater than 0.8 Cq were excluded.

3.4.6 Measuring protein concentrations

Serum concentrations of IL-6, IL-10, and soluble FasL were measured by cytometric bead array (CBA) flex sets (BD Biosciences, USA) as described previously [55]. Prior to analysis, samples were diluted 1:4 in assay diluent, measured in duplicate using a FACSCanto™ II flow cytometer (BD Biosciences, USA), and analysed using FCAP Array™ v3.0 software (Soft Flow, Hungary). The lower limits of detection were: 1.13 pg/mL IL-6; 0.092 pg/mL IL-10; and 1.838 pg/mL FasL, as defined by the manufacturer.

Serum concentrations of MMP9, TREM1, and S100A9, and plasma concentrations of MyD88 were measured using ELISA (DuoSet® R&D Systems, USA)
according to the manufacturer’s instructions. The lower limits of detection were: 22.06 pg/mL MMP9; 33.15 pg/mL TREM1; 22.06 pg/mL S100A9; and 220.6 pg/mL MyD88. Intra- and inter-assay CVs for the ELISAs were 1.8% and 9.3% for MMP9, 4.9% and 13.0% for TREM1, 2.9% and 18.5% for S100A9, and 2.5% and 18.3% for MyD88 respectively.

3.4.7 **Statistical Analysis**

All mRNA variables were log transformed to normalise the distribution of results; however, due to small sample sizes, non-parametric tests were used for comparisons between groups. Specifically, differences across groups were assessed using Kruskal-Wallis tests, with subsequent pairwise comparisons performed using Mann-Whitney tests. With the protein assays, a large number of samples were below the detection limits. As such, we tested for differences in the proportion of detectable samples in each group using Fisher’s exact tests. Differences in protein concentrations between groups were tested for using linear or Tobit regression models as appropriate, and only when more than 50% of samples had detectable protein concentrations. Due to the exploratory nature of the study, no adjustments were made for multiple comparisons, and p-values < 0.05 were considered significant. All statistical analyses were performed using Stata v 12.1 (StataCorp, USA).

3.5 **Results**

3.5.1 **Cohort characteristics**

This study cohort consisted of 69 patients with a confirmed diagnosis of anaphylaxis, 36 (52%) of these had severe reactions. The patient demographics and
Chapter 3

Clinical characteristics for each severity group are presented in Table 3.1. The majority of patients were female, accounting for 38 (55%) reactions. The average age of patients was 40 years old, ranging from 16 – 85 years. Both food and drug triggers each accounted for roughly one third of reactions, with most moderate reactions being food-induced and most severe reactions being drug-induced. The average time between the onset of symptoms and the first blood sample was 81 minutes, ranging from 10 – 215 minutes.

3.5.2 The cytokines IL-6, IL-10, and OSM are elevated during anaphylaxis

The raw mRNA expression data of the cytokines, and other gene targets, is presented in Figure 3.1. There were no differences in IL6 mRNA expression between the three groups (healthy controls, moderate, and severe anaphylaxis) at the time of enrolment (T0) (p = 0.160), or after one (T1) (p = 0.453) or three hours (T3) (p = 0.503). At T0, IL10 mRNA expression was lower in moderate anaphylaxis than both controls or severe anaphylaxis (p = 0.004 and p < 0.001, respectively). At T1, IL10 expression was higher in severe anaphylaxis than either healthy controls or moderate anaphylaxis (p = 0.001 and p < 0.001, respectively). Expression was also higher in severe anaphylaxis than healthy controls at T3 (p < 0.001). OSM expression was higher at T0 in severe than moderate anaphylaxis (p < 0.001). At T1, OSM was higher in severe anaphylaxis than healthy controls (p = 0.003). Finally, at T3, OSM was higher in both moderate and severe anaphylaxis than controls (p < 0.001 and p = 0.008, respectively).

Following measurement of protein concentrations, many samples returned results below the limit of detection. The numbers of measureable samples for each
protein have been summarised in Table 3.2, according to group and time point. IL-6 and IL-10 concentrations were undetectable in all healthy control samples tested. At T0, IL-6 concentrations were detectable in a higher proportion of anaphylaxis patients than controls (p = 0.001), with severe patients more so than moderate patients (p = 0.015). IL-6 was also detectable in a higher proportion of patient samples than controls at T1 and T3 (p < 0.001 for both comparisons), with no difference between severities (T1 p = 0.087 and T3 p = 0.204). In those with detectable levels, IL-6 concentrations at T0 were 49.3-fold (95% CI: 4.1, 595.0) higher in severe patients than moderate patients (p = 0.003). At T1, concentrations were 13.7-fold (95% CI: 2.1, 89.8) higher in severe patients than moderate patients (p = 0.007). At T3 there were no differences in concentration between the two reaction severities (p = 0.097). IL-10 was also detectable more often in patients than controls at all time points (T0 p = 0.008, T1 p = 0.001, and T3 p = 0.011) with no difference between reaction severities (T0 p = 0.06, T1 p = 0.065, and T3 p = 0.398). Due to the high proportion of samples with concentrations below the limit of detection for the assay, we were unable to test for differences in IL-10 concentrations. OSM protein concentrations were not measured due to limited sample availability.

### 3.5.3 S100A8 and S100A9 are elevated during anaphylaxis

At T0, there was no difference in the mRNA expression of either S100A8 or S100A9 between the three groups (p = 0.315 and p = 0.527, respectively) (Figure 3.1). However, by T1, expression of S100A8 was significantly higher in severe patients than controls (p < 0.001); and by T3, significantly higher expression was observed in both moderate and severe patients compared to controls (p < 0.001 for both tests). Expression of S100A9 mRNA was higher at T1 in both moderate and severe patients.
compared to controls (p = 0.006 and p < 0.001, respectively). This pattern was still observed at T3 (p < 0.001 for both tests).

The S100A8 and S100A9 proteins are commonly expressed as a heterodimer, known as calprotectin, and consequently their protein levels typically trend together [161]. For this reason, and the limited availability of serum/plasma for analysis, we selected only one of these, S100A9, for protein measurement. The concentration of S100A9 in all 20 healthy controls was below the limit of the assay. Significantly more patients had detectable concentrations of S100A9 at the time of arrival in the ED and after one and three hours (T0 p = 0.001, T1 p < 0.001, and T3 p = 0.001) (Table 3.2). We were unable to test for differences in S100A9 concentrations between moderate and severe patients due to the large proportion of samples with undetectable concentrations.

### 3.5.4 MMP9 expression is increased during anaphylaxis

At enrolment, MMP9 mRNA expression was lower in moderate anaphylaxis than healthy controls (p = 0.001) with no other significant differences between groups (Figure 3.1). After one hour, MMP9 expression was higher in severe patients than healthy controls (p < 0.001). By T3, MMP9 expression was higher in both severe and moderate patients than in healthy controls (p < 0.001 for both tests).

All control and patient samples had detectable MMP9 protein concentrations as shown in Figure 3.2A. There was no difference in MMP9 protein concentrations between healthy controls, moderate, and severe anaphylaxis at T0 (p=0.074). At T1, concentrations were 1.8-fold (95% CI: 1.2, 2.7) higher in moderate patients than controls (p = 0.005) and 3.1-fold (95% CI: 2.1, 4.6) higher in severe patients than controls.
controls (p < 0.001). MMP9 concentrations were 1.7-fold (95% CI: 1.2, 2.5) higher in severe patients than moderate patients (p = 0.004). At T3, MMP9 concentrations were 3.5-fold (95% CI: 2.5, 4.8) higher in moderate anaphylaxis and 4.0-fold (95% CI: 2.9, 5.4) higher in severe anaphylaxis than controls (p < 0.001 for both tests). Concentrations were not significantly different between reaction severities at T3 (p = 0.377).

3.5.5 FasL mRNA is downregulated during anaphylaxis

Expression of FASL mRNA was higher at T0 in moderate and severe patients than healthy controls (p = 0.003 and p = 0.026, respectively), with no significant difference between the two reaction severities (p = 0.165) (Figure 3.1). There were no differences in FASL expression between the three groups at T1 (p = 0.101). However, the expression of FASL at T3 was lower in both moderate and severe patients than controls (p < 0.001 and p = 0.001, respectively).

A higher proportion of healthy controls had detectable soluble FasL protein concentrations than anaphylaxis patients at all three time points (T0 p = 0.025, T1 p = 0.048, and T3 p = 0.001) (Table 3.2). Whilst no differences were apparent between reaction severities at T0 (p = 0.333) or T1 (p = 0.451), at T3 a higher proportion of severe patients had detectable soluble FasL concentrations than moderate patients (p = 0.021). There were no differences in concentrations between groups at any time point (T0 p = 0.091, T1 p = 0.464, and T3 p = 0.055) (Figure 3.2B).
3.5.6  **TLR4 and MyD88 mRNA is upregulated during anaphylaxis**

At T0, there was no difference in TLR4 expression between patients and controls (Figure 3.1). At T1, expression was higher in severe patients than controls (p < 0.001); and at T3, expression was higher in both moderate and severe patients than controls (p = 0.001 and p < 0.001, respectively). There were no differences in MYD88 expression between patients and controls at T0 (p = 0.094) or T1 (p = 0.632) (Figure 3.1). However at T3, MYD88 expression was higher in severe anaphylaxis than healthy controls (p = 0.004).

Due to sample availability and assay quality, only MyD88 protein was measured. There were no differences in the proportion of samples with detectable soluble MyD88 concentrations between groups at any time point (T0 p = 0.913, T1 p = 0.695, and T3 p = 1.000) (Table 3.2). Due to the large proportion of samples with undetectable MyD88 protein concentrations, we were unable to test for differences in concentrations between groups at any time point.

3.5.7  **TREM1 mRNA and protein levels increase during anaphylaxis**

At T0, there was an overall significant difference between the TREM1 mRNA expression of healthy controls, moderate, and severe anaphylaxis patients (p = 0.044). There were visible, but not significant, reductions in expression for both patient groups when compared with controls (Figure 3.1). At T1, there were no significant differences between the groups (p = 0.354). However at T3, TREM1 expression in both moderate and severe anaphylaxis was higher than healthy controls (p = 0.003 and p < 0.001, respectively).
Soluble TREM1 protein was detectable in all but one sample, which was from a severe patient at T0. At both T0 and T1, there were no differences in soluble TREM1 concentrations between groups (T0 \( p = 0.073 \) and T1 \( p = 0.066 \)) (Figure 3.2C). At T3, concentrations in severe anaphylaxis were 1.4-fold (95% CI: 1.1, 1.7) higher than in controls (\( p = 0.014 \)). There was no difference between moderate anaphylaxis and controls, or between reaction severity groups (\( p = 0.243 \) and \( p = 0.192 \), respectively).

3.5.8 **CD64 expression is elevated during anaphylaxis**

At T0, no differences in CD64 mRNA were apparent due to anaphylaxis (\( p = 0.921 \)) (Figure 3.1). After one hour, CD64 mRNA expression was higher in severe patients than either healthy controls or moderate patients (\( p = 0.006 \) and \( p = 0.009 \), respectively). The same pattern was observed at T3 with greater expression in severe patients than either healthy controls or moderate anaphylaxis (\( p < 0.001 \) and \( p = 0.010 \), respectively).

3.6 **Discussion**

This study investigated a panel of markers involved in innate immunity, neutrophil activation, and apoptosis in patients with anaphylaxis and healthy controls. We have presented evidence of differences associated with anaphylaxis in all the selected targets at either the mRNA or protein levels. IL10, OSM, S100A8, S100A9, MMP9, TLR4, MYD88, TREM1, and CD64 were all upregulated during anaphylaxis, whilst FASL was downregulated. The protein analyses identified increased IL-6, IL-10, S100A9, MMP9, and soluble TREM1 concentrations, and reduced soluble FasL concentrations in anaphylaxis patients when compared to healthy individuals. Taken
together, these results provide support for the hypothesis that innate immune pathways and myeloid cells such as neutrophils are activated during human anaphylaxis.

We have shown elevated IL10 and OSM expression and increased IL-6 and IL-10 protein concentrations during anaphylaxis, when compared to healthy controls. The mRNA results validate the findings of our microarray analysis and the protein results support those we have previously reported in a different cohort of anaphylaxis patients [55, 149]. These findings are also supported by observations from other groups as described in the literature. Elevated IL-6 concentrations are evident in allergic asthma patients [162, 163], a condition that shares many similarities with anaphylaxis. OSM concentrations were also elevated in asthmatics, following allergen challenge but not saline challenge [164, 165]. IL-6 and its family member OSM are pro-inflammatory cytokines, whilst IL-10 is an anti-inflammatory cytokine. They are released from a number of cell types including mast cells, macrophages, and neutrophils [152-154]. Due to the pro-inflammatory functions of IL-6 and OSM, their release during a reaction likely contributes to the symptoms of anaphylaxis. In contrast, IL-10 has been found to reduce anaphylaxis following challenge of sensitized mice [166-168]. The upregulation and release of IL-10 evident in our cohort likely represents an attempt by the immune system to limit the severity of symptoms and assist with recovery. The severity of a reaction could be, in part, determined by the relative concentrations of the pro- and anti-inflammatory cytokines released, with IL-6/OSM dominance resulting in poorer outcomes and IL-10 dominance improving symptom resolution. The increases we observed in these cytokines during anaphylaxis highlight the involvement of innate immune cell activation.
During anaphylaxis, $S100A8$ and $S100A9$ expression was elevated in patients compared to controls, particularly evident as the reactions progressed. We also observed a marked increase in S100A9 protein in patients, and by association the same is likely true for S100A8. These results validate the previous microarray findings, with the largest increase in mRNA evident three hours after enrolment [149]. S100A8 and S100A9 are present at high levels in neutrophil cytosol and to a lesser extent in other phagocytes [169]. Their intracellular functions include calcium binding and scavenging of reactive oxygen species [155, 169]. Activated neutrophils export large amounts of S100A8 and S100A9, both separately and as the heterodimer calmodulin, with neutrophils considered their primary producers [169]. Our observations of elevated $S100A8$ and $S100A9$ mRNA, and serum S100A9 protein during anaphylaxis are suggestive of neutrophil activation. Once in the extracellular environment, these proteins function as danger-associated molecular patterns (DAMPs) and chemotactants to recruit additional neutrophils. Their DAMP functions amplify inflammatory responses via binding to TREM1 and TLR4/MyD88 on the surface of target cells [155, 161], all of which were also elevated during anaphylaxis in this study. Therefore, our findings are not only suggestive of neutrophil activation, but also support neutrophil activation as a mechanism for amplifying the inflammatory signal during anaphylaxis.

We observed elevated expression of $MMP9$ mRNA and increased MMP9 protein concentrations in anaphylaxis patients, compared to controls, validating the microarray results. MMP9 is an enzyme produced by an array of inflammatory cells including neutrophils, macrophages, and lymphocytes [157, 170, 171]. It performs a key role in tissue remodelling and inflammatory cell trafficking by degrading the
extracellular matrix and basement membrane [172, 173]. Elevated MMP9 has been reported following exacerbation of allergic asthma and in patients with allergic bronchopulmonary aspergillosis (a hypersensitivity reaction to fungus) [174, 175]. In addition, allergen challenge in a mouse model was associated with elevated serum MMP9 [176]. Particularly in light of the elevated S100A8/S100A9 observed in our study, the increases in MMP9 are also suggestive of neutrophil activation. Elevated MMP9 in anaphylaxis may cause extracellular matrix degradation, allowing inflammatory cells to diffuse into tissues and trigger symptoms such as erythema and angioedema.

Whilst initially elevated, FASL expression decreased over the course of anaphylaxis, and soluble FasL protein was reduced in patients at all time points. FasL is a membrane-bound ligand for fas that plays an important role in the regulation of apoptosis and, in some cases, necrosis [177-179]. Following shedding from the cell surface by matrix metalloproteinases (MMPs), soluble FasL can still bind to fas; however, this no longer results in apoptosis [159]. Thereby, soluble FasL acts as a competitive inhibitor of membrane-bound FasL, and acts to minimise apoptosis. We propose that the reduction in FASL mRNA reflects increased translation of transcripts into surface FasL as immune cells are activated and subsequently prepared for apoptosis. In addition, the reduction in soluble FasL supports a pro-apoptotic environment, which would be necessary to clear inflammatory cells following anaphylaxis.

Elevated expression of TLR4 and MYD88 was observed during anaphylaxis. However, no significant differences were observed in MyD88 protein concentrations,
and TLR4 protein was not measured. TLR4 is a member of a large family of pattern recognition receptors that are expressed on an array of innate immune cells [180, 181]. MyD88 is an adaptor protein for TLR4, that promotes signalling through this receptor [182, 183]. Signalling through TLR4/MyD88 can be initiated via binding of DAMPs including S100A8/S100A9. Our observations of elevated TLR4 and MYD88, coupled with upregulation of S100A8 and S100A9 suggest this signalling pathway as a mechanism for amplifying innate immune signals during anaphylaxis.

Elevated TREM1 mRNA was evident three hours after arrival with anaphylaxis, validating the previous microarray findings. In addition, soluble TREM1 concentrations were also increased during anaphylaxis, a result more prominent in severe rather than moderate patients. TREM1 is expressed on the surface of neutrophils, monocytes, and macrophages and plays a regulatory role in innate immune responses during infection and inflammation [184, 185]. Binding of ligands to surface TREM1 triggers the cell to express additional activation receptors, thereby amplifying inflammatory responses [186]. It has been shown to co-operate with TLR4/MyD88 following binding with S100A8/S100A9 to enhance inflammatory responses [169, 187]. As with FasL, membrane-bound TREM1 is cleaved from the cell surface by MMPs [188]. Indeed we observed similar trends in soluble TREM1 and MMP9 concentrations. Soluble TREM1 may reduce inflammation by binding to TREM1 ligands in the serum, preventing them from binding to membrane-bound TREM1 [188]. Differential regulation of TREM1 during anaphylaxis has not been reported by others; however, increased expression has been observed in patients with the inflammatory condition, atopic dermatitis [189]. We propose that TREM1 is shed from the cell surface following cell activation during
Chapter 3

anaphylaxis, perhaps in response to elevated MMP9 concentrations, as a mechanism for dampening systemic inflammation.

In this study, we have described the upregulation of $CD64$ expression during anaphylaxis. This gene encodes the CD64 protein, also known as FcγRI, which is a high-affinity IgG receptor present on the surface of human neutrophils, monocytes, and macrophages [190]. In mouse models, binding of IgG to CD64 induces systemic anaphylaxis, in addition to other inflammatory disorders such as autoimmune arthritis and airway inflammation [190]. Elevated levels of surface and soluble CD64 have been observed in rheumatoid arthritis [191]. Upregulation of $CD64$ mRNA is expected to correlate with increased surface expression, although this would need to be confirmed using flow cytometry or microscopy. Additional CD64 on cell surfaces increases the likelihood of sensitisation through binding of allergen-specific IgG via the proposed IgG-mediated anaphylaxis pathway.

The findings of this study have provided support for the involvement of the innate immune system during anaphylaxis, particularly the activation of granulocytes such as neutrophils. In particular, we observed increases in S100A8 and S100A9, which are released in large quantities following neutrophil activation. These molecules signal additional cells via binding to surface receptors such as TLR4/MyD88 and TREM1, all of which were also upregulated during anaphylaxis. These proteins are all expressed by neutrophils, representing a potential mechanism for widespread systemic neutrophil activation. Products released from neutrophils such as MMP9, also upregulated during anaphylaxis in this study, and other potent antimicrobials can cause non-specific tissue damage. Therefore, the widespread activation of neutrophils may not only amplify the
inflammatory signal systemically, but also contribute to the physiological symptoms of anaphylaxis. Further analysis into the surface protein expression of these receptors by flow cytometry or microscopy would improve our understanding of the mechanisms driving receptor production, expression, and cleavage. Additionally, studies investigating the roles of the innate immune cells, such as neutrophils, during anaphylaxis may open up new avenues for improved treatments, prevention, and screening to identify at-risk individuals.

Acknowledgements

The authors appreciate and acknowledge the research nursing staff at Royal Perth, Fremantle, Armadale, and Austin hospitals, including Ellen MacDonald (Clinical Nurse Manager), for identifying patients for the study and collecting and processing blood samples. The authors also appreciate Mrs Claire Neil for her expert assistance in optimising the laboratory methods. The authors acknowledge the facilities, scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, University of Western Australia, a facility funded by the University, State and Commonwealth Governments. Prof. Simon Brown is supported by a NHMRC Career Development Fellowship, and laboratory work was supported by the RPH Medical Research Foundation. Funding bodies played no role in study design, data collection, or analysis.

Author contributions

All authors reviewed and approved the final manuscript. AF contributed to the study concept and design, performed the laboratory experiments, contributed to data analysis, and drafted the manuscript. EB contributed to the data analysis and manuscript
preparation. SFS contributed to the study concept and design, and assisted in obtaining funding support. DMF contributed to the collection of data. SB contributed extensive statistical knowledge to assist in data analysis and interpretation. SGAB contributed to the study concept and design, obtaining funding support, and collection of data.

Conflicts of interest

The authors declare that they have no conflicts of interest.
### 3.7 Tables

Table 3.1: Patient demographics and clinical observations

<table>
<thead>
<tr>
<th></th>
<th>Moderate anaphylaxis</th>
<th>Severe anaphylaxis</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>33</td>
<td>36</td>
<td>69</td>
</tr>
<tr>
<td><strong>Age (years), mean (SD)</strong></td>
<td>36 (15.4)</td>
<td>44 (15.6)</td>
<td>40 (15.9)</td>
</tr>
<tr>
<td><strong>Male gender, n (%)</strong></td>
<td>15 (45)</td>
<td>16 (44)</td>
<td>31 (45)</td>
</tr>
<tr>
<td><strong>Cause</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug, n (%)</td>
<td>7 (21)</td>
<td>19 (53)</td>
<td>26 (38)</td>
</tr>
<tr>
<td>Food, n (%)</td>
<td>18 (54)</td>
<td>5 (14)</td>
<td>23 (33)</td>
</tr>
<tr>
<td>Insect, n (%)</td>
<td>3 (9)</td>
<td>3 (8)</td>
<td>6 (9)</td>
</tr>
<tr>
<td>Other/Unknown, n (%)</td>
<td>5 (15)</td>
<td>9 (25)</td>
<td>14 (20)</td>
</tr>
<tr>
<td><strong>Onset to enrolment (mins), mean (SD)</strong></td>
<td>72 (37)</td>
<td>89 (51)</td>
<td>81 (45)</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any skin feature, n (%)</td>
<td>32 (97)</td>
<td>36 (100)</td>
<td>68 (99)</td>
</tr>
<tr>
<td>Any GI feature, n (%)</td>
<td>11 (33)</td>
<td>18 (50)</td>
<td>29 (42)</td>
</tr>
<tr>
<td>Any respiratory feature, n (%)</td>
<td>32 (97)</td>
<td>29 (81)</td>
<td>61 (88)</td>
</tr>
<tr>
<td>Hypoxemia, n (%)</td>
<td>-</td>
<td>5 (14)</td>
<td>5 (7)</td>
</tr>
<tr>
<td>Cyanosis, n (%)</td>
<td>-</td>
<td>10 (28)</td>
<td>10 (14)</td>
</tr>
<tr>
<td>Any cardiovascular feature, n (%)</td>
<td>7 (21)</td>
<td>35 (97)</td>
<td>42 (61)</td>
</tr>
<tr>
<td>Hypotension, n (%)</td>
<td>-</td>
<td>26 (72)</td>
<td>26 (38)</td>
</tr>
<tr>
<td>LOC/Collapse, n (%)</td>
<td>-</td>
<td>12 (33)</td>
<td>12 (17)</td>
</tr>
<tr>
<td><strong>Treatments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine^, n (%)</td>
<td>29 (88)</td>
<td>36 (100)</td>
<td>65 (94)</td>
</tr>
<tr>
<td>Pre-hospital, n (%)</td>
<td>8 (24)</td>
<td>18 (50)</td>
<td>26 (38)</td>
</tr>
<tr>
<td>Steroids, n (%)</td>
<td>19 (58)</td>
<td>27 (75)</td>
<td>46 (67)</td>
</tr>
<tr>
<td>Fluids, n (%)</td>
<td>16 (48)</td>
<td>27 (75)</td>
<td>43 (62)</td>
</tr>
<tr>
<td><em><em>Positive histamine</em> (&gt;1.2ng/mL), n (%)</em>*</td>
<td>13 (52)</td>
<td>21 (60)</td>
<td>34 (57)</td>
</tr>
<tr>
<td><strong>Positive MCT</strong> (711.4 ng/mL, or ΔMCT &gt;2ng/mL), n (%)**</td>
<td>16 (52)</td>
<td>32 (89)</td>
<td>48 (72)</td>
</tr>
</tbody>
</table>

GI, gastrointestinal; LOC, loss of consciousness; MCT, mast cell tryptase; ΔMCT, difference between highest and lowest observed MCT concentrations.

Skin features = erythema, urticaria, periorbital oedema, angioedema

GI features = nausea, vomiting, abdominal/pelvic pain, incontinence

Respiratory features = dyspnoea, stridor, wheeze, chest/throat tightness, hypoxemia, cyanosis

Cardiovascular features = dizziness, diaphoresis, confusion, hypotension, LOC, collapse

^ = epinephrine administered at any stage
Note, due to sample availability:
* Histamine was measured by ELISA in 25 moderate and 35 severe patients at enrolment
** MCT was measured by ImmunoCAP® in 31 moderate and 36 severe patients at enrolment
Table 3.2: Proportion of samples with detectable protein concentrations for IL-6, IL-10, S100A9, soluble FasL, and MyD88.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Healthy Control</th>
<th>Moderate</th>
<th>Severe</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
<td>T1</td>
<td>T3</td>
<td>T0</td>
</tr>
<tr>
<td>IL-6</td>
<td>0/20</td>
<td>9/33</td>
<td>15/32</td>
<td>9/30</td>
</tr>
<tr>
<td>IL-10</td>
<td>0/20</td>
<td>3/33</td>
<td>6/32</td>
<td>6/30</td>
</tr>
<tr>
<td>S100A9</td>
<td>0/20</td>
<td>9/23</td>
<td>7/23</td>
<td>7/17</td>
</tr>
<tr>
<td>FasL</td>
<td>18/20</td>
<td>18/33</td>
<td>19/32</td>
<td>13/30</td>
</tr>
<tr>
<td>MyD88</td>
<td>8/20</td>
<td>11/23</td>
<td>12/23</td>
<td>7/17</td>
</tr>
</tbody>
</table>

Bolded p-values (< 0.05) were considered significant.

p-values 1, 3, and 5 represent testing across the 3 groups at T0, T1, and T3 respectively.

p-values 2, 4, and 6 represent testing between moderate and severe anaphylaxis at T0, T1, and T3 respectively. Performed where the p-value across the 3 groups < 0.05.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>Amplicon length (bp)</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL10</td>
<td>GGCTACGGCGCTGTCATCGATT</td>
<td>GCATTCTTCACCTGCTCCACGG</td>
<td>70</td>
<td>64</td>
</tr>
<tr>
<td>IL6</td>
<td>GCAGCAAGAGGCACTGGGCAAGA</td>
<td>CCAAGCAAGTCTCCCTCAAATCC</td>
<td>99</td>
<td>68</td>
</tr>
<tr>
<td>TREM1</td>
<td>AACTGTGACCCAAGCTCCACCCA</td>
<td>GTTGAAACACCGGAACCCTGATGAT</td>
<td>106</td>
<td>64</td>
</tr>
<tr>
<td>CD64</td>
<td>TGGTGCGAGCGCTGCCACAGA</td>
<td>AACTGGAGGCAAGCAGACTTGAAGC</td>
<td>80</td>
<td>67</td>
</tr>
<tr>
<td>MYD88</td>
<td>AGCATTGAGGAGGATTGCCA</td>
<td>GGCCACCTGTAAGGCTTCT</td>
<td>75</td>
<td>63</td>
</tr>
<tr>
<td>MMP9</td>
<td>TTCAGGGAGACGCCATTTT</td>
<td>AACCGATTTGGAACCACGAC</td>
<td>73</td>
<td>60</td>
</tr>
<tr>
<td>FASLG</td>
<td>ATAGGCCACCCCAGTCCACCC</td>
<td>TGGAACCTGTCTTTAAATGGCCAC</td>
<td>70</td>
<td>67</td>
</tr>
<tr>
<td>S100A8</td>
<td>TATCAGGAAAAAGGGTGCGAGAC</td>
<td>TGCCACGCCCCATCTTTATCA</td>
<td>109</td>
<td>62</td>
</tr>
<tr>
<td>S100A9</td>
<td>CGCGGTACTCTCTCGAGCA</td>
<td>GCCGCAGGCTCACAGATAT</td>
<td>107</td>
<td>63</td>
</tr>
<tr>
<td>TLR4</td>
<td>GCCCTGCGTGAGGTGTCGCC</td>
<td>GAGAAGGGGAGGTTGGCGGGAT</td>
<td>84</td>
<td>66</td>
</tr>
<tr>
<td>OSM</td>
<td>CGCTGCTCATCCTGGGCAGA</td>
<td>CGCGGTACTCTTTCCGAGCA</td>
<td>84</td>
<td>66</td>
</tr>
<tr>
<td>RPS18</td>
<td>TCTTCAGTCGTCCTCAGGTCAGTCT</td>
<td>TGAGGATGAGGTTGGAAACGTG</td>
<td>167</td>
<td>60</td>
</tr>
<tr>
<td>HPRT1</td>
<td>TGCACATTGCGCAAAAACAATGCA</td>
<td>GGTCCTTTCTCCACAGCAAGCT</td>
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<td>60</td>
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<tr>
<td>YWHAZ</td>
<td>ACTTTTGGTACATTGGCTTCAA</td>
<td>CCGCCAGGACAAAACCAGTAT</td>
<td>94</td>
<td>60</td>
</tr>
</tbody>
</table>
3.8 **Figures**

![Figure 3.1: Gene expression during anaphylaxis.](image)

Quantitative PCR was performed on mRNA extracted from peripheral blood leukocytes of moderate (MOD, n=11) and severe (SEV, n=15) anaphylaxis patients at the time of enrolment in the emergency department (T0), and again after one (T1) and three (T3) hours. Gene expression data are presented as calibrated normalised relative quantities (CNRQ). Boxplots identify the median, upper and lower quartiles, and the range. Dotted lines indicate the median CNRQ of healthy controls (n=20). Due to the number and complexity of comparisons, significant differences between groups are described in the results text.
Figure 3.2: Protein concentrations during anaphylaxis.
A) MMP9, B) soluble Fas ligand (FasL), and C) soluble TREM1 in serum/plasma from moderate (n = 33) and severe (n = 36) anaphylaxis patients at the time of admission to the emergency department (T0), and again after one (T1) and three (T3) hours. Boxplots identify the median, upper and lower quartiles, and the range. Dotted lines indicate the median concentration of healthy controls (n = 20) of: 304.1 ng/mL MMP9; 14.3 pg/mL FasL; and 182.3 pg/mL TREM1. Due to the number and complexity of comparisons, significant differences between groups are described in the results text.
Chapter 4  

Small nucleolar RNA networks and genes involved in NK cytotoxicity and Th1/Th2 signalling are upregulated during human anaphylaxis

4.1  Background

The previous chapter described a series of experiments that aimed to validate the results of a microarray performed earlier in our laboratory. The aforementioned microarray investigated differential gene expression in a cohort of six healthy controls and six moderate patients with reactions to a variety of triggers. In the present study, high quality RNA samples were available from a larger group of patients, including those with severe reactions. To date, no previous studies have investigated mRNA expression changes associated with both moderate and severe anaphylaxis by microarray. The present study also narrowed down the possible triggers to only foods or drugs, to minimise the heterogeneity of cases. Additionally, for the first time, we compared the genomic profiles of anaphylaxis patients to those of sepsis and head trauma patients. This allowed investigation into immune pathways that may be unique to anaphylaxis rather than ubiquitous inflammatory changes. This chapter has been prepared as an article for submission, as follows.
Title: Small nucleolar RNA networks and genes involved in NK cytotoxicity and Th1/Th2 signalling are upregulated during human anaphylaxis

Short title: Genomic responses driving anaphylaxis severity

Abbie Francis¹,², Niamh M. Troy³, Ramesh Ram⁴,⁵, Erika Bosio¹,², Shelley F. Stone¹,², Daniel M. Fatovich¹,²,⁶, Grant Morahan⁴,⁵, Simon G.A. Brown¹,²,⁶,⁷, Anthony Bosco³

1. Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Perth, WA, Australia
2. Discipline of Emergency Medicine, School of Medicine, Faculty of Health and Medical Sciences, University of Western Australia, Perth, WA, Australia
3. Telethon Kids Institute, University of Western Australia, Perth, WA, Australia
4. Centre for Diabetes Research, Harry Perkins Institute of Medical Research, Nedlands, WA, Australia
5. Centre of Medical Research, University of Western Australia, Nedlands, WA, Australia
6. Emergency Department, Royal Perth Hospital, Perth, WA, Australia
7. Emergency Department, Royal Hobart Hospital, Hobart, TAS, Australia

Key words: Anaphylaxis, gene expression profiling, microarray, network analysis, snoRNA, NK cytotoxicity.

Corresponding Author: Abbie Francis, Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Level 6 MRF Building, Rear 50 Murray Street, Perth, Western Australia 6000.

Phone: +61 8 9224 0356 Email: Abbie.Francis@uwa.edu.au
Abbreviations
CAMERA, Correlation Adjusted MEan RAnk; CISS, Critical Illness and Shock Study; ED, emergency department; FDR, false discovery rate; HGNC, HUGO Gene Nomenclature Committee; IFN, interferon; IL, interleukin; IQR, interquartile range; NIAID/FAAN, National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network; NK, natural killer; PBL, peripheral blood leukocyte; RIN, RNA integrity number; snoRNA, small nucleolar RNA; Th, T helper; TNF, tumour necrosis factor; WGCNA, weighted gene coexpression network analysis.

4.2 Abstract

Background
Regardless of the immunogenic trigger, there is limited knowledge of the processes underlying the rapid amplification of localised allergic responses to life-threatening anaphylaxis within minutes. We hypothesized that peripheral blood leukocytes (PBLs) play a role.

Objective
To characterise genomic responses in PBLs during moderate and severe reactions and identify gene expression networks specific to anaphylaxis.

Methods
PBLs were collected from patients with moderate (n=5) and severe (n=10) anaphylaxis at emergency department arrival, in addition to sepsis (n=20) and head trauma (n=10) patients, and healthy controls (n=10). RNA was extracted (PAXgene™) and high quality samples were labelled and hybridized to Human Gene 2.1 ST microarrays.
(Affymetrix®). Differences between groups were investigated using 2-way LIMMA tests. Gene expression patterns were analysed using pathway, network, and upstream regulator analyses (Ingenuity, GeneMANIA, and CAMERA).

**Results**

Genomic responses in anaphylaxis were characterised by the upregulation of natural killer (NK) and T helper (Th) cell activation pathways and gene networks. Upstream regulator analysis identified 10 candidate drivers of these responses, 7 were predicted to be activated and 3 inhibited during anaphylaxis. Most of the activated drivers were cytokines (IL-2, IL-15, IL-12, IFN-α, and IFN-λ). When comparing these responses to sepsis and head trauma profiles, NK and Th cell activation signatures were stronger in patients with anaphylaxis. Coexpression network analysis identified the upregulation of an extensive network of small nucleolar RNAs (snoRNAs) during anaphylaxis. This network was not upregulated in the other inflammatory conditions, suggesting a unique role for snoRNAs in anaphylaxis.

**Conclusion**

Upregulation of NK and Th cell activation pathways and snoRNA networks are hallmark features of anaphylaxis in humans. This represents a significant departure from our current understanding of anaphylaxis and supports a role for a leukocyte-cytokine activation cascade in the rapid amplification of immune responses.
4.3 Introduction

Regardless of the initial immunogenic trigger, there is limited knowledge of how localised allergic reactions become rapidly amplified, potentially leading to death within minutes of exposure. It is also unknown why only some sensitised individuals experience anaphylaxis whilst others do not, regardless of whether their mast cells activate/degranulate. These questions have been difficult to answer in humans for a number of reasons. Key steps in the activation process occur locally at the site of initial contact with the triggering agent. This localised release of mediators, which may be critically involved in amplifying the immune response, could be undetectable systemically [103]. In addition, spontaneous anaphylaxis is notoriously difficult to research, particularly during the acute phase, due to the unpredictable nature of reactions and the time taken to attend emergency departments (EDs). We hypothesize that a cascade of leukocyte activation in the peripheral blood rapidly amplifies the localised response.

To investigate these questions and further our understanding of human anaphylaxis, we developed an unbiased, genomics-based strategy. This entailed comparative profiling of gene expression patterns by microarray in peripheral blood leukocytes (PBLs) from anaphylaxis patients collected upon ED arrival, or from healthy controls [149]. We analysed the data using network analysis [192] and causal inference algorithms [193] to provide a holistic view of the underlying mechanisms, and identify the putative causal pathways driving responses. Employing this methodology, we previously demonstrated that genomic responses during anaphylaxis were characterized by upregulation of innate inflammatory gene networks [149]. In the present study, we built on these previous findings by investigating a larger patient cohort that included
both moderate and severe reactions. We also focussed on the early phase of the response by investigating changes evident upon ED arrival, in most cases before any treatment or intervention. In addition, we aimed to investigate the mechanisms specific to anaphylaxis, as opposed to those simply characteristic of inflammation. To achieve this, we compared the gene expression patterns of anaphylaxis patients to those of other inflammatory conditions (i.e. head trauma and sepsis).

We report for the first time the upregulation of genes that mediate natural killer (NK) cell cytotoxicity, T helper type 1 (Th1) and Th2 activation, and cytokine signalling pathways during human anaphylaxis. These activation signals were also stronger in anaphylaxis than other inflammatory conditions. In addition, we have identified the upregulation of a network of snoRNAs, a response that was unique to anaphylaxis. These findings provide a unique insight into the pathogenesis of anaphylaxis in humans.

4.4 Methods

4.4.1 Study population

Adult patients with features of acute anaphylaxis were recruited in five Australian EDs between September 2010 and April 2015 as part of our ongoing Critical Illness and Shock Study (CISS) [143]. As emergency care took priority, waiver of initial consent was approved under the provision of paragraph 2.3.6 of the National Health and Medical Research Council Ethical Conduct guidelines (2007). Fully informed written consent was obtained as soon as possible and patients were given the option of declining further involvement and having all samples already collected destroyed. Ethics
approval, including waiver of initial consent, was obtained from the Human Research Ethics Committees at each site (Royal Perth Hospital, Fremantle Hospital, Armadale Kelmscott Memorial Hospital: EC 2009/080; South West Health Campus: 2012:31; Austin Hospital: H2012/04477).

Inclusion criteria were age $\geq$ 16 years and the presence of National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network (NIAID/FAAN) clinical criteria for a diagnosis of anaphylaxis [1] with features present in two or more organ systems at the time of enrolment. As special sample handling was required, this was a convenience sample, taken only when research staff (research nurse and/or investigator) was available to supervise procedures. Severe anaphylaxis was defined by the presence of hypotension (systolic blood pressure < 90 mmHg), hypoxaemia ($\text{SpO}_2 \leq 92\%$ and/or cyanosis), or neurological compromise (confusion, collapse, or loss of consciousness). The remainder of cases were classified as moderate anaphylaxis. Mild reactions did not meet CISS criteria and were not recruited.

To investigate anaphylaxis specific changes, we also collected peripheral blood samples from patients presenting to the ED with sepsis ($n = 20$) and head trauma ($n = 10$). These patients were similarly recruited through CISS. Inclusion criteria for these patients have been described previously [143, 194]. Sepsis was defined as either uncomplicated infection ($n = 10$) or severe sepsis/septic shock ($n = 10$) based on the Surviving Sepsis Campaign 2012 definition [195]. CISS ethics approvals also included allowance for the collection of samples from healthy controls, which were recruited through community advertisement.
4.4.2 Sample collection and storage

Peripheral blood samples were collected from all patients upon presentation to the ED. RNA was stabilized in PAXgene™ tubes (PreAnalytiX GmbH, Switzerland). Tubes were placed immediately at 4°C, transferred to -20°C within 72 hours, before final storage at -80°C.

4.4.3 Gene expression profiling

Total RNA was extracted using the PAXgene™ Blood RNA Extraction Kits (PreAnalytiX GmbH, Switzerland) by automation with a QIAcube instrument (Qiagen, USA). The purity and integrity of the RNA samples were assessed on a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and a 2100 Bioanalyzer (Agilent, USA). Total RNA samples of very high quality (median (interquartile range, IQR) 260/280 absorbance ratio: 2.07 (0.04); and median (IQR) RNA integrity number (RIN): 8.9 (1.3)) were shipped on dry ice to the Ramaciotti Centre for Gene Function Analysis. Samples were labelled and hybridized to Affymetrix® Human Gene 2.1 ST microarrays (Affymetrix®, Thermo Fisher Scientific, USA). The raw microarray data files are available from the Gene Expression Omnibus repository (accession: GSE69063).

4.4.4 Microarray data analysis

The microarray data was pre-processed in the R Project for Statistical Computing (R) environment (The R Foundation, USA) employing the Robust Multi-array Average (RMA) algorithm [196]. A custom chip description file (hugene21sthsentrezg, version = 18) was used to map probe sets to genes based on current genome annotation [197]. The quality of the microarray data was assessed using
the R package array quality metrics [198]. As the anaphylaxis, sepsis, and head trauma microarrays were performed separately, each batch of results was individually normalised and ComBat software (Boston University, USA) was used to adjust for batch effects (Figure S4.1) [199-201].

Due to the small sample sizes, non-parametric tests were used to investigate differences in gene expression. Differences between groups were analysed using LIMMA tests, with each comparison run using 1000 permutations. Permutation p-values were adjusted for multiple testing employing the false discovery rate (FDR) method [202]. Comparisons with an absolute \( \log_2 \) fold change (FC) > 0.6 and FDR \( p < 0.05 \) were considered differentially expressed. Heatmap figures were produced using Morpheus software (Broad Institute, USA).

Identification of canonical pathways and upstream regulators, and gene network analysis were performed using Ingenuity Pathway Analysis software v1.07 (Qiagen, USA) [193, 203]. The MCODE clustering algorithm was used on the GeneMANIA database (Cytoscape, USA) to identify and characterize sub-networks of differentially expressed genes. Gene symbols were mapped using the HUGO Gene Nomenclature Committee (HGNC) identifiers and fold-change was treated as actual fold-change rather than \( \log_2 \) fold-change. The Core Analysis function was run using the default settings, with the species specified as humans. Benjamini-Hochberg adjusted p-values were used in canonical pathway analysis.

A coexpression network was constructed employing the weighted gene coexpression network analysis (WGCNA) algorithm in the R environment [204]. This
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identified gene coexpression networks in PBLs underlying anaphylaxis and grouped them into modules. These modules were analysed for enrichment of biological pathways using the R package clusterProfiler [205]. Correlation Adjusted MEan RAnk gene set analysis (CAMERA) was used to investigate the significance of modules in anaphylaxis when compared to healthy controls [206]. The anaphylaxis coexpression modules were similarly investigated for differential expression in the sepsis and head trauma cohorts when compared to the relevant healthy controls run with each batch (n = 10, for each batch).

4.5 Results

4.5.1 Characteristics of the study population

The anaphylaxis microarray cohort consisted of 15 cases with RNA samples of sufficient quality and quantity for analysis, including 10 severe reactions. Clinical information for each patient is presented in Table 4.1. Patients were between 29 – 69 years of age (median = 35) and were primarily female (10 reactions, 66%). All patients were treated with epinephrine. Beta-lactam antibiotics were the primary trigger for severe reactions (n = 6, 60%). Non-steroidal anti-inflammatory drugs (NSAIDs) and foods accounted for equal proportions of the remaining severe reactions. All reactions of moderate severity were caused by food. Two moderate reactions (40%) and 8 severe reactions (80%) had MCT and/or histamine concentrations above healthy control cut-offs (peak MCT > 11.4 ng/mL and/or delta-MCT ≥ 2 ng/mL; peak histamine ≥ 1.2 ng/mL) [51, 55].
The clinical characteristics of the sepsis and head trauma patients are presented in Tables S4.1 and S4.2. The sepsis cases were subclassified as either uncomplicated infection (n = 10) or severe sepsis/septic shock (n = 10) based on standard criteria [195]. Sepsis patients were between 22 – 85 years of age (median = 67), with a roughly equal sex distribution (11, 55% male). Two patients (10%) succumbed to their infections. The head trauma patients were between 17 – 80 years of age (median = 27) and were primarily male (8, 80%). Half of these patients had unsurvivable injuries.

4.5.2 Timing from illness onset to sample collection did not effect gene expression patterns

Due to differences in the time taken to arrive at an ED for treatment, the timing between symptom onset and blood sampling was variable in the anaphylaxis population. This ranged from 15 min to 2 hours and 22 minutes (median = 65 minutes). To investigate the impact of this variation on the data we employed a linear model. This correlated gene expression patterns at ED arrival with the time lag from the onset of symptoms to blood collection. There was no significant association identified (FDR p = 0.99).

4.5.3 Genomic responses during anaphylaxis

4.5.3.1 Gene expression patterns in anaphylaxis were markedly different from healthy controls

Analysis of genes that were differentially expressed between anaphylaxis patients and healthy controls demonstrated that 510 genes were upregulated and 421 genes were downregulated (FDR p < 0.05 and absolute log\textsubscript{2} fold change > 0.6). The
heatmap presented in Figure 4.1 illustrates the patterns of differential expression in each anaphylaxis patient and healthy control. The top 40 most significantly upregulated and top 40 most significantly downregulated genes are shown, listed by gene relatedness. Figure 4.1 clearly demonstrates differences in genomic responses during anaphylaxis when compared to healthy controls. The gene expression data for a subset of these genes, the top 12 based on significance, is shown in Figure 4.2. For several of these genes (e.g. DDIT4 and GK5), there is an apparent trend with reaction severity, although larger sample sizes are required to investigate significance. Of these 12 genes, 10 were upregulated and 2 were downregulated in anaphylaxis compared to controls. The expression of the most significantly altered gene, CD69, was upregulated by 4.4-fold in anaphylaxis patients (FDR p = 7.4 x 10^{-6}). This gene encodes the transmembrane receptor protein CD69, which is rapidly induced on leukocytes following activation, and has been implicated in several inflammatory diseases [207]. The expression of the most significantly downregulated gene, DNM3, was 2.2-fold lower in anaphylaxis than controls (FDR p = 1.8 x 10^{-5}). DNM3 encodes the dynamin-3 protein, which is involved in GTP binding, microtubule binding, vesicular trafficking, and endocytosis [208].

### 4.5.3.2 Principal immune cell activation pathways are enriched in anaphylaxis at ED arrival

The differentially expressed genes in anaphylaxis patients compared to controls were interrogated using pathway analysis to identify the biological pathways most strongly represented. This analysis identified 11 significant canonical pathways that were driving responses at ED arrival (FDR p < 0.05) (Table 4.2). The top three enriched pathways were: natural killer (NK) cell signalling (FDR p = 4.4 x 10^{-8}); crosstalk between dendritic cells (DCs) and NK cells (FDR p = 1.3 x 10^{-4}); and macropinocytosis
signalling (FDR p = 1.4 \times 10^{-4}). T cells were also well represented in the pathway analysis with three of the other significant pathways directly identifying T helper cell activation. Additionally, three activated pathways identified inositol phosphate signalling, which functions in cell growth, migration, and survival.

4.5.3.3 The top gene network in anaphylaxis highlights NK cell and T helper cell activation

Next, we employed network enrichment analysis (Ingenuity Pathway Analysis, Qiagen) to interrogate the differentially expressed genes and identify closely related gene networks that are strongly represented in anaphylaxis. The top gene network had a score of 36 and included 29 focus molecules (Figure 4.3). The primary functions of this network are to control cell-to-cell signalling and interactions, haematological system development and function, and immune cell trafficking. A large number of the upregulated targets in this top network are associated with NK cells (CLEC2D, KLRB1, KLRC4, KLRD1, KLRF1, KLRK1, HCST, NCR1, NCR3, PRF1, and ZAP70). Activation of their relevant proteins stimulates NK cell activation. Additionally, several upregulated targets are associated with T helper cells (CD52, FYN, HAVCR2, HCST, STAT4, and ZAP70), indicating activation of these cell types. The downregulated targets in this top network include those involved in endocytic membrane trafficking (STON2 and ITSN1) and platelet activation (PTFAR). A further 24 gene networks were identified during this analysis. They shared the following common functions: cell-to-cell signalling; cell death and survival; inflammatory response; cell-mediated immune response; cellular movement; and cell growth and proliferation.
4.5.3.4 The observed gene expression patterns are driven primarily by cytokine activation

To infer the putative molecular drivers that may be initiating the observed gene expression patterns, we utilised upstream regulator analysis [193]. This was conducted by interrogating the differentially expressed genes for enrichment of sets of target genes, that based on prior experimental evidence, are known to be driven by a given candidate regulator. An overlap p-value was calculated, which measures the enrichment of target genes in the data for a given regulator. An activation Z-score was also calculated, which measures the pattern match between the observed gene expression changes (up/down regulation) and the predicted pattern from the literature. At ED arrival, 10 candidate drivers of anaphylactic responses were identified (overlap p < 0.05 and absolute Z-score > 2), 7 of these were activated and 3 were inhibited (Table 4.3). The activated drivers were the cytokines interleukin (IL)-2, IL-15, IL-21, interferon (IFN)-α, and IFN-λ, as well as PDZ and LIM domain protein 2 (PDLIM2, involved in cell attachment), and toll-like receptor 9 (TLR9, involved in innate and adaptive immunity). The inhibited drivers were programmed cell death protein 1 (PDCD1, controls apoptosis and down-regulates immune responses), runt-related transcription factor 1 (RUNX1, regulates haematopoiesis), and CD3 (involved in T cell activation).

4.5.4 Differences in genomic responses that were unique to anaphylaxis

4.5.4.1 Gene expression patterns in anaphylaxis were strikingly different from other inflammatory conditions

The second aim of this study was to investigate whether the alterations in gene expression demonstrated during anaphylaxis were specific to anaphylaxis or simply characteristic of systemic inflammation. We performed identical microarray analyses of
samples from sepsis and head trauma patients. Differences in gene expression between anaphylaxis and these other inflammatory conditions were investigated using LIMMA tests to complete two-way comparisons. The results demonstrated significant differences in gene expression between anaphylaxis and the other inflammatory conditions. This suggests that unique sets of genes are modulated during the anaphylactic response. There were 227 genes upregulated and 77 genes downregulated in anaphylaxis compared to sepsis and head trauma patients (FDR p < 0.05 and absolute log₂ fold change > 0.6). The patterns of differential expression of all 304 genes are shown in Figure 4.4. This heatmap highlights the clear differences in genomic responses between the patient groups.

4.5.4.2 T cell activation pathways and networks characterise genomic responses in anaphylaxis but not sepsis or head trauma

Following pathway analysis, 61 canonical pathways that were unique to anaphylaxis were identified at ED arrival (FDR p < 0.05) (Table 4.4), 18 of these were highly significant (FDR p < 0.001). The top three enriched pathways were: iCOS-iCOSL signalling in T helper cells (FDR p = 4.0 x 10⁻¹²); T cell receptor signalling (FDR p = 7.9 x 10⁻¹²); and calcium-induced T lymphocyte apoptosis (FDR p = 2.0 x 10⁻¹⁰). T cell activation is the dominant feature of the significant pathways, with 15 directly related to T cells including the top 11 most significant. Interestingly, 8 of the 11 pathways previously identified as enriched in anaphylaxis patients compared to controls were also significantly upregulated when compared to other inflammatory conditions. These included NK cell signalling (FDR p = 2.1 x 10⁻⁶) and the crosstalk between NK cells and dendritic cells (FDR p = 2.1 x 10⁻²).
Network enrichment analysis (GeneMANIA) identified two key highly significant sub-networks linking genes upregulated exclusively in anaphylaxis (Figure 4.5). The first sub-network had a score of 17.3 and included 19 distinct significant functions (FDR p < 0.05). The most significant role of this sub-network was T cell activation (FDR p = 1.0 x 10^{-11}). The remaining functions were related to the positive regulation of leukocyte activation and signalling. The second sub-network had a score of 12.4 and encompassed 28 significant functions (FDR p < 0.05). The most significant function of this sub-network was the antigen receptor-mediated signalling pathway (FDR p = 1.8 x 10^{-5}). Six of the proposed functions involved T cell activation or signalling. Of note, Fc receptor (Ig) and Fcε (IgE) receptor (which can bind allergen-specific antibodies) signalling pathways were also significant (FDR p = 3.5 x 10^{-3} and FDR p = 2.0 x 10^{-2}, respectively).

4.5.4.3 Differential gene expression between anaphylaxis and other inflammatory conditions are driven by TGM2 and OSCAR

Despite the large number of differentially expressed genes and associated pathways between anaphylaxis and other inflammatory conditions, only two molecular drivers were identified using upstream regulator analysis (Ingenuity Pathway Analysis, Qiagen) (overlap p < 0.05 and absolute Z-score > 2). The first, transglutaminase 2 (TGM2) was activated in anaphylaxis (p = 2.1 x 10^{-6}) and plays a role in apoptosis, wound healing, inflammation, and extracellular matrix stabilisation [209]. The second, osteoclast-associated immunoglobulin-like receptor (OSCAR) was also activated in anaphylaxis (p = 4.2 x 10^{-4}). OSCAR is involved in the regulation of the innate and adaptive immune responses, oxidative stress-mediated atherogenesis, and monocyte adhesion [210].
4.5.5 Coexpression network analysis identified upregulation of a gene module enriched with small nucleolar RNAs in anaphylaxis but not sepsis or head trauma

The above findings provide a knowledge-based view of the data, and whilst this is powerful it is also biased. To obtain an unbiased, holistic view of the data, we employed coexpression network analysis [192]. The network was constructed by interrogating gene coexpression patterns across all of the samples in the data set. The resulting network comprised 2,705 genes that were organized into eight modules based on their coexpression. These modules include genes of diverse functionality, and have been grouped together by colour. Pathways analyses demonstrated that the modules were enriched for coherent biological functions (Figure 4.6A). The significance of each pathway in the module is indicated by the dot colour with dark red being the most significant. The proportion of the total pathway members that are present in each module is indicated by the size of the dot.

The coexpression network graphs in Figure 4.6B display the significance of the differences between patients and healthy controls for each module. The range bound by the dotted lines indicates the non-significant FDR p-value range of $1.000 - 0.010$. Boxplots that lie above this range indicate the module is differentially expressed (FDR $p < 0.01$) in that patient group compared to their respective healthy controls. These figures do not indicate the direction of differential expression (upregulation or downregulation), which was subsequently investigated for each significant module. This analysis showed that at ED arrival, the red module was significantly differentially expressed in anaphylaxis patients (FDR $p = 0.007$) (Figure 4.6B: graph A). Subsequent investigation revealed that the red module was upregulated in patients. This module contained 152 genes, and strikingly, 88 of these genes were small nucleolar RNAs (snoRNAs).
SnoRNAs are classified into two major structural classes: C/D box snoRNAs and H/ACA box snoRNAs [211]. This gene module contained 58 C/D box and 23 H/ACA box snoRNAs, in addition to 7 small Cajal body-specific RNAs (a minor subclass of snoRNAs). Pathway analysis demonstrated that the red module was enriched with genes involved in chromatin organization, cell cycle, and the cellular response to stress. The yellow module was also significantly differentially expressed in anaphylaxis at ED arrival (FDR p = 0.009), with subsequent investigation showing this was downregulated in anaphylaxis. The yellow module was enriched for genes involved in developmental biology, axon guidance, and membrane trafficking.

We then tested these anaphylaxis coexpression networks for differential expression in sepsis or head trauma patients compared to healthy controls. The patterns of significance for the different modules were very similar for both sepsis groups and the head trauma cohort. This pattern was clearly different to that previously observed in the anaphylaxis cohort. The blue module was strongly upregulated in patients with head trauma (FDR p = 0.001, Figure 4.6B: graph D). Pathway analysis showed that this module was primarily involved in interferon signalling and lipid metabolism. There was also a trend for upregulation of the blue module in patients with both uncomplicated sepsis (FDR p = 0.10, Figure 4.6B: graph B) and severe sepsis (FDR p = 0.07, Figure 4.6B: graph C). Interestingly, both the red (snoRNA-enriched) and yellow modules were not significantly altered in either of the other inflammatory conditions.

4.6 Discussion

Currently there is a lack of understanding of the processes underlying systemic anaphylaxis. As such, we employed genomic profiling of PBLs collected from patients
during acute anaphylaxis to identify potential genetic drivers of the response. Using this approach, we identified differential expression of an extensive array of genes, early on during the acute phase of anaphylaxis. These genes were involved in a variety of signalling pathways and have implicated a number of PBL cell types including both innate and adaptive immune cells (e.g. B cells, monocytes, macrophages, and dendritic cells). Pathways involving NK cells and Th1/Th2 cells were the most frequently identified during our pathway and upstream regulator analyses, and importantly, these pathways were activated in anaphylaxis at ED arrival. We also identified the upregulation of an extensive network of snoRNAs. Furthermore, these responses were clearly different to those associated with other types of inflammation, namely sepsis and head trauma. These findings support the involvement of NK cells and T helper cells during the acute phase of human anaphylaxis, and suggest a unique role for snoRNAs.

The involvement of NK cells in anaphylaxis has been previously proposed and is supported by some experimental models [212-216]. However, their role is not well understood. NK cells constitute a significant component of circulating cells, accounting for approximately 10% of peripheral blood leukocytes [217]. Once activated, NK cells primarily function to mediate cytotoxicity and produce cytokines, in particular high levels of tumour necrosis factor (TNF) and IFNγ, which amplify inflammatory responses [217, 218]. NK cells exhibit their cytotoxic role through the release of perforin and granzymes A and B [217], with the genes for these products all upregulated during anaphylaxis in this study. These molecules create pores in the cell membrane and induce programmed cell death of target cells [218]. The release of TNF and IFNγ, which are elevated in patient serum [55], from activated NK cells during anaphylaxis would likely result in activation of additional PBLs to amplify the inflammatory signal.
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The activation of the NK system can occur via a number of pathways. Our data suggests that during anaphylaxis NK cell activation is driven by the activation/release of IL-2, IL-15, and IL-21. These cytokines are produced by cell types including activated mast cells, Th2 cells, phagocytes, and dendritic cells [95], all of which were implicated in this study. Alternatively, NK cell activation could occur due to exposure to the allergen itself. Sensitisation to allergen typically occurs through the binding of allergen-specific antibodies, sIgE and sIgG, to IgE/IgG receptors on effector cells. NK cells do not express IgE receptors, however they do express the IgG receptors CD16 and CD32 [219-221]. In vitro and in vivo mouse models have demonstrated that both IgG and IgE can bind these receptors, which activates NK cells [212, 213]. In healthy non-allergic individuals, the extent of NK cell activity correlated with total serum IgE levels [222]. Taken together, these findings imply that sensitised individuals have highly active NK cells, which can respond to allergen exposure.

The functions and implications of NK cell activation during anaphylaxis are unclear. However, in mouse models of allergy, depletion or deficiency of NK cells attenuates the production of Th2 cytokines, allergen-specific IgE, and eosinophilic airway inflammation [215, 216]. However, NK cell depletion had no effect on the fall of rectal temperatures, used as a surrogate marker of anaphylactic shock [223]. It is possible that the release of mediators from NK cells directly contributes to symptoms of anaphylaxis, particularly respiratory symptoms. However, there is more evidence to suggest their role is in signal amplification [55, 217, 218]. We propose that cytokines derived from activated mast cells at the site of allergen exposure trigger the activation of the NK system. Activated NK cells then produce high levels of TNF and IFNγ, which
activate additional innate immune cells and amplify the response. Thereby, NK cells provide a plausible mechanism for the activation of a leukocyte-cytokine cascade during anaphylaxis. Detailed studies will be required to further elucidate the role of NK cells in human anaphylaxis.

The second major finding of this study was the upregulation of both Th1 and Th2 activation pathways in anaphylaxis, compared to healthy controls and other types of inflammation. Whilst both Th1 and Th2 cells are CD4+ T cells, Th1 cells are defined by their production of IFNγ, and Th2 cells by their production of IL-4 [224, 225]. Th1 cells have strong catalytic activity and primarily function in host defence against intracellular pathogens. Excessive or prolonged Th1 activation can have adverse effects related to their cytotoxic and phagocytic activation functions [224]. Allergic responses are typically considered to be Th2 driven. However, a Th1-dominant cytokine profile has been observed in allergic drug reactions [214, 226] and bee venom-sensitized patients during specific immunotherapy treatment [227, 228]. In addition, elevated IFNγ has been observed during anaphylaxis [55], although this could be derived from other cellular sources such as NK cells.

Whilst the function of Th1 cell activation during anaphylaxis is unclear, Th2 cells have a well-established role during allergen sensitisation. Allergen exposure has been shown to induce recruitment and activation of Th2 cells, causing the production of cytokines such as IL-4, IL-5, and IL-13 [229, 230]. These cytokines stimulate B cells to produce allergen-specific IgE antibodies, a critical step in the classical IgE-mediated anaphylaxis pathway [231]. It is plausible that the allergen exposure that triggers an anaphylactic reaction also induces additional Th2 activation, as we have observed.
The consequences of allergen-induced Th2 cell activation are not limited to sensitisation and the production of sIgE. Th2-derived cytokines have also been implicated in the pathophysiology of airway inflammation via induction of eosinophilia, the hyper-production of mucus, and fibrosis [232-234]. Promotion of Th1 responses causes a subsequent inhibition of Th2 responses, and this has been shown to attenuate allergy and asthma [234]. In our study, we observed upregulation of both the Th1 and Th2 pathways during the early phase of anaphylaxis. It is unexpected that Th1 cell activation in particular was so strong in anaphylaxis compared to sepsis, considering their role in pathogen removal (e.g. bacteria and viruses). Perhaps the inappropriate and excessive activation of both Th1 and Th2 cells is a hallmark feature that differentiates anaphylaxis from “appropriate” responses to allergen. Cytokine release from these cells could also contribute to the symptoms of anaphylaxis, particularly respiratory symptoms. Extensive future investigation is required to clarify the role of T helper cells and the potential dysregulation of T helper cell responses during anaphylaxis.

The other major finding of this study was the activation of snoRNAs during the early phase of anaphylaxis, which was not evident in other inflammatory conditions. SnoRNAs are short (60-300 nucleotides), non-coding RNA molecules, which are located in the nucleolus, a specific compartment of the nucleus that primarily synthesises ribosomes [235]. SnoRNAs act as guides for the posttranslational modifications of these ribosomes [211]. The two main types of snoRNAs are C/D box and H/ACA box snoRNAs, which guide 2′-O-ribose methylation and pseudouridylation, respectively [211]. Although these modifications represent housekeeping roles in the cell, snoRNAs may also contribute to disease processes. This could occur via guiding
mRNA splicing events, or by functioning as precursors for microRNAs, which regulate the translation of target mRNAs [236, 237].

Our analysis showed that the snoRNA module was enriched for genes involved in the regulation of the cell cycle, chromatin organization, and the response to cellular stress. Interestingly, the nucleolus can function as a sensor of cellular stress in response to a broad range of stimuli (e.g. hypoxia, DNA damage, heat shock, serum starvation, osmotic stress, and viral infection) [235]. Generalised and localised hypoxia is known to occur during anaphylaxis, as observed in our patient cohort. Therefore, it is likely that this hypoxic stress is detected by the nucleolus of PBLs, which then triggers the observed upregulation of snoRNAs as a consequence of anaphylaxis. However, hypoxic stress is also frequently observed in sepsis and head trauma patients, and the snoRNA-enriched module was not upregulated in these groups. This suggests the snoRNAs may be playing a more causative role in anaphylaxis. Indeed, specific snoRNAs in the anaphylaxis-associated network (e.g. SNORD32A, SNORD33, SNORD35A) have been directly implicated in the stress response, contributing to lipotoxic and oxidative stresses [238-240]. Upregulation of the snoRNA network in anaphylaxis could contribute to the physiological symptoms by enhancing oxidative damage. Detailed future investigation is required to understand the regulation, specific functions, and implications of snoRNAs in anaphylaxis. This observed upregulation of an extensive network of snoRNAs highlights an intriguing and previously unknown role for these noncoding RNAs in allergy.

This study has identified NK and T helper cell activation, and upregulation of an array of snoRNAs during anaphylaxis, more so than in other inflammatory conditions.
These findings support our hypothesis of a leukocyte-derived cytokine activation cascade underpinning the rapid amplification of immune responses following allergen exposure. Future studies investigating the roles of these cells and snoRNAs might identify new targets to improve therapies or pinpoint differences that drive susceptibility to anaphylaxis.

Limitations

This study has limitations that should be acknowledged. The genomic profiling studies were performed on whole blood, thus the cellular source(s) of the gene expression signals is unclear. The timing between symptom onset and ED presentation was highly variable. However, we investigated the impact of this and found that the timing had no significant effect on the gene expression patterns of patients. In our population, beta-lactam antibiotics triggered an unusually high proportion of severe reactions and moderate reactions were induced only by foods. We have previously demonstrated that medications are more likely to cause severe anaphylaxis [241], however the representation in this cohort was unusual. Despite these limitations, the findings from our study shed a unique level of insight into the pathogenesis of anaphylaxis, and point towards novel avenues for future research in this area.

Acknowledgements

We would like to acknowledge the research nurses and clinicians for identifying cases and enrolling patients. The authors also appreciate the generous support from the Laboratory for Cancer Medicine at the Harry Perkins Institute of Medical Research for access to data analysis software. This work was supported by grants from the US Food Allergy and Anaphylaxis Network (FAAN) and the Royal Perth Hospital Medical
Research Foundation. AB is supported by a BrightSpark Foundation McCusker Fellowship. SGAB is supported by a NHMRC Career Development Fellowship Award ID1023265. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.
### Tables

#### Table 4.1: Anaphylaxis reaction features.

<table>
<thead>
<tr>
<th>Age/Sex</th>
<th>Time (mins)*</th>
<th>Treatment</th>
<th>Trigger</th>
<th>Clinical features</th>
<th>MCT peak (delta) (ng/mL)</th>
<th>Histamine (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moderate anaphylaxis (n = 5)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66F</td>
<td>55</td>
<td>Epinephrine Steroid Inhaled bronchodilator</td>
<td>Food (shellfish)</td>
<td>Erythema, periorbital oedema, dyspnoea, wheeze</td>
<td>21.7 (6.4)</td>
<td>7.28</td>
</tr>
<tr>
<td>33M</td>
<td>45</td>
<td>Epinephrine IV fluid Steroid Antihistamine</td>
<td>Food (pine nuts)</td>
<td>Urticaria, vomiting, chest/throat tightness</td>
<td>7.0 (1.5)</td>
<td>N/A</td>
</tr>
<tr>
<td>30F</td>
<td>88</td>
<td>Epinephrine Steroid</td>
<td>Food (nuts)</td>
<td>Angioedema, dyspnoea, chest/throat tightness</td>
<td>4.3 (0.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>34F</td>
<td>55</td>
<td>Epinephrine IV fluid Steroid</td>
<td>Food (peanut)</td>
<td>Angioedema, wheeze, nausea, chest/throat tightness, abdominal/pelvic pain</td>
<td>5.2 (1.2)</td>
<td>0.30</td>
</tr>
<tr>
<td>29M</td>
<td>100</td>
<td>Epinephrine Steroid</td>
<td>Food (seafood)</td>
<td>Erythema, periorbital oedema, angioedema, dyspnoea, chest/throat tightness, stridor, wheeze, nausea</td>
<td>24.8 (20.0)</td>
<td>3.32</td>
</tr>
<tr>
<td><strong>Severe anaphylaxis (n = 10)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38F</td>
<td>142</td>
<td>Epinephrine IV fluid Antibiotic (cefoxime)</td>
<td>Urticaria, wheeze, hypotension</td>
<td></td>
<td>68.8 (24.0)</td>
<td>61.27</td>
</tr>
<tr>
<td>31F</td>
<td>68</td>
<td>Epinephrine IV fluid Potassium Antibiotic (cephalexin)</td>
<td>Erythema, periorbital oedema, chest/throat tightness, diaphoresis, dyspnoea, wheeze, nausea, hypotension</td>
<td></td>
<td>11.6 (7.0)</td>
<td>N/A</td>
</tr>
<tr>
<td>Age</td>
<td>Illness Duration</td>
<td>Treatment 1</td>
<td>Treatment 2</td>
<td>Symptoms</td>
<td>MCT 1 (SD)</td>
<td>MCT 2 (SD)</td>
</tr>
<tr>
<td>-----</td>
<td>------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>43F</td>
<td>115</td>
<td>Epinephrine</td>
<td>NSAID (aspirin)</td>
<td>Erythema, urticaria, angioedema, chest/throat tightness, hypotension</td>
<td>15.6 (10.3)</td>
<td>3.45</td>
</tr>
<tr>
<td>45M</td>
<td>65</td>
<td>Epinephrine</td>
<td>Antibiotic (amoxicillin)</td>
<td>Erythema, nausea, abdominal/pelvic pain, hypotension</td>
<td>14.1 (0.4)</td>
<td>1.28</td>
</tr>
<tr>
<td>69F</td>
<td>18</td>
<td>Epinephrine</td>
<td>Food (uncertain)</td>
<td>Erythema, angioedema, dyspnoea, chest/throat tightness, cyanosis, hypoxemia</td>
<td>7.7 (0.7)</td>
<td>1.17</td>
</tr>
<tr>
<td>35F</td>
<td>60</td>
<td>Epinephrine</td>
<td>Food (fruit)</td>
<td>Erythema, stridor, wheeze, collapse</td>
<td>1.2 (0.3)</td>
<td>0.06</td>
</tr>
<tr>
<td>58M</td>
<td>60</td>
<td>Epinephrine</td>
<td>Antibiotic (cephalexin)</td>
<td>Erythema, urticaria, periorbital oedema, dyspnoea, chest/throat tightness, hypotension, confusion</td>
<td>22.6 (15.2)</td>
<td>58.0</td>
</tr>
<tr>
<td>29F</td>
<td>15</td>
<td>Epinephrine</td>
<td>Antibiotic (amoxicillin)</td>
<td>Erythema, urticaria, periorbital oedema, dizziness, hypotension</td>
<td>15.1 (3.9)</td>
<td>9.81</td>
</tr>
<tr>
<td>49F</td>
<td>30</td>
<td>Epinephrine</td>
<td>NSAID (diclofenac)</td>
<td>Erythema, diaphoresis, hypotension, collapse, dyspnoea, stridor, nausea, vomiting, abdominal/pelvic pain, cyanosis, hypoxemia</td>
<td>24.8 (20.0)</td>
<td>3.32</td>
</tr>
<tr>
<td>35M</td>
<td>97</td>
<td>Epinephrine</td>
<td>Antibiotic (amoxicillin)</td>
<td>Erythema, urticaria, angioedema, dyspnoea, nausea, dizziness, diaphoresis, chest/throat tightness, cyanosis, hypotension, confusion, collapse</td>
<td>19.7 (16.6)</td>
<td>44.1</td>
</tr>
</tbody>
</table>

IV, intravenous; MCT, mast cell tryptase; NA, not available; NSAID, non-steroidal anti-inflammatory drug; LOC, loss of consciousness.

*Time from illness onset to first blood sample collected in the ED
Table 4.2: Pathways analyses of genes that were differentially regulated in anaphylaxis at ED arrival compared to healthy controls.

<table>
<thead>
<tr>
<th>Pathway name</th>
<th>FDR p-value</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Killer Cell Signalling</td>
<td>4.37E-08</td>
<td>0.20</td>
</tr>
<tr>
<td>Crosstalk between Dendritic Cells and Natural Killer Cells</td>
<td>1.32E-04</td>
<td>0.18</td>
</tr>
<tr>
<td>Macropinocytosis Signalling</td>
<td>1.35E-04</td>
<td>0.19</td>
</tr>
<tr>
<td>Th1 Pathway</td>
<td>2.63E-03</td>
<td>0.13</td>
</tr>
<tr>
<td>Th1 and Th2 Activation Pathway</td>
<td>4.68E-03</td>
<td>0.11</td>
</tr>
<tr>
<td>3-phosphoinositide Biosynthesis</td>
<td>2.19E-02</td>
<td>0.10</td>
</tr>
<tr>
<td>Tec Kinase Signalling</td>
<td>3.55E-02</td>
<td>0.10</td>
</tr>
<tr>
<td>Superpathway of Inositol Phosphate Compounds</td>
<td>4.90E-02</td>
<td>0.09</td>
</tr>
<tr>
<td>Th2 Pathway</td>
<td>4.90E-02</td>
<td>0.10</td>
</tr>
<tr>
<td>Role of Tissue Factor in Cancer</td>
<td>4.90E-02</td>
<td>0.11</td>
</tr>
<tr>
<td>Integrin Signalling</td>
<td>4.90E-02</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table 4.3: Candidate molecular drivers of anaphylaxis genomic responses at ED arrival.

<table>
<thead>
<tr>
<th>Upstream regulator</th>
<th>Predicted activation state</th>
<th>Activation z-score</th>
<th>p-value of overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDCD1</td>
<td>Inhibited</td>
<td>-2.31</td>
<td>1.04E-08</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Inhibited</td>
<td>-2.20</td>
<td>1.19E-07</td>
</tr>
<tr>
<td>IL2</td>
<td>Activated</td>
<td>2.45</td>
<td>5.34E-06</td>
</tr>
<tr>
<td>IL21</td>
<td>Activated</td>
<td>2.34</td>
<td>5.63E-06</td>
</tr>
<tr>
<td>IL15</td>
<td>Activated</td>
<td>2.23</td>
<td>2.70E-05</td>
</tr>
<tr>
<td>CD3</td>
<td>Inhibited</td>
<td>-2.56</td>
<td>5.61E-05</td>
</tr>
<tr>
<td>Interferon alpha</td>
<td>Activated</td>
<td>2.36</td>
<td>1.05E-03</td>
</tr>
<tr>
<td>PDLIM2</td>
<td>Activated</td>
<td>2.53</td>
<td>3.44E-03</td>
</tr>
<tr>
<td>IFNL1</td>
<td>Activated</td>
<td>2.45</td>
<td>3.56E-02</td>
</tr>
<tr>
<td>TLR9</td>
<td>Activated</td>
<td>2.22</td>
<td>4.93E-02</td>
</tr>
</tbody>
</table>
Table 4.4: Pathways analyses of genes that were differentially regulated in anaphylaxis compared to sepsis and head trauma at ED arrival.

<table>
<thead>
<tr>
<th>Pathway Name</th>
<th>FDR p-value</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>iCOS-iCOSL Signalling in T Helper Cells</td>
<td>3.98E-12</td>
<td>0.15</td>
</tr>
<tr>
<td>T Cell Receptor Signalling</td>
<td>7.94E-12</td>
<td>0.15</td>
</tr>
<tr>
<td>Calcium-induced T Lymphocyte Apoptosis</td>
<td>2.04E-10</td>
<td>0.20</td>
</tr>
<tr>
<td>CD28 Signalling in T Helper Cells</td>
<td>6.61E-10</td>
<td>0.12</td>
</tr>
<tr>
<td>Th2 Pathway</td>
<td>4.57E-09</td>
<td>0.10</td>
</tr>
<tr>
<td>PKC(\alpha), Signalling in T Lymphocytes</td>
<td>6.46E-09</td>
<td>0.11</td>
</tr>
<tr>
<td>Th1 and Th2 Activation Pathway</td>
<td>6.61E-09</td>
<td>0.09</td>
</tr>
<tr>
<td>Th1 Pathway</td>
<td>7.59E-08</td>
<td>0.10</td>
</tr>
<tr>
<td>CTLA4 Signalling in Cytotoxic T Lymphocytes</td>
<td>3.63E-07</td>
<td>0.11</td>
</tr>
<tr>
<td>Role of NFAT in Regulation of the Immune Response</td>
<td>3.80E-07</td>
<td>0.08</td>
</tr>
<tr>
<td>Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes</td>
<td>3.80E-07</td>
<td>0.13</td>
</tr>
<tr>
<td>Phospholipase C Signalling</td>
<td>1.07E-06</td>
<td>0.06</td>
</tr>
<tr>
<td>Natural Killer Cell Signalling</td>
<td>2.09E-06</td>
<td>0.09</td>
</tr>
<tr>
<td>Primary Immunodeficiency Signalling</td>
<td>1.05E-05</td>
<td>0.16</td>
</tr>
<tr>
<td>NF-\kappa B Signalling</td>
<td>1.29E-05</td>
<td>0.07</td>
</tr>
<tr>
<td>Nur77 Signalling in T Lymphocytes</td>
<td>3.39E-05</td>
<td>0.13</td>
</tr>
<tr>
<td>CCR5 Signalling in Macrophages</td>
<td>1.58E-04</td>
<td>0.10</td>
</tr>
<tr>
<td>Systemic Lupus Erythematosus Signalling</td>
<td>5.01E-04</td>
<td>0.05</td>
</tr>
<tr>
<td>Haematopoiesis from Pluripotent Stem Cells</td>
<td>1.55E-03</td>
<td>0.12</td>
</tr>
<tr>
<td>T Helper Cell Differentiation</td>
<td>1.74E-03</td>
<td>0.09</td>
</tr>
<tr>
<td>Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis</td>
<td>1.86E-03</td>
<td>0.04</td>
</tr>
<tr>
<td>Type I Diabetes Mellitus Signalling</td>
<td>2.40E-03</td>
<td>0.07</td>
</tr>
<tr>
<td>Macropinocytosis Signalling</td>
<td>3.63E-03</td>
<td>0.07</td>
</tr>
<tr>
<td>Phagosome Formation</td>
<td>3.63E-03</td>
<td>0.06</td>
</tr>
<tr>
<td>OX40 Signalling Pathway</td>
<td>3.80E-03</td>
<td>0.09</td>
</tr>
<tr>
<td>Glucocorticoid Receptor Signalling</td>
<td>3.80E-03</td>
<td>0.04</td>
</tr>
<tr>
<td>Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells</td>
<td>4.07E-03</td>
<td>0.13</td>
</tr>
<tr>
<td>Tec Kinase Signalling</td>
<td>6.17E-03</td>
<td>0.05</td>
</tr>
<tr>
<td>Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses</td>
<td>6.61E-03</td>
<td>0.05</td>
</tr>
<tr>
<td>TREM1 Signalling</td>
<td>1.07E-02</td>
<td>0.07</td>
</tr>
<tr>
<td>Protein Citrullination</td>
<td>1.20E-02</td>
<td>0.40</td>
</tr>
<tr>
<td>HGF Signalling</td>
<td>1.58E-02</td>
<td>0.05</td>
</tr>
<tr>
<td>Leukocyte Extravasation Signalling</td>
<td>1.82E-02</td>
<td>0.04</td>
</tr>
<tr>
<td>Communication between Innate and Adaptive Immune Cells</td>
<td>1.82E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>fMLP Signalling in Neutrophils</td>
<td>1.82E-02</td>
<td>0.05</td>
</tr>
<tr>
<td>Pathway</td>
<td>p-value</td>
<td>q-value</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>IL-3 Signalling</td>
<td>1.82E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>Prolactin Signalling</td>
<td>1.82E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>Altered T Cell and B Cell Signalling in Rheumatoid Arthritis</td>
<td>1.82E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>Granulocyte Adhesion and Diapedesis</td>
<td>1.91E-02</td>
<td>0.04</td>
</tr>
<tr>
<td>Molecular Mechanisms of Cancer</td>
<td>2.00E-02</td>
<td>0.03</td>
</tr>
<tr>
<td>LPS-stimulated MAPK Signalling</td>
<td>2.00E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>HER-2 Signalling in Breast Cancer</td>
<td>2.09E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>Crosstalk between Dendritic Cells and Natural Killer Cells</td>
<td>2.09E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>Cdc42 Signalling</td>
<td>2.09E-02</td>
<td>0.05</td>
</tr>
<tr>
<td>cAMP-mediated Signalling</td>
<td>2.09E-02</td>
<td>0.04</td>
</tr>
<tr>
<td>G-Protein Coupled Receptor Signalling</td>
<td>2.09E-02</td>
<td>0.03</td>
</tr>
<tr>
<td>MSP-RON Signalling Pathway</td>
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<td>0.07</td>
</tr>
<tr>
<td>Dendritic Cell Maturation</td>
<td>2.63E-02</td>
<td>0.04</td>
</tr>
<tr>
<td>ErbB Signalling</td>
<td>2.75E-02</td>
<td>0.05</td>
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<tr>
<td>IL-12 Signalling and Production in Macrophages</td>
<td>3.24E-02</td>
<td>0.04</td>
</tr>
<tr>
<td>Virus Entry via Endocytic Pathways</td>
<td>3.31E-02</td>
<td>0.05</td>
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<td>Thrombopoietin Signalling</td>
<td>3.39E-02</td>
<td>0.06</td>
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<td>Eicosanoid Signalling</td>
<td>3.39E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>Melatonin Signalling</td>
<td>4.07E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>Glioma Signalling</td>
<td>4.07E-02</td>
<td>0.05</td>
</tr>
<tr>
<td>ErbB4 Signalling</td>
<td>4.17E-02</td>
<td>0.06</td>
</tr>
<tr>
<td>Hepatic Cholestasis</td>
<td>4.37E-02</td>
<td>0.04</td>
</tr>
<tr>
<td>Glycogen Degradation III</td>
<td>4.68E-02</td>
<td>0.15</td>
</tr>
<tr>
<td>Integrin Signalling</td>
<td>4.79E-02</td>
<td>0.03</td>
</tr>
<tr>
<td>p38 MAPK Signalling</td>
<td>4.90E-02</td>
<td>0.04</td>
</tr>
<tr>
<td>Age/Sex</td>
<td>Diagnosis</td>
<td>Organism*</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td></td>
<td><strong>Infection with SIRS/uncomplicated sepsis (n = 10)</strong></td>
<td></td>
</tr>
<tr>
<td>78M</td>
<td>Hepatic abscess</td>
<td></td>
</tr>
<tr>
<td>83M</td>
<td>Wound infection</td>
<td><em>S. Pneumonia</em></td>
</tr>
<tr>
<td>57M</td>
<td>Cholangitis</td>
<td></td>
</tr>
<tr>
<td>85F</td>
<td>Cellulitis</td>
<td><em>P. Aeruginosa</em></td>
</tr>
<tr>
<td>72F</td>
<td>Pelvic abscess</td>
<td></td>
</tr>
<tr>
<td>22F</td>
<td>Skin abscess</td>
<td><em>S. Aureus</em></td>
</tr>
<tr>
<td>57M</td>
<td>Urosepsis</td>
<td><em>E. Coli</em></td>
</tr>
<tr>
<td>52F</td>
<td>Pneumonia</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Severe sepsis/septic shock (n = 10)</strong></td>
<td></td>
</tr>
<tr>
<td>66F</td>
<td>Cellulitis</td>
<td></td>
</tr>
<tr>
<td>59M</td>
<td>Pneumonia</td>
<td><em>S. Pneumonia</em></td>
</tr>
<tr>
<td>75M</td>
<td>Urosepsis</td>
<td><em>K. Pneumoniae</em></td>
</tr>
<tr>
<td>74F</td>
<td>Septic shock presumed from radiation colitis</td>
<td><em>S. Pyogenes</em></td>
</tr>
<tr>
<td>Age</td>
<td>Diagnosis</td>
<td>Organism</td>
</tr>
<tr>
<td>-----</td>
<td>------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>47M</td>
<td>S aureus sepsis</td>
<td><em>S. Aureus</em></td>
</tr>
<tr>
<td>66F</td>
<td>Urosepsis</td>
<td><em>E. Coli</em></td>
</tr>
<tr>
<td>80M</td>
<td>Gram negative sepsis</td>
<td><em>Capnocytophaga</em></td>
</tr>
<tr>
<td>69F</td>
<td>Leg ulcer</td>
<td><em>S. Aureus</em></td>
</tr>
<tr>
<td>71F</td>
<td>Meningococcal sepsis</td>
<td><em>N. Meningitis</em></td>
</tr>
<tr>
<td>56M</td>
<td>Necrotizing fasciitis</td>
<td></td>
</tr>
</tbody>
</table>

*Organism isolated from sample collected within first 24h of admission.

#Sequential Organ Failure Assessment (SOFA) score peak in first 72 hours.
Table S4.2: Clinical characteristics of head trauma patients.

<table>
<thead>
<tr>
<th>Age/Sex</th>
<th>Cause</th>
<th>Time (mins)*</th>
<th>GCS</th>
<th>INR</th>
<th>NISS</th>
<th>NISS (% Head)</th>
<th>Died</th>
</tr>
</thead>
<tbody>
<tr>
<td>80F</td>
<td>Fall</td>
<td>239</td>
<td>3T</td>
<td>1.2</td>
<td>35</td>
<td>97</td>
<td>Yes</td>
</tr>
<tr>
<td>41M</td>
<td>MVA</td>
<td>195</td>
<td>3T</td>
<td>1.3</td>
<td>75</td>
<td>100</td>
<td>Yes</td>
</tr>
<tr>
<td>62F</td>
<td>Assault</td>
<td>208</td>
<td>4T</td>
<td>3.3</td>
<td>57</td>
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</tr>
<tr>
<td>30M</td>
<td>MBA</td>
<td>65</td>
<td>3</td>
<td>1.0</td>
<td>66</td>
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</tr>
<tr>
<td>17M</td>
<td>MVA</td>
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<td>3</td>
<td>1.4</td>
<td>66</td>
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</tr>
<tr>
<td>20M</td>
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<td>3</td>
<td>1.6</td>
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<tr>
<td>23M</td>
<td>MVA</td>
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<td>3T</td>
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<tr>
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<td>29</td>
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</tr>
<tr>
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<td>3T</td>
<td>1.1</td>
<td>22</td>
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<tr>
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<td>160</td>
<td>3T</td>
<td>1.0</td>
<td>17</td>
<td>76</td>
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</tr>
</tbody>
</table>

MVA, motor vehicle accident; MBA, motorbike accident; NISS, New Injury Severity Score; GCS, Glasgow Coma Score; INR, International Normalised Ratio.

* Time – time from injury to blood sample collection in the emergency department.
Chapter 4

4.8 Figures
Figure 4.1: Differential gene expression patterns in anaphylaxis at ED arrival.
Peripheral blood samples were collected from anaphylaxis patients (moderate anaphylaxis: n = 5; severe anaphylaxis: n = 10) upon arrival at the emergency department (ED) and from healthy controls (n = 10). RNA was extracted and gene expression patterns were profiled on Affymetrix® Human Gene 2.1 ST microarrays. Statistical analyses were performed to identify gene expression patterns that were differentially expressed between healthy controls and anaphylaxis patients (LIMMA). The heatmap figure was produced using Morpheus software (Broad Institute). Data for samples are in columns and genes are in rows with the phylogenetic tree indicating their relatedness. The colour scale indicates the relative expression of the gene in each sample from low expression (-3, dark blue) to high expression (+3, dark red).
Figure 4.2: Gene expression levels of the top 12 most significantly altered genes in anaphylaxis patients at ED arrival.

Gene expression levels from peripheral blood mononuclear cell samples collected at ED presentation were profiled on Affymetrix® Human Gene 2.1 ST microarrays and differences between anaphylaxis patients and controls were investigated using LIMMA tests. The data presents the normalised expression of the 12 most significantly differentially expressed genes in each of the healthy controls (CTRL: n = 10), moderate anaphylaxis (MOD: n = 5) and severe anaphylaxis patients (SEV: n = 10).
Following causal network analysis (Ingenuity Pathway Analysis) of differentially regulated genes in anaphylaxis patients, the above network (score 36) included 29 focus molecules. Primary functions included cell-to-cell signalling and interaction, haematological system development and function, and immune cell trafficking. Targets coloured red are upregulated and blue targets are downregulated in anaphylaxis compared to controls. Darker colours indicate higher absolute Z-scores. Grey targets are proposed to be involved but were not differentially regulated in the dataset. Solid lines represent direct interactions and dashed lines represent indirect interactions, with arrowheads representing activating interactions and blunted lines representing inhibition.
Figure 4.4: Differential gene expression between patients with anaphylaxis and sepsis and head trauma patients.

Peripheral blood samples were collected from moderate (n = 5) and severe (n = 10) anaphylaxis, uncomplicated (n = 10) and severe (n = 10) sepsis, and head trauma (n = 10) at ED arrival. RNA was extracted from PBLs and gene expression patterns were
profiled on Affymetrix® Human Gene 2.1 ST microarrays. Statistical analyses were performed to identify gene expression patterns that were differentially expressed between anaphylaxis patients and those with sepsis or head trauma (LIMMA 2-way comparison). The heatmap figure was produced using Morpheus software (Broad Institute). Data for samples are in columns and genes are in rows with the phylogenetic tree indicating their relatedness. The colour scale indicates the relative expression of the gene in each sample from low expression (-3, dark blue) to high expression (+3, dark red).
Sub-networks were identified using network analysis (GeneMANIA, Cytoscape) of differentially regulated genes in anaphylaxis patients compared to sepsis and head trauma patients (LIMMA 2-way comparison). Targets indicated in dark grey were differentially regulated in the dataset, whilst those in white were predicted to be involved although not significantly altered in our dataset. The primary functions of these sub-networks are: A) T cell activation (FDR $p = 1.0 \times 10^{-11}$) and B) antigen receptor-mediated signalling pathway (FDR $p = 1.8 \times 10^{-5}$).
Figure 4.6A: Biological pathways enriched in the gene coexpression modules.

WGCNA was employed to identify gene coexpression networks in peripheral blood underlying anaphylaxis, and the modules were analysed for enrichment of biological pathways using the R package ClusterProfiler.
Figure 4.6B: A module enriched with snoRNAs (red) was upregulated with in anaphylaxis but not sepsis or head trauma at ED arrival.

WGCNA was employed to identify gene coexpression networks in peripheral blood underlying anaphylaxis. A) Genes associated with anaphylaxis were identified by LIMMA, and the negative log\(_{10}\) transformed p-values were plotted as boxplots on a module-by-module basis. Correlation Adjusted MEan RAnk gene set analysis (CAMERA) demonstrated that the red module was significantly upregulated in anaphylaxis at ED arrival (FDR p = 0.007) and the yellow module was significantly downregulated (FDR p = 0.009). B) Uncomplicated sepsis, C) severe sepsis, and D) head trauma data was analysed similarly for comparison. There were no significantly altered modules in either of the sepsis severity groups. The blue module was significantly upregulated in head trauma (FDR p = 0.001).
Figure S4.1: Correction for batch effects.

ComBat analysis showing that batch effects have been appropriately corrected for between anaphylaxis, sepsis, and head trauma microarrays. This is indicated by the alignment of the density plots and the linearity of the Q-Q plot in the corrected data (top) compared with the uncorrected data (bottom).
Neutrophil activation during acute human anaphylaxis: analysis of MPO and sCD62L

5.1 Background

The results of chapters three and four highlighted the importance of the innate immune system in the acute phase of anaphylaxis and, in particular, have suggested a role for neutrophils. The upregulation of several proteins expressed by neutrophils, although not exclusively, including S100A9, MMP9, and TREM1 strongly suggests neutrophil activation occurs during the acute phase of anaphylaxis. The following chapter specifically investigates neutrophils, for the first time, during acute human anaphylaxis. Activation markers were measured in samples from patients during acute reaction progression, treatment, and resolution. The experimental work for this chapter has been published in the Journal of Clinical and Experimental Allergy. The article is presented as published following a brief introduction about the development, structure, and physiological functions of neutrophils and their role in pathology.

5.1.1 Neutrophils

Neutrophils are polymorphonuclear granulocytes, named due to their lobular nuclei and extensive number of cytoplasmic granules. The process of neutrophil development begins in the bone marrow where, under the influence of growth factors and cytokines, pluripotent hematopoietic cells differentiate into myeloblasts [242]. These are a developmental cell type committed to becoming granulocytes, primarily neutrophils in addition to eosinophils, basophils, and monocytes [242]. Neutrophils are terminally differentiated cells, 9 – 15 µm in diameter, that have a short lifespan.
(typically less than 2 days) and rapid turnover (~10^{11} neutrophils are produced and removed each day) [243]. They are an important component of the innate immune response, acting as a first line of defence against invading bacteria and other microorganisms [244, 245]. Neutrophils are vital for adequate immune function and are the predominant infiltrating cell type present in the cellular phase of the acute inflammatory response [242, 244].

5.1.1.1 Structure

Neutrophils, either constitutively or inducibly, express many different classes of surface receptors. This allows them to respond to innate and adaptive immune signals as well as the inflammatory environment itself. They express several types of innate immune receptors including all known toll-like receptors (TLRs) excluding TLR3, and can therefore directly recognize pathogens and damaged tissue [246-249]. Receptors that facilitate cell-to-cell signalling, such as receptors for leukotrienes, and chemokines are also expressed [250-252]. Neutrophils may also respond directly to allergic signals due to their expression of IgE and IgG receptors (FcεRI, FcγRI, and FcγRIIB) [98, 253, 254]. Adhesion molecule receptors, selectins and integrins, mediate interactions between neutrophils and blood vessel walls to initiate transendothelial migration [255, 256]. Finally, the presence of cytokine receptors (e.g. receptors for IL-6, IL-10, granulocyte-colony stimulating factor (G-CSF), and IFNγ) allows neutrophils to respond to signals from other leukocytes [68, 71, 257-259].

As myeloblasts mature into neutrophils, they synthesize proteins that are sorted into, and contained within, different granules [260]. These granules are formed through a continuous process whereby vesicles bud from the Golgi apparatus and fuse,
producing granular structures [261, 262]. As a neutrophil matures, it sequentially alters its transcriptional profile causing the granule contents to change, thereby producing different granule classes with overlapping mediator contents [245, 261]. There are three such granule classes: (1) azurophilic, or primary, granules; (2) specific, or secondary granules; and (3) gelatinase, or tertiary, granules. Azurophilic granules are the largest and are formed first during the neutrophil maturation process. They contain large amounts of myeloperoxidase (MPO), in addition to neutrophil elastase (NE), defensins, lysozyme, proteinase 3, cathepsin G, and an array of other antimicrobial agents [261]. Specific granules are formed next and are smaller than azurophilic granules. They do not contain MPO, characterized instead by large amounts of lactoferrin, which also performs antibacterial functions [261, 263]. These also contain other antibacterial compounds including neutrophil gelatinase-associated lipocalin (NGAL), some matrix metalloproteases (MMPs), and lysozyme [262]. Gelatinase granules are formed last and are the smallest [260]. They primarily store a number of MMPs, such as MMP9 (gelatinase) and MMP2 (leukolysin) [261]. Finally, in addition to granules, neutrophils also contain highly mobilisable secretory vesicles that serve primarily as a reservoir for plasma membrane receptors. These include receptors for lipopolysaccharide (LPS), complement factors, urokinase-type plasminogen activator, immune complexes, and chemo-attractants [245, 263].

5.1.1.2 Recruitment

Most neutrophil products have the potential to cause non-specific damage to healthy tissue, for example through oxidative damage. Therefore, their recruitment and activation must be tightly controlled. The neutrophil recruitment cascade is mediated by the sequential interaction of neutrophil surface receptors with ligands induced on the
surface of the activated (inflamed) endothelium [264]. The classical multistep adhesion cascade proceeds as follows: (1) initial attachment of the neutrophil to the endothelium, referred to as capture; (2) rolling of the neutrophil along the endothelium; (3) firm adhesion of the neutrophil to the endothelium with accompanying neutrophil cell spreading; and (4) transmigration of the neutrophil into the tissue, where activation leads to phagocytosis, degranulation and the formation of neutrophil extracellular traps (NETs) (Figure 5.1) [245].

Inflammation stimulates and activates endothelial cells, which is detected by neutrophils in the circulation that continually probe blood vessel walls [245]. Neutrophils then tether to the activated vessel walls, mediated by the interaction between CD62L (L-selectin) and P-selectin glycoprotein ligand-1 (PSGL-1) binding to selectins present on the activated endothelial cells [265, 266]. Firm adhesion is achieved by four mechanisms: (1) flattening out over the endothelium to interact with more adhesive proteins; (2) greater use of selectins that form bonds which strengthen with increasing force; (3) formation of membrane tethers that slow the neutrophil down; and (4) formation of membrane slings which extend in front of the cell to slow it down further [263]. Upon arrival at an endothelial cell junction, a complex interaction between the neutrophil selectins/integrins and their endothelial partners, results in transmigration through the endothelial junction [267]. Neutrophils then navigate the basement membrane, a protein mesh consisting largely of laminins and collagen type IV, after which they follow chemotactic gradients to reach the site of inflammation [242, 268]. Activated neutrophils also generate chemotactic signals to recruit additional immune cells to the site of injury [269].
Blood flow

Capture → Rolling → Firm adhesion → Transmigration

Activated endothelium

Legend
- Granules and secretory vesicles
- PSGL-1
- CD62L
- Selectins (E- / P-)
- Integrins
- ICAM-1
- Granule contents (e.g. MPO, NE, lactoferrin, MMPs, cytokines)
- Bacteria
- DNA
- Histones
Figure 5.1: Recruitment and functions of neutrophils.

Neutrophils are directed towards the site of inflammation through interactions between E-selectins/P-selectins on the activated endothelium and selectin ligands (PSGL-1 and CD62L) on the neutrophil surface, termed capture. The force of the blood flow causes the neutrophil to roll along the endothelial surface, interacting with additional selectins, which slows it down until firm adhesion is achieved. This is facilitated through binding of neutrophil integrins to endothelial intercellular adhesion molecule (ICAM)-1. The neutrophil then transmigrates into the tissue and follows chemotactic gradients to reach the site of inflammation where it becomes activated. Activated neutrophils can contribute to pathogen killing through degranulation, phagocytosis, and the NET formation (NETosis).
5.1.1.3 Activation

Neutrophils are partially activated during their transit through the endothelium, with complete activation occurring at the site of inflammation, in response to pro-inflammatory stimuli [245, 263]. A large array of stimuli can contribute to neutrophil activation including bacterial components (e.g. LPS), cytokines (e.g. TNFα), chemokines (e.g. IL-8), and growth factors (e.g. GM-CSF) [247, 248, 270-272]. Neutrophils are typically nonresponsive to a single stimulus, but initial exposure to one stimulus can enhance the response to subsequent stimuli. This typically occurs via increased expression of surface receptors following their mobilisation from granules [273-277]. For example, exposure to LPS induces assembly of the NADPH oxidase machinery on the plasma membrane, and subsequent N-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulation then activates this machinery [278]. This effect, referred to as priming, allows for greater control over activation and more rapid responses to future stimuli [275, 279, 280]. In contrast to receptor priming, another critical feature of the activation process is the desensitization to previously encountered ligands. Stimulation by one chemoattractant often results in endocytosis of the corresponding receptor, thereby leading to desensitization to repeated stimulation with the same molecule [281, 282].

Neutrophils respond to activation with phagocytosis, production of reactive oxygen species (ROS) and NETs, degranulation, and the release of cytokines and chemokines. Phagocytosis is a process also utilized by other cell types (e.g. macrophages and monocytes) to engulf and destroy foreign debris and pathogens [244, 283]. Foreign particles are recognized by surface receptors (e.g. TLRs) and internalized by the cell membrane into a vacuole termed a phagosome [242, 244, 249]. The foreign
matter is then removed via degradation or by stimulating apoptosis [283]. During phagocytosis, neutrophils assemble an electron transport chain called NADPH oxidase. This consumes molecular oxygen from outside of the cell, and generates reactive oxygen species (ROS) such as superoxide (O$_2^−$) and hydrogen peroxide (H$_2$O$_2$), in a process termed the respiratory burst [278, 284, 285]. Neutrophils also contribute to pathogen killing through the release of NETs, which are decondensed chromatin strands enriched with antimicrobial proteins [286-288]. They are formed during NETosis, an active form of cell death, and capture and destroy many types of microbes [245, 289]. A similar process also occurs in other phagocytes, including macrophages, monocytes, and mast cells, under certain stimuli [290, 291]. Once activated, neutrophils rapidly degranulate causing granule contents to be released into the surrounding tissue [261]. These molecules perform antimicrobial and antifungal functions and act as signals to stimulate recruitment of additional neutrophils and immune cells [242, 245]. Unfortunately, they also contribute to oxidative tissue damage, degradation of the extracellular matrix, and subsequent pro-inflammatory mediator release [108, 292-298]. Cytokine and chemokine production by neutrophils is largely influenced by the stimulatory conditions, allowing neutrophils to target their response [299, 300].

Following activation, neutrophils are killed via apoptosis, which not only reduces the overall number, but also reduces further neutrophil recruitment [245, 301]. As neutrophils undergo apoptosis, they produce signals that attract phagocytes and signify their need for removal via engulfment [301]. Neutrophils can influence whether macrophages differentiate to a predominantly pro- or anti-inflammatory state, and phagocytosis of apoptotic neutrophils encourages an anti-inflammatory macrophage
phenotype [302]. This drives the production of anti-inflammatory cytokines such as TGFβ and IL-10, thereby further resolving inflammation [301].

5.1.1.4 Homeostasis

Neutrophils are the most abundant leukocyte, making up ~70% of all peripheral blood leukocytes. However, their overall numbers and activation status are tightly regulated due to their potential for non-specific tissue damage [242, 245]. Neutrophils in the blood are partitioned between a circulating pool and a marginating pool. The circulating pool is present in large blood vessels and the central stream of small vessels. The marginating pool is transiently held in narrow capillaries in the absence of inflammation [303]. A store of neutrophils is also withheld in bone marrow following maturation [242]. The main factors controlling the total neutrophil population in the blood are their: (1) rate of production; (2) storage in and exit from the bone marrow; and (3) survival in and clearance from the blood [263]. Neutrophil numbers appear to be partly controlled simply by the amount of available space in the bone marrow, referred to as density-dependent neutrophil-mass sensing [304]. Feedback loops are in place to regulate the total neutrophil population, including the IL-23–IL-17–G-CSF feedback loop [263]. Chemokines control the passage of neutrophils into circulation and maintain the bone marrow pool ready for release in case of infection [242]. Indeed, the number of circulating neutrophils rapidly increases during infection and some diseases, due to release from bone marrow stores [242]. Haematopoietic progenitors can increase the rate of neutrophil production in response to an inflammatory or pathogenic challenge. They proliferate upon sensing pathogenic molecules through a host of innate immune receptors such as the TLRs and nucleotide oligomerisation domain (NOD)-like receptors [305]. The combination of neutrophil consumption through fighting pathogens
and exposure of progenitors to pathogens could lead to a synergistic increase in neutrophil generation during such emergency situations [263, 306]. Ultimately, neutrophil homeostasis is likely to be influenced by all of these processes in a complex, interrelated network.

5.1.1.5 Dysfunction and Implication in Disease

In addition to their vital role in inflammation and defence against pathogens, neutrophils can also support disease progression particularly in autoimmune disorders and certain cancers. As well as direct tissue damage, neutrophil-derived ROS have the potential to initiate tumour formation via genotoxic stress and the induction of genomic instability [307]. Neutrophils are frequently found to have undesirable roles, with clinical studies indicating that neutrophil infiltration into tumours is associated with poorer prognosis [308, 309]. Some cancers (e.g. lung tumours) even appear to actively recruit neutrophils through production of IL-8, a strong chemo attractant for neutrophils [310]. The pro-tumour function of neutrophils operates at multiple levels, including via the production of angiogenic factors, enhancement of metastasis, and suppression of the antitumor immune response [245].

Inappropriate neutrophil numbers, which can be transient or persistent, are also associated with pathology. For example, reduced numbers of circulating neutrophils (neutropenia) is associated with an increased risk and frequency of severe infections [311]. Elevated circulating neutrophil counts (neutrophilia) may be physiological (e.g. transient neutrophilia during acute inflammation) or pathological (e.g. chronic neutrophilic leukaemia, or chronic idiopathic neutrophilia) [244, 312, 313]. Dysregulated neutrophil cell death and/or clearance often accompany autoimmune
syndromes (e.g. systemic lupus erythematosus, rheumatoid arthritis, and vasculitis) and may play a major role in disease pathogenesis [314, 315].

There are also a number of disorders associated with abnormal neutrophil function, rather than number. Neutrophils from patients with chronic granulomatous disease, MPO deficiency, or glutathione synthetase deficiency all have reduced or absent capacity for respiratory burst, thereby diminishing their ability to respond to pathogens. As a result, they present with frequent bacterial and fungal infections, and associated granulomas [311]. Another concern is leukocyte adhesion deficiencies in which neutrophils are unable to adhere and transmigrate through the endothelium, minimising their effectiveness in wound healing and pathogen defence [316]. Disorders affecting chemotaxis, degranulation, and phagocytosis are rare but do also occur [311].

5.1.1.5.1 Role in Anaphylaxis

Neutrophils have been shown to play a significant role in anaphylaxis in mice. These studies showed that neutrophil depletion completely inhibited both active and passive anaphylaxis. However, subsequent administration of mouse or human neutrophils restored anaphylactic potential [101]. This demonstrated that neutrophils have a critical and indispensible role in the development of anaphylaxis following allergen challenge. Other studies using a transgenic mouse model focussed on non-IgE mediated anaphylaxis. They found that the expression of human high-affinity IgG receptor, FcγRI (CD64), on neutrophils is responsible for systemic anaphylaxis via triggering the release of PAF [190]. Taken together, these mouse models identify an importance for neutrophils in both IgE- and IgG-mediated anaphylaxis, suggesting that their role may lie in amplifying, rather than initiating, the reaction.
Using mouse models to study neutrophils, in the context of anaphylaxis or otherwise, is not ideal as there are significant interspecies differences. Firstly, neutrophils are much less abundant in the circulation of mice, making up only 30% of leukocytes compared with approximately 70% in humans [245], suggesting a different level of physiological importance in mice versus humans. There are also differences in both the surface receptor expression and granular contents between mouse and human neutrophils [98]. It is unclear whether neutrophils perform the same functions via the same processes in mouse and human physiology/pathology, and as such the relevance of these studies to the human condition is uncertain.

There has been minimal previous investigation into the involvement of neutrophils during human anaphylaxis. A microarray performed in our laboratory prior to the commencement of this thesis found evidence of neutrophil activation signatures in peripheral blood leukocytes from moderate anaphylaxis patients taken during their reaction [149]. The mRNA and protein work presented in chapter 3 validated these findings, supporting the hypothesis that neutrophil activation occurs during the acute phase of anaphylaxis. Another group has also shown evidence to suggest that neutrophils and IgG may be involved in human anaphylaxis. In patients with a history of anaphylaxis triggered by lipid transfer proteins (LTP), increased levels of specific anti-LTP IgG1 and IgG3 were observed, and expression of genes coding for FcγRI (CD64) was also increased [317]. They also observed evidence of enhanced neutrophil activity, with elevated levels of ROS and reactive nitrogen species [317].
These previous studies provide preliminary support for the hypothesis that neutrophils are involved during anaphylaxis. They provide justification for further investigation into neutrophil activation and function during acute human anaphylaxis. The remainder of this thesis aims to further examine neutrophil activation during anaphylaxis by measuring specific activation markers in serum.
Title: Neutrophil activation during acute human anaphylaxis: analysis of MPO and sCD62L

Short title: Neutrophil activation in human anaphylaxis

Abbie Francis¹, ², Erika Bosio¹, ², Shelley F Stone¹, ², Daniel M Fatovich¹, ², ³, Glenn Arends¹, ², ³, ⁴, Yusuf Nagree¹, ⁴, ⁵, Stephen PJ Macdonald¹, ², ³, ⁶, Hugh Mitenko¹, ⁷, Mani Rajee¹, ⁸, Sally Burrows⁹, and Simon GA Brown¹, ², ³, ¹⁰

1. Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Perth, WA, Australia
2. Discipline of Emergency Medicine, School of Primary, Aboriginal and Rural Health Care, University of Western Australia, Perth, WA, Australia
3. Emergency Department, Royal Perth Hospital, Perth, WA, Australia
4. Emergency Department, Fiona Stanley Hospital, Murdoch, WA, Australia
5. Emergency Department, Fremantle Hospital, Fremantle, WA, Australia
6. Emergency Department, Armadale Kelmscott Memorial Hospital, Mount Nasura, WA, Australia
7. Emergency Department, South West Health Campus, Bunbury, WA, Australia
8. Emergency Department, Austin Hospital, Heidelberg, VIC, Australia
9. School of Medicine & Pharmacology, University of Western Australia, Perth, WA, Australia
10. Emergency Department, Royal Hobart Hospital, Hobart, TAS, Australia

Key words: anaphylaxis, basic mechanisms, clinical immunology, granulocyte, neutrophil, peripheral blood leukocyte
Corresponding author: Abbie Francis, Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Level 6 MRF Building, Rear 50 Murray Street, Perth, Western Australia 6000.

Phone: +61 8 9224 0356 Email: Abbie.Francis@uwa.edu.au

Abbreviations
AH, Austin Hospital; AKMH, Armadale Kelmscott Memorial Hospital; CI, confidence interval; CISS, Critical Illness and Shock Study; ED, emergency department; FH, Fremantle Hospital; IQR, interquartile range; MCT, mast cell tryptase; MLE, Maximum Likelihood Estimation; MPO, myeloperoxidase; NIAID/FAAN, National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network; RPH, Royal Perth Hospital; sCD62L, soluble L-selectin; SWHC, South West Health Campus; TREM-1, triggering receptor expressed on myeloid cells-1.

5.2 Abstract

Background
The mechanisms involved in the amplification of the mast cell response during anaphylaxis are unclear. Mouse models of anaphylaxis demonstrate the critical involvement of neutrophils. These innate immune cells are highly abundant in peripheral blood and can be rapidly activated to trigger both local and systemic inflammation.

Objective
To investigate neutrophil activation in peripheral blood during acute human anaphylaxis.
Chapter 5

Methods

Patients presenting to the Emergency Department with anaphylaxis underwent blood sampling upon enrolment and at up to three subsequent time points. Traditional anaphylaxis biomarkers, histamine and mast cell tryptase, were measured by ELISA and ImmunoCAP respectively. Plasma myeloperoxidase concentrations were measured by ELISA, serum soluble CD62L concentrations by cytometric bead array, and both compared to healthy controls.

Results

In 72 patients, 37 (51%) had severe anaphylaxis, 33 (60%) were histamine positive, and 47 (70%) were mast cell tryptase positive. At enrolment, myeloperoxidase concentrations were 2.9- (95% CI: 1.3, 6.5) and 5.0- (95% CI: 2.4, 10.5) fold higher in moderate and severe patients respectively, compared with healthy controls, and remained stable over the first 5 hours following symptom onset. At enrolment, soluble CD62L was 29% (95% CI: 19, 38) and 31% (95% CI: 22, 40) lower in moderate and severe patients respectively, than healthy controls, and was stable over the first 5 hours. There were no associations between myeloperoxidase or soluble CD62L concentrations and either histamine or mast cell tryptase concentrations.

Conclusions and Clinical Relevance

These results provide compelling evidence for the involvement of neutrophils during acute human anaphylaxis, suggesting they are activated early in the reaction, regardless of mast cell activation. This important finding increases our understanding of the basic
mechanisms of anaphylaxis, a necessary precursor to improving treatment and prevention.

5.3 **Introduction**

Allergies are a growing health concern with an increase in the number of children experiencing adverse food reactions, and adults having reactions to drugs, reported over the last 20 years [150, 151]. Anaphylaxis is a serious and potentially life-threatening allergic reaction that affects multiple organ systems, characterized by hives/rash, bronchospasm, and collapse [1]. Mild reactions involve only the skin and subcutaneous tissue, moderate reactions involve additional organ systems, and the presence of hypotension, hypoxia, or neurological compromise at any point indicates a severe reaction [119].

In humans, IgE-mediated anaphylaxis requires sensitization whereby the initial exposure to an allergen stimulates the production of allergen-specific IgE, which bind to IgE receptors on the surface of effector cells, such as mast cells. On subsequent exposure, the allergen binds to these IgE resulting in cross-linkage, which activates tissue mast cell populations. This results in the local release of an array of pro- and anti-inflammatory mediators, cytokines and chemokines. This response is measured systemically by increases in traditional markers of anaphylaxis, histamine and mast cell tryptase (MCT), which are widely reported to correlate with reaction severity [5, 6, 47, 51, 52, 55, 318]. The half-life of MCT in the bloodstream is approximately 2 hours [141], considerably longer than that of histamine, which reportedly returns to baseline within 60 minutes of reaction onset [52, 141]. Therefore, MCT is the preferred clinical biomarker for anaphylaxis.
The processes that rapidly amplify the initial antigenic stimulation of mast cells into a potentially life-threatening systemic reaction remain unclear, although research by our group and others suggest that neutrophils may play an important role. Neutrophils have been shown to be important in mouse models of anaphylaxis wherein depletion of neutrophils completely inhibited anaphylaxis, whilst replenishment with either mouse or human neutrophils restored anaphylactic potential [101]. In addition, a microarray investigation of human anaphylaxis performed in our laboratory identified the upregulation of major inflammatory pathways including the toll-like receptor and triggering receptor expressed on myeloid cells-1 (TREM-1) pathways, which suggested early involvement of the innate immune system and neutrophil activation [149]. Upon activation, neutrophils release neutrophil elastase, collagenase, and gelatinase which have been implicated in the degradation of the extracellular matrix [319, 320], as well as lactoferrin, which up-regulates neutrophil adhesion molecules and delays apoptosis [321, 322]. Activated neutrophils also produce and/or release cytokines such as IL-1, IL-6, IL-8, IL-12, tumor necrosis factor α, and transforming growth factor β, which may amplify anaphylaxis by activating additional neutrophils and other immune cells [244]. Therefore, it is likely that neutrophil activation may be one mechanism driving the rapid systemic activation of the immune system as seen in anaphylaxis. To date, no studies have examined neutrophil activation in human patients experiencing active symptoms of acute anaphylaxis.

Neutrophil activation is most commonly assessed by the measurement of activation markers in serum and plasma such as myeloperoxidase (MPO) [108], soluble L-selectin (sCD62L) [323], elastase [324], IL-8 [324, 325], and S100A12 [326, 327]. MPO is an enzyme confined within neutrophil azurophilic granules and released upon
their activation [108]. Elevated MPO levels in peripheral blood have been widely reported as a marker of neutrophil activation and degranulation, and the concentration of MPO provides a measure of the degree of activation [108, 328, 329]. CD62L is an adhesion molecule present on the surface of most leukocytes including neutrophils, and is involved in the initial attachment to endothelial walls at the site of inflammation [244, 330]. Upon activation, CD62L proteins are enzymatically cleaved from the neutrophil surface, releasing sCD62L into the circulation [323, 331]. This shedding enables the translocation of the β2 integrin CD11b to the cell surface, required for tight binding to the endothelium [332]. Soluble CD62L levels are elevated in some inflammatory disease states and reduced in others, despite showing similar evidence for immune cell activation. Elevated sCD62L concentrations have been observed in systemic lupus erythematosus [333], acute myeloblastic leukaemia and insulin-dependent diabetes mellitus [334], whilst reduced sCD62L has been associated with poor outcomes in trauma [335-337] and acute lymphoblastic leukaemia [338]. This suggests the sCD62L shedding/binding process is influenced by a number of factors and may be disease specific. Since neither MPO nor sCD62L have been investigated in patients with anaphylaxis or allergy, particularly during an active reaction, this study aimed to measure concentrations of these markers during anaphylaxis; to identify variations from healthy control levels; and investigate associations with reaction severity.

### 5.4 Materials and Methods

#### 5.4.1 Patient recruitment

Study participants and healthy controls were enrolled in our prospective, observational Critical Illness and Shock Study (CISS) between September 2010 and
April 2015 in the emergency departments (EDs) of five Australian hospitals: Royal Perth Hospital (RPH), Perth WA; Fremantle Hospital (FH), Fremantle WA; Armadale Kelmscott Memorial Hospital (AKMH), Mount Nasura WA; South West Health Campus (SWHC), Bunbury WA; and Austin Hospital (AH), Heidelberg VIC. The CISS methodology has been previously described [143]. Briefly, patients enrolled in CISS meet a case definition of critical illness, and then undergo serial blood sampling and clinical data collection at protocolled time points. CISS enrolment criteria exclude mild (skin-only) allergic reactions.

5.4.2 Ethics approval and consent

Ethics approval was obtained from the Human Research Ethics Committees at each hospital (RPH, FH, AKMH: EC 2009/080; SWHC: 2012:31; AH: H2012/04477). As the need for emergency care took priority, waiver of initial consent was approved under the provision of paragraph 2.3.6 of the National Health and Medical Research Council Ethical Conduct guidelines (2007). Once treatment was started, fully informed written consent was obtained as soon as possible and patients were given the option of declining further involvement and having all research samples collected up to that point destroyed.

5.4.3 Case selection

We selected cases that; (i) satisfied a clinical definition of anaphylaxis based on National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network (NIAID/FAAN) criteria [1], and (ii) had blood sampling initiated within 180
minutes of symptom onset. Healthy controls for this study were selected to match the age and sex distributions of the patient cohort.

### 5.4.4 Case classification

In order to investigate changes associated with disease severity, cases were reviewed by three clinical investigators (SB, DF, and GA) and classified into two severity groups, moderate and severe anaphylaxis, based on clinical features according to standard consensus criteria [119]. *Severe anaphylaxis* was defined as the presence of hypoxemia (SpO₂ ≤ 92% or cyanosis), hypotension (systolic blood pressure < 90 mm Hg), and/or neurological compromise (confusion, collapse, or loss of consciousness) at any stage. *Moderate anaphylaxis* was defined as meeting the definition of anaphylaxis and involving multiple organ systems, in the absence of hypoxemia, hypotension, or neurological compromise. This clinical classification was undertaken separately and blinded to the laboratory analyses.

### 5.4.5 Blood sampling and storage

Blood samples were collected as soon as practicable after enrolment criteria were met in the ED (T0), between 1-2 (T1), and 3-6 (T3) hours post enrolment, and finally within 3 hours of discharge. At each time point, 2 x 4 mL EDTA plasma tubes and 1 x 3.5 mL serum tube were collected, processed and aliquots of plasma and serum stored immediately at -80°C until analysis.
5.4.6  **Histamine measurement**

Histamine concentrations were measured in neat patient plasma in duplicate using validated, pre-coated histamine ELISA plates according to the manufacturer’s standard protocol (IBL International, Hamburg, Germany). The lower limit of detection of plasma histamine was 0.014 ng/mL. A positive result was defined as a peak plasma histamine concentration greater than 1.2 ng/mL, the 99th percentile of healthy control samples measured using this technique in our laboratory during a previous study [55]. The pre-coated and validated histamine ELISA plates met the quality standards set by the manufacturer, and intra assay CVs were 4.6%.

5.4.7  **Mast cell tryptase measurement**

The concentration of MCT in neat patient sera was measured using the ImmunoCAP® system according to the manufacturer’s recommended protocol. Concentrations higher than 11.4 ng/mL were considered positive, as defined by the 95th percentile of healthy controls determined by the manufacturer (Phadia (now Thermo Fisher Scientific), Uppsala, Sweden). In addition, as determined by our previous study, an MCT result was also considered positive where a difference (i.e. delta-MCT) of 2.0 ng/mL or more was evident between the minimum and maximum values for each patient [51]. The lower limit of detection for serum MCT was 0.707 ng/mL.

5.4.8  **Myeloperoxidase measurement**

Plasma MPO concentrations were measured in duplicate using ELISA according to the manufacturer’s standard protocol (Duoset® R&D Systems, Minneapolis, USA). Samples were diluted 1:100, 1:150, or 1:200, as required, in Reagent Diluent (1%
BSA/PBS, pH 7.2 – 7.4, 0.2µM filtered) prior to analysis. The lower limit of detection for plasma MPO was 0.0442 ng/mL. Intra and inter assay CVs for MPO ELISAs were 3.4% and 14.3%, respectively; recovery was 107% and linearity was 112%.

5.4.9 Soluble CD62L measurement

Serum concentrations of sCD62L were measured using a cytometric bead array flex set (BD Biosciences, San Jose, USA) as previously described [55]. Prior to analysis for sCD62L, serum samples were diluted 1:40 and 1:80, or 1:100 and 1:200, as necessary. The final result for each sample was calculated as the mean concentration after correction for the appropriate dilution factors. Samples were measured using a BD FACSCanto™ II flow cytometer (BD Biosciences, San Jose, USA) and analysed using FCAP Array™ v3.0 software (Soft Flow, Pecs, Hungary). The lower limit of detection for serum sCD62L was 0.020 ng/mL.

5.4.10 Statistical analysis

Patient demographics and clinical data are presented as mean (SD) or count (%) for continuous or class variables, respectively. Pearson’s $\chi^2$ tests were used to analyse differences in proportions and two-sample t tests were used to examine differences in means for age and time since symptom onset between severity groups. Mediator concentrations were log transformed to normalize the distribution of results. Linearity of continuous covariates was examined using multivariate regression splines and fractional polynomials. Random effects linear regression models with Maximum Likelihood Estimation (MLE) were employed to analyse continuous outcomes measured over time. MLE is a technique that retains those with incomplete data thus avoiding complete case
bias and is known to produce unbiased estimates when data are missing at random. It uses a combination of complete and incomplete data to produce the most likely estimate for the whole sample. Due to undetectable concentrations in some samples, random effects Tobit regression models were used for histamine, MCT, and MPO to account for the censored data. Models initially included the interaction of group and time (in categories) to test for unadjusted differences between groups at each time point. To investigate patterns over time within and between groups, the time categories were replaced with minutes since symptom onset. Models were then adjusted for age, sex, and reaction trigger. Results are presented as the fold change (the ratio of two geometric means) and the corresponding 95% confidence interval (CI). Associations between mediators were also investigated using random effects linear regression and random effects Tobit regression models as appropriate, adjusted for age, sex, and reaction trigger. P-values <0.05 were deemed significant, no adjustments were made to the significance level to account for multiple comparisons due to the exploratory nature of this study. Statistical analysis was performed with Stata v.12.1 (StataCorp, College Station, USA).

5.5 Results

5.5.1 Cohort characteristics

This study cohort comprised 72 patients that met the clinical definition of anaphylaxis, 37 (51%) of these were classified as severe reactions. The patient demographics and clinical characteristics are described in Table 5.1. Patients with moderate anaphylaxis were younger (p = 0.007). Moderate reactions were primarily triggered by food sensitivities whilst the majority of severe cases were adverse reactions
to drugs. Eight (11%) reactions were triggered by insect venom and only four (6%) were summative anaphylaxis with physical triggers (food + exercise). The median time from onset of symptoms to study enrolment was 73.5 minutes (interquartile range (IQR): 45.5), ranging from 10 minutes to 155 minutes, and was similar for both groups (p = 0.480). A higher proportion of severe patients received pre-hospital epinephrine and fluid therapy (p = 0.007 and p = 0.009, respectively).

5.5.2 **Histamine and mast cell tryptase are elevated in anaphylaxis patients**

Plasma histamine concentrations were positive at any time point in 33 (60%) of all anaphylaxis patients (52% of moderate patients and 66% of severe patients) (Table 5.1). A summary of the raw histamine concentrations with unadjusted comparisons at each timepoint between reaction severity groups is shown in Figure 5.2A. At the time of enrolment in the study, histamine concentrations were 5.8-fold (95% CI: 1.2, 27.1) higher in severe patients compared to those with moderate reactions (p = 0.025). Histamine levels decreased by 20% (95% CI: 10, 28) each hour since the onset of symptoms (p < 0.001), and there was no difference in trend between the two reaction severities (p = 0.618) (Figure 5.3A).

Serum MCT concentrations were positive in 47 (70%) of anaphylaxis patients and significantly more severe reactions had elevated MCT than moderate reactions with 32 (89%) and 15 (48%), respectively (p < 0.001) (Table 5.1). A summary of the raw MCT concentrations with unadjusted comparisons at each timepoint according to reaction severity is shown in Figure 5.2B. At enrolment, concentrations of MCT were 2.6-fold (95% CI: 1.6, 4.1) higher in severe patients than in moderate patients (p < 0.001). MCT concentrations decreased by 5.5% (95% CI: 4.5, 6.4) per hour since
symptom onset (p < 0.001), with no difference in trend between the two severities (p = 0.811) (Figure 5.3B).

5.5.3 *Myeloperoxidase was consistently elevated during anaphylaxis*

The raw MPO concentrations at each timepoint are summarised according to reaction severity in Figure 5.2C, with unadjusted comparisons between severities as indicated. In healthy controls (n = 23), the median (IQR) MPO concentration was 16.24 (12.08) ng/mL. At enrolment, MPO concentrations were 2.9-fold (95% CI: 1.3, 6.5) higher in moderate patients compared with healthy controls (p = 0.008), and 5.0-fold (95% CI: 2.4, 10.5) higher in severe patients than healthy controls (p < 0.001). At enrolment, MPO concentrations were not significantly different between moderate and severe patients, or between patients who received epinephrine pre-hospital and those who did not (p = 0.143 and p = 0.248, respectively). There were no significant changes in MPO concentrations over time since the onset of symptoms (p = 0.737), with a similar trend observed in both moderate and severe anaphylaxis (p = 0.636) (Figure 5.3C).

5.5.4 *Soluble CD62L was consistently reduced during anaphylaxis*

A summary of the sCD62L concentrations at each timepoint by reaction severity is displayed in Figure 5.2D, with unadjusted comparisons between severities as indicated. In healthy controls (n = 20), the median (IQR) sCD62L concentration was 708.8 (157.8) ng/mL. Upon enrolment, sCD62L was 29% (95% CI: 19, 38) lower in moderate patients than healthy control levels (p < 0.001), and 31% (95% CI: 22, 40) lower in severe patients than controls (p < 0.001), and no difference was apparent
between moderate and severe patients \( (p = 0.829) \). Soluble CD62L concentrations were also not significantly different at enrolment between patients based on whether they had already been administered epinephrine pre-hospital \( (p = 0.279) \). Serum sCD62L levels remained stable over time \( (p = 0.113) \), and there was no difference in trend \( (p = 0.118) \) between moderate and severe reactions (Figure 5.3D).

### 5.5.5 MPO is elevated and sCD62L is reduced in patients regardless of mast cell activation

There was no significant difference in plasma MPO concentrations in patients with and without a positive result for histamine and/or MCT \( (p = 0.051) \) (Figure 5.4A). MPO concentrations were not associated with histamine concentrations \( (p = 0.107) \) or MCT concentrations \( (p = 0.101) \). There were no differences in sCD62L concentration in patients with and without mast cell activation \( (p = 0.859) \) (Figure 5.4B). There were no associations between sCD62L and histamine \( (p = 0.545) \) or MCT \( (p = 0.144) \). There was also no association between MPO concentration and sCD62L concentration \( (p \geq 0.771) \).

### 5.6 Discussion

This study assessed neutrophil activation in acute human anaphylaxis through the measurement of MPO and sCD62L concentrations in samples taken from patients over the course of their anaphylactic reaction. We identified evidence of mast cell activation, namely elevated histamine or MCT concentrations, in the majority of anaphylaxis patients. Concentrations of MPO in this cohort were significantly elevated in patients with anaphylaxis when compared to healthy controls, regardless of reaction
severity. This result was apparent early in the reaction and was sustained during patient observation, treatment, and symptom resolution in the ED. MPO was also higher in severe patients than in moderate patients over the course of their reaction. Reduced sCD62L concentrations were evident in almost all anaphylaxis patients, regardless of reaction severity, when compared with healthy controls, and this was sustained over time since reaction onset. MPO concentrations were elevated and sCD62L concentrations were reduced similarly in patients with evidence of mast cell activation compared to those without. This study provides compelling evidence for the involvement of neutrophils during acute human anaphylaxis. Furthermore, this neutrophil activation occurs alongside mast cell activation, suggesting that neutrophils are actively involved during IgE-mediated anaphylaxis, perhaps in the amplification of the immune response.

This study is unique in that samples were obtained prospectively from patients during the acute course of anaphylaxis in the ED, enabling a real-time assessment of the immunological changes occurring over time. In this cohort, the majority of moderate reactions were triggered by foods and drugs were the primary trigger of severe reactions, a trend consistent with a previous sub-study of the CISS [241]. Other studies have identified a greater risk of death from reactions triggered by drugs than food- and insect-triggered anaphylaxis [339, 340]. The median interval between symptom onset and study enrolment showed that the initial blood sampling occurred within the acute stage of the reaction and, most importantly, whilst the physiological symptoms of anaphylaxis were still present. It is unlikely that the results are attributable to something other than an anaphylactic presentation.
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The precise role neutrophils might play in anaphylaxis is unclear, in particular whether neutrophil activation contributes to symptoms and reaction severity, or is simply a consequence of the reaction. However, as many neutrophil products, including MPO, elastase, and matrix metalloproteinase 9 (MMP9), cause non-specific tissue damage and symptoms akin to those observed during anaphylaxis (e.g. erythema, angioedema, and hypotension) [244, 328, 329, 341], it is likely that neutrophil activation contributes to the physiological changes in anaphylaxis, with higher concentrations of MPO (and other neutrophil products) leading to more severe symptoms. Indeed, animal and *ex vivo* studies suggested that neutrophils play a critical role in anaphylaxis, and that their activation is not a non-consequential by-product of the allergic response [101]. However, a direct clinical investigation during the acute phase of human anaphylaxis has not been reported. Additionally, neutrophils express receptors for both IgE and IgG [253, 254] and can theoretically directly respond to the allergen. It is possible that patients who experience more severe reactions have neutrophils that are more sensitised to the allergen, or simply in greater numbers, thereby driving a stronger inflammatory response. Alternatively, reactions that are more severe (particularly those with hypotension) are typically associated with a greater activation of mast cells.

Reduced serum concentration of sCD62L appears to be a ubiquitous and sustained response during anaphylaxis. Neutrophil activation is typically associated with CD62L shedding, which is required for the cells to traverse the endothelium. A previous study demonstrated evidence of CD62L shedding following the *ex vivo* culture of neutrophils from allergic patients after stimulation with their sensitizing antigen [255]. The serum concentrations of sCD62L in patients with widespread immune activation
but not anaphylaxis do not follow a predictable trend; levels are elevated in some conditions and reduced in others [333-338]. Interestingly, sCD62L levels are also reduced in trauma, a condition arguably similar to anaphylaxis in that there is a rapid insult to the immune system from an external source, as opposed to a chronic condition or one that has developed more slowly. In our study, the reduction in sCD62L occurs even though the neutrophils are activated. It is possible that although the neutrophils are activated, they are not traversing the endothelium and therefore are not shedding any CD62L. Alternatively, circulating sCD62L could be non-specifically bound or masked by other proteins, bound to endothelial cells [335-338], or caught up in complexes generated by the acute anaphylactic response. In a condition such as anaphylaxis where the immune system is rapidly overstimulated, the peripheral blood is a complex cocktail of cytokines, chemokines, and other proteins. As such, it is possible that the true measureable concentration of sCD62L in the serum is suppressed.

This study provides the first evidence that neutrophils are activated during human anaphylaxis by showing elevation of MPO. Further studies are needed to investigate: the mechanism leading to neutrophil activation in the context of anaphylaxis; whether neutrophils contribute to the initiation and/or amplification of the immune response; and the potential effect of neutrophil activation on clinical manifestations.

Limitations

The CISS criteria only captured patients who were significantly unwell and as a result, patients with mild allergic reactions were not recruited. Furthermore, patient sampling was dependent on the availability of the enrolling nurse/physician and their
experience with the study protocol. While early sampling times post-enrolment were adhered to, discharge samples were more difficult to obtain, affecting the number of patient samples available at this sampling time. However, we utilized MLE in our longitudinal analysis, which is known to overcome the issue of selection bias when data are missing at random. Future studies will be strengthened by the inclusion of patients encompassing the full spectrum of allergic disease and in convalescence.

Acknowledgements

The authors appreciate and acknowledge the research nursing staff at Royal Perth, Fremantle, Armadale, Bunbury, and Austin hospitals, including Ellen MacDonald (Clinical Nurse Manager), for identifying patients for the study and collecting and processing blood samples. The authors also appreciate Mrs Claire Neil for her expert assistance in optimising the laboratory methods. The authors acknowledge the facilities, scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, University of Western Australia, a facility funded by the University, State and Commonwealth Governments. Prof. Simon Brown is supported by a NHMRC Career Development Fellowship, and laboratory work was supported by the RPH Medical Research Foundation. Funding bodies played no role in study design, data collection, or analysis.

Author contributions

All authors reviewed and approved the final manuscript. AF contributed to the study concept and design, performed the laboratory experiments, contributed to data analysis, and drafted the manuscript. EB contributed to the data analysis and manuscript preparation. SFS contributed to the study concept and design, and obtaining funding.
support. DMF, GA, YN, SPJM, HM, and MR contributed to the collection of data. SB contributed extensive statistical knowledge to assist in data analysis and interpretation. SGAB contributed to the study concept and design, obtaining funding support, and collection of data.

Conflicts of interest

The authors declare that they have no conflicts of interest.
### 5.7 Tables

#### Table 5.1: Patient demographics and clinical observations of anaphylaxis patients.

<table>
<thead>
<tr>
<th></th>
<th>Moderate</th>
<th>Severe</th>
<th>P value</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>35</td>
<td>37</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td><strong>Age (years), mean (SD)</strong></td>
<td>33 (13)</td>
<td>43 (15)</td>
<td>0.007</td>
<td>38 (15)</td>
</tr>
<tr>
<td><strong>Male gender, n (%)</strong></td>
<td>18 (51.4)</td>
<td>16 (43.2)</td>
<td>0.487</td>
<td>34 (47.2)</td>
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<tr>
<td><strong>Cause</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug, n (%)</td>
<td>6 (17.1)</td>
<td>20 (54.1)</td>
<td>26 (36.1)</td>
<td></td>
</tr>
<tr>
<td>Food, n (%)</td>
<td>19 (54.3)</td>
<td>6 (16.2)</td>
<td>25 (34.7)</td>
<td></td>
</tr>
<tr>
<td>Insect, n (%)</td>
<td>5 (14.3)</td>
<td>3 (8.1)</td>
<td>0.003</td>
<td>8 (11.1)</td>
</tr>
<tr>
<td>Physical (including food + exercise), n (%)</td>
<td>2 (5.7)</td>
<td>2 (5.4)</td>
<td>4 (5.6)</td>
<td></td>
</tr>
<tr>
<td>Other/unknown, n (%)</td>
<td>3 (8.6)</td>
<td>6 (16.2)</td>
<td>9 (12.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Onset to enrolment (mins), mean (SD)</strong></td>
<td>71 (34)</td>
<td>77 (36)</td>
<td>0.480</td>
<td>74 (35)</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Any skin feature, n (%)</td>
<td>34 (97.1)</td>
<td>37 (100)</td>
<td>0.300</td>
<td>71 (98.6)</td>
</tr>
<tr>
<td>Any GI feature, n (%)</td>
<td>13 (37.1)</td>
<td>18 (48.7)</td>
<td>0.324</td>
<td>31 (43.1)</td>
</tr>
<tr>
<td>Any respiratory feature, n (%)</td>
<td>34 (97.1)</td>
<td>29 (78.4)</td>
<td>0.016</td>
<td>63 (87.5)</td>
</tr>
<tr>
<td><strong>Hypoxemia, n (%)</strong></td>
<td>5 (13.5)</td>
<td>1 (2.8)</td>
<td>5 (6.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Cyanosis, n (%)</strong></td>
<td>5 (24.3)</td>
<td>4 (11.1)</td>
<td>9 (12.5)</td>
<td></td>
</tr>
<tr>
<td>Any cardiovascular feature, n (%)</td>
<td>8 (22.9)</td>
<td>36 (97.3)</td>
<td>&lt;0.001</td>
<td>44 (61.1)</td>
</tr>
<tr>
<td><strong>Hypotension, n (%)</strong></td>
<td>2 (5.7)</td>
<td>26 (70.3)</td>
<td>26 (36.1)</td>
<td></td>
</tr>
<tr>
<td><strong>LOC/Collapse, n (%)</strong></td>
<td>13 (35.1)</td>
<td>13 (35.1)</td>
<td>13 (18.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Treatments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine^, n (%)</td>
<td>31 (88.6)</td>
<td>36 (97.3)</td>
<td>0.145</td>
<td>67 (93.1)</td>
</tr>
<tr>
<td>Pre-hospital, n (%)</td>
<td>8 (22.9)</td>
<td>20 (54.1)</td>
<td>0.007</td>
<td>28 (38.9)</td>
</tr>
<tr>
<td>Steroids, n (%)</td>
<td>20 (57.1)</td>
<td>28 (75.7)</td>
<td>0.095</td>
<td>48 (66.7)</td>
</tr>
<tr>
<td>Fluids, n (%)</td>
<td>16 (45.7)</td>
<td>28 (75.7)</td>
<td>0.009</td>
<td>44 (61.1)</td>
</tr>
<tr>
<td><em><em>Positive histamine</em> (&gt;1.2ng/mL), n (%)</em>*</td>
<td>12 (52.2)</td>
<td>21 (65.6)</td>
<td>0.315</td>
<td>33 (60.0)</td>
</tr>
<tr>
<td><strong>Positive MCT</strong> (11.4 ng/mL, or ΔMCT &gt;2ng/mL), n (%)</td>
<td>15 (48.4)</td>
<td>32 (88.9)</td>
<td>&lt;0.001</td>
<td>47 (70.2)</td>
</tr>
</tbody>
</table>

GI, gastrointestinal, LOC, loss of consciousness, MCT, mast cell tryptase, ΔMCT, difference between highest and lowest observed MCT concentrations. Bold P values (< 0.05) were considered significant. Skin features: erythema, urticaria, periorbital oedema, and angioedema. GI features: nausea, vomiting, abdominal/pelvic pain, and incontinence. Respiratory features: dyspnea, stridor, wheeze, chest/throat tightness, hypoxemia, and cyanosis. Cardiovascular features: dizziness, diaphoresis, confusion, hypotension, LOC, and collapse. ^ = epinephrine administered at any stage.
Due to sample availability: * Histamine was measured in 23 moderate and 32 severe patients at enrolment; ** MCT was measured in 31 moderate and 36 severe patients at enrolment.
5.8 Figures

Figure 5.2: Histamine, MCT, MPO, and sCD62L protein concentrations during anaphylaxis.

A) Histamine was measured in moderate (T0 n = 23, T1 n = 22, T3 n = 17, and discharge n = 4) and severe (T0 n = 32, T1 n = 31, T3 n = 25, and discharge n = 7) patients. The dashed line indicates 1.2 ng/mL, the 99th percentile of healthy controls. B) Mast cell tryptase was measured in moderate (T0 n = 31, T1 n = 29, T3 n = 25, and discharge n = 7) and severe (T0 n = 36, T1 n = 35, T3 n = 33, and discharge n = 12) patients. The dashed line indicates 11.4 ng/mL, the 95th percentile of healthy controls. C) Myeloperoxidase was measured in moderate (T0 n = 27, T1 n = 26, T3 n = 20, and discharge n = 3) and severe (T0 n = 36, T1 n = 35, T3 n = 29, and discharge n = 9) patients. The dashed line indicates 16.24 ng/mL, the median concentration of healthy controls (n = 23). D) Soluble CD62L concentrations was measured in moderate (T0 n = 31, T1 n = 30, T3 n = 28, and discharge n = 7) and severe (T0 n = 33, T1 n = 33, T3 n = 32, and discharge n = 12) patients. The dashed line indicates 708.8 ng/mL, the median concentration of healthy controls (n = 20).
Note: Boxplots identify the median, upper and lower quartiles, and the range. Raw values are plotted and p values are derived using random effects linear or Tobit regression models testing the ratio of the two means following log transformation.
Figure 5.3: Changes in histamine, mast cell tryptase (MCT), myeloperoxidase (MPO), and soluble CD62L (sCD62L) concentrations over the course of anaphylactic reactions.

Margins plots show the predicted concentrations (back transformed from log variables) over the first 5 hours since the onset of symptoms in moderate (dotted) and severe (solid) patients based on the appropriate random effects linear or Tobit regression models (adjusted for age, sex, and trigger). **A)** The dashed line indicates 1.2 ng/mL, the 99th percentile of healthy controls. **B)** The dashed line indicates 11.4 ng/mL, the 95th percentile of healthy controls. **C)** The dashed line indicates 16.24 ng/mL, the median concentration of healthy controls (n = 23). **D)** The dashed line indicates 708.8 ng/mL, the median concentration of healthy controls (n = 20).
Mast cell activation status was deemed positive where histamine and/or mast cell tryptase (MCT) concentrations were above the 99th and 95th percentiles of healthy controls, respectively, or if delta-MCT was at least 2 ng/mL. A) The dashed line indicates 16.24 ng/mL, the median concentration of healthy controls (n = 23). B) The dashed line indicates 708.8 ng/mL, the median concentration of healthy controls (n = 20). Boxplots identify the median, upper and lower quartiles, and the range.
Chapter 6  General Discussion

This research challenges the current dogma of an IgE-mediated pathway, involving the activation of mast cells, as the predominant immune mechanism of anaphylaxis. Here we show no activation of mast cells, MCT or histamine release occurs in 30% of anaphylactic patients. By measuring changes in mRNA expression and protein concentrations in serum/plasma we demonstrate consistent evidence of neutrophil activation in anaphylaxis, regardless of reaction severity, trigger, or mast cell activation status. The improved understanding of cellular drivers of anaphylaxis presented in this thesis will underpin novel approaches to its monitoring and treatment.

The overarching aim of this thesis was to improve our knowledge of the immune mechanisms underlying anaphylaxis. Despite the rising incidence of anaphylaxis in both adults and children, and the remarkable advances in immunological research, we still do not fully understand the mechanisms of anaphylaxis. It has been well established that the adaptive immune system, in particular T cells and B cells, are involved in the production of allergen-specific antibodies. These antibodies have been shown to bind to IgE receptors on the surface of mast cells and their circulating counterparts, basophils in a process described as sensitisation. On a subsequent exposure, allergen binds to the allergen-specific IgE, resulting in activation of these cells and the release of pro-inflammatory mediators, such as mast cell tryptase (MCT). This process is referred to as the IgE-mediated anaphylaxis pathway. However, mast cells are tissue-specific and basophils account for less than 1% of circulating peripheral blood leukocytes. Therefore, it is highly unlikely that these cells are solely responsible for the overwhelming rapid systemic inflammation observed during anaphylaxis. In addition, activation via an IgE-mediated pathway is not evident in a considerable proportion of
patients. We observed no detectable changes in markers of mast cell activation, MCT and/or histamine, in ~30% of patients. Also, particularly in cases of drug-induced anaphylaxis, patients may react on their first exposure, without being previously sensitised.

Regardless of the initial immunogenic trigger, the mechanisms that rapidly amplify a localised immune response to allergens, in only some individuals, are poorly understood. In addition, it is not known why patients present with such remarkable variations in symptoms and reaction severity, even within the same patient on different exposure episodes. Two comprehensive microarray studies performed in our laboratory, highlighted the involvement of innate immune activation pathways during anaphylaxis. Aside from mast cells and basophils, innate immune cells include macrophages, monocytes, neutrophils, eosinophils, dendritic cells, and natural killer (NK) cells. The central hypothesis of this thesis is that of the innate cells, neutrophils are the standout candidate likely to be activated and critically involved during anaphylaxis. We proposed that their involvement probably occurs during the amplification process of a localised immunogenic signal.

There are several key features of neutrophils that provide support for this hypothesis. Firstly, they are not only the most abundant innate immune cell, but also the most abundant of all peripheral blood leukocytes, greatly outnumbering other cell types, making them a plausible candidate for systemic signal dissemination. Secondly, neutrophil activation can occur rapidly after exposure to an array of stimuli, including signals from neighbouring cells. Mediators that are released from mast cells and basophils following allergen exposure can activate neutrophils in vitro and in vivo.
Therefore, it is highly likely that neutrophils are activated during reactions triggered by the IgE-mediated pathway. Thirdly, activated neutrophils can also be an important source of mediators that have been identified as elevated during anaphylaxis, which can then in turn activate additional neutrophils. Fourth, in addition to overall innate immune pathways, the microarrays mentioned previously also identified the upregulation of neutrophil activation pathways during anaphylaxis. Lastly, the presence of neutrophils, but not mast cells or monocytes/macrophages, was shown to be critical for anaphylaxis to occur in a mouse model [101]. This suggests that neutrophil activation is required for the development of anaphylaxis and also that mast cell activation is not a necessary precursor step.

This thesis details an investigation into the immune mechanisms of anaphylaxis, with an initial broad exploration into innate immunity and then a more specific focus on neutrophils. The findings of a microarray study performed in previous work was validated in chapter 3 and expanded upon in chapter 4 to investigate differences specific to anaphylaxis. The key findings suggested the involvement of the IL-6, IL-10, TLR, interferon, granulocyte adhesion, and phagocytosis-related signalling pathways. Subsequent analyses in a larger cohort of anaphylaxis patients confirmed the differential expression of “hub genes” and key drivers of the two microarrays. Specifically, the mRNA targets \textit{IL10, OSM, S100A8, S100A9, MMP9, TLR4, MYD88, TREM1}, and \textit{CD64} were all upregulated. Elevated concentrations of IL-6, IL-10, S100A9, MMP9, and soluble TREM1 proteins were also detectable systemically in anaphylaxis patients compared to controls. Figure 6.1 demonstrates the candidate cells of the innate immune system and identifies the potential sources of these genes/proteins elevated during anaphylaxis. Neutrophils can produce almost all of the targets identified in this thesis,
and as they greatly outnumber the other cell types in peripheral blood, they are most likely the primary source of the targets measured. For example, although other cell types produce and export S100A8/A9, neutrophils are considered to be the primary cellular source of these proteins. This is due to the large amount of S100A8/A9 that each neutrophil can export, relative to other cell types, as well as the sheer number of neutrophils in peripheral blood.

Although they are unlikely to contribute significant concentrations of S100A9, MMP9, and soluble TREM1, other cell types can also be sources of these proteins. Therefore, we then aimed to confirm neutrophil activation during anaphylaxis by investigating a neutrophil-specific product. The enzyme myeloperoxidase (MPO) is contained within azurophilic granules of neutrophils and is released into the extracellular environment, specifically following neutrophil activation. MPO is frequently used as a marker of neutrophil activation, and the relative concentration of extracellular MPO indicates the degree of activation. Plasma MPO concentrations were measured in a large cohort of anaphylaxis patients and compared to healthy controls. MPO was consistently elevated in patients regardless of reaction severity, trigger, or mast cell activation status. This result, recently published in *Clinical and Experimental Allergy*, confirmed for the first time that neutrophils are activated during human anaphylaxis.
Figure 6.1: Differentially expressed mRNA and protein targets during anaphylaxis and their potential innate immune cells sources.

This figure illustrates the different types of circulating innate immune cells and whether they have been reported in the literature to express the mRNA/protein targets shown to be upregulated during anaphylaxis in this thesis.
The activation of neutrophils during anaphylaxis represents a mechanism for the rapid amplification of an initial immune signal. Activated neutrophils produce an array of cytokines, chemokines, proteins, and enzymes that can activate neighbouring neutrophils and other immune cells resulting in an inflammatory storm. Therefore, the activation of a small number of neutrophils directly at the site of allergen exposure could, very quickly, lead to exponential activation of billions of neutrophils throughout the body. In addition, we have demonstrated that the systemic activation of neutrophils is not dependent upon an IgE-mediated/mast cell pathway triggering the reaction. Hence, neutrophils could be responsible for the amplification of allergic signals from alternative pathways such as IgG-mediated anaphylaxis and complement generation.

Neutrophil activation during anaphylaxis may not only be acting to rapidly amplify an allergic signal. Following activation, each of the billions of neutrophils exports enzymes such as MPO and MMP9. These enzymes normally assist in host defence and wound healing through their antimicrobial functions. However, they have also been shown to cause oxidative and degradative damage to normal/healthy tissues when released in excess into the periphery. For these reasons, it is likely that neutrophil activation during anaphylaxis also has a detrimental effect, contributing to the physiological symptoms and reaction severity. As such, the inhibition or reduction of neutrophil activation represents a novel target for future therapies. However, as adequate neutrophil function is critical for the prevention of infection and for effective wound healing, permanently dampening neutrophil function would be harmful. Systemic neutrophil depletion has been effective in reducing tissue damage in severe acute pancreatitis, another condition with rapid systemic neutrophil involvement [342]. In addition, neutrophil-targeted therapies have shown promise in the treatment of
ischaemic stroke [343]. Transient inhibition of neutrophil activation and function following allergen exposure might prevent progression of symptoms or reduce reaction severity. Extensive in vitro and in vivo experimental work would be required to develop and test this theory.

It has been well established that not all individuals who experience mast cell activation at the site of allergen exposure progress to systemic anaphylaxis. For example, following most bee stings, the victim suffers localised mast cell activation and inflammation, characterised by redness and swelling at the sting site. In these circumstances, perhaps a lack of neutrophil activation is the reason why they do not progress to systemic anaphylaxis. There may be a fundamental difference between the neutrophils of people who suffer anaphylaxis and those who exhibit “appropriate” responses to allergens. Genetic studies might uncover a specific neutrophil “fingerprint” in individuals prone to anaphylaxis. There could be an underlying mutation causing the neutrophils of allergy sufferers to be hyper-reactive to allergic stimuli. In addition, future studies using microscopy and cell culture experiments could investigate phenotypic changes/differences in neutrophils from anaphylactic patients during a reaction. This could be compared to cells from the same patients once their neutrophil population had been completely replaced following recovery (> 2 weeks) and also to non-allergic controls. We would investigate differences in neutrophil morphology, mediator production, surface receptor expression, activation potential, and degranulation speed following stimulation. These studies might identify features specific to neutrophils from sufferers of anaphylaxis that could explain the rapid systemic spread from a localised reaction. The ultimate goal of research is disease prevention, which is particularly important with anaphylaxis, as the first reaction can be
fatal. To achieve this, we would need to develop a screening tool to detect either genetic or phenotypic features that underlie susceptibility, perhaps based on neutrophils. With the incidence of allergies and anaphylaxis on the rise, this is a research priority.

During this thesis, I have presented evidence confirming that neutrophil activation occurs during acute human anaphylaxis. This includes changes in mRNA expression and protein concentrations in serum/plasma. Neutrophils may play a key role in the amplification of the localised allergic response. This could have potential clinical implications in contributing to symptoms and reaction severity.
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