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Title: Neutrophil activation during acute human anaphylaxis: analysis of MPO and sCD62L

Short title: Neutrophil activation during human anaphylaxis

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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.

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.

Key words:

Anaphylaxis; basic mechanisms; clinical immunology; granulocyte; neutrophil; peripheral blood leukocyte

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

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 [1, 2]. Anaphylaxis is a serious and potentially life-threatening allergic reaction that affects multiple organ systems, characterized by hives/rash, bronchospasm, and collapse [3]. 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 [4].

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 [5-11]. The half-life of MCT in the bloodstream is approximately 2 hours [12], considerably longer than that of histamine which reportedly returns to baseline within 60 minutes of reaction onset [7, 12]. 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 [13]. 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 [14]. Upon activation, neutrophils release neutrophil elastase, collagenase, and gelatinase which have been implicated in the degradation of the extracellular matrix [15, 16], as well as lactoferrin, which up-regulates neutrophil adhesion molecules and delays apoptosis [17, 18]. 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 [19]. 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) [20], soluble L-selectin (sCD62L) [21], elastase [22], IL-8 [22, 23], and S100A12 [24, 25]. MPO is an enzyme confined within neutrophil azurophilic granules and released upon their activation [20]. 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 [20, 26, 27]. 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 [19, 28]. Upon activation, CD62L proteins are enzymatically cleaved from the neutrophil surface, releasing sCD62L into the circulation [21, 29]. This shedding enables the translocation of the β_2 integrin CD11b to the cell surface, required for tight binding to the endothelium [30]. sCD62L 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 [31], acute myeloblastic leukaemia and insulin-dependent diabetes mellitus [32], whilst reduced sCD62L has been associated with poor outcomes in trauma [33-35] and acute lymphoblastic leukaemia [36]. 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.

Materials and Methods

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 (ED)s 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 [37]. 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.

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.

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 [3], 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.

Case classification

In order to investigate changes associated with disease severity, cases were reviewed by two clinical investigators (SB and GA) and classified into two severity groups, moderate and severe anaphylaxis, based on clinical features according to standard consensus criteria [4]. *Severe anaphylaxis* was defined as the presence of hypoxia (cyanosis or SpO_2 ≤92%), hypotension (systolic blood pressure <90 mmHg), 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 hypoxia, hypotension, or neurological compromise. This clinical classification was undertaken separately and blinded to the laboratory analyses.

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.

Histamine measurement

Histamine concentrations were measured in neat patient plasma in duplicate using validated, pre-coated histamine ELISA according to the manufacturer's standard protocol (IBL International, 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 [6]. The pre-coated and validated histamine ELISA plates met the quality standards set by the manufacturer, and intra assay CVs were 4.6%.

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, 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 [5]. The lower limit of detection for serum MCT was 0.707 ng/mL.

Myeloperoxidase measurement

Plasma MPO concentrations were measured in duplicate using ELISA according to the manufacturer's standard protocol (Duoset R&D Systems, 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%.

Soluble CD62L measurement

Serum concentrations of sCD62L were measured using a cytometric bead array flex set (BD Biosciences, USA) as previously described [6]. 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 (BD Biosciences, USA) and analysed using FCAP Array v3.0 software (Soft Flow, Hungary). The lower limit of detection for serum sCD62L was 0.020 ng/mL.

Statistical analysis

Patient demographics and clinical data are presented as mean (SD) or count (%) for continuous or class variables, respectively. Pearson's χ^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 regressions 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, USA).

Results

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 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 adrenaline and fluid therapy (p=0.007 and p=0.009, respectively).

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 1). A summary of the raw histamine concentrations with unadjusted comparisons at each timepoint between reaction severity groups is shown in Figure 1A. At the time of enrolment in the study, histamine

concentrations were 5.8- (95% CI: 1.2, 27.1) fold higher in severe patients compared to those with moderate reactions (p=0.025). Histamine levels decreased by 20% (95% CI: 10, 28) every hour since the onset of symptoms (p<0.001), with no difference in trend between the two reaction severities (p=0.618) (Figure 2A).

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 1). A summary of the raw MCT concentrations with unadjusted comparisons at each timepoint according to reaction severity is shown in Figure 1B. At enrolment, concentrations of MCT were 2.6- (95% CI: 1.6, 4.1) fold 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 2B).

Myeloperoxidase was consistently elevated during anaphylaxis

The raw MPO concentrations at each timepoint are summarised according to reaction severity in Figure 1C, 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 (95% CI: 1.3, 6.5) fold higher in moderate patients compared with healthy controls (p=0.008), and 5.0 (95% CI: 2.4, 10.5) fold 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 adrenaline 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 2C).

Soluble CD62L was consistently reduced during anaphylaxis

A summary of the sCD62L concentrations at each timepoint by reaction severity is displayed in Figure 1D, 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). sCD62L concentrations were also not significantly different at enrolment between patients based on whether they had already been administered adrenaline 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 2D).

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 3A). 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 3B). 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≥0.771).

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 whilst drugs were the primary trigger of severe reactions, a trend consistent with a previous sub-study of the CISS [38]. Other studies have identified a greater risk of death from reactions triggered by drugs than food- and insect-triggered anaphylaxis [39, 40]. 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.

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) [19, 26, 27, 41], 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 [13]. 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 [42, 43] and can therefore 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 [44]. 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 [31-36]. 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 [33-36], 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.

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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.

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Legends to Figures

Figure 1: 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: 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 2: 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).

Figure 3: Concentrations of myeloperoxidase and soluble CD62L in anaphylaxis patients with/without evidence of mast cell activation (as determined by histamine and mast cell tryptase (MCT) concentrations). **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).

Note: p-values are derived using random effects linear regression models testing the ratio of the two means following log transformation.

Table 1: Patient demographics and clinical observations

	Moderate anaphylaxis	**Severe anaphylaxis**	**P value**	**All**
n	35	37		72
Age (years), mean (SD)	33 (13)	43 (15)	**0.007**	38 (15)
Male gender, n (%)	18 (51.4)	16 (43.2)	0.487	34 (47.2)
Cause				
Drug, n (%)	6 (17.1)	20 (54.1)		26 (36.1)
Food, n (%)	19 (54.3)	6 (16.2)		25 (34.7)
Insect, n (%)	5 (14.3)	3 (8.1)	**0.003**	8 (11.1)
Physical (including food + exercise), n (%)	2 (5.7)	2 (5.4)		4 (5.6)
Other/Unknown, n (%)	3 (8.6)	6 (16.2)		9 (12.5)
Onset to enrolment (mins), mean (SD)	71 (34)	77 (36)	0.480	74 (35)

Symptoms				
Any skin feature, n (%)	34 (97.1)	37 (100)	0.300	71 (98.6)
Any GI feature, n (%)	13 (37.1)	18 (48.7)	0.324	31 (43.1)
Any respiratory feature, n (%)	34 (97.1)	29 (78.4)	**0.016**	63 (87.5)
Hypoxemia, n (%)	-	5 (13.5)	-	5 (6.9)
Cyanosis, n (%)	-	9 (24.3)	-	9 (12.5)
Any cardiovascular feature, n (%)	8 (22.9)	36 (97.3)	**<0.001**	44 (61.1)
Hypotension, n (%)	-	26 (70.3)	-	26 (36.1)
LOC/Collapse, n (%)	-	13 (35.1)	-	13 (18.1)
Treatments				
Adrenaline^, n (%)	31 (88.6)	36 (97.3)	0.145	67 (93.1)
Pre-hospital, n (%)	8 (22.9)	20 (54.1)	**0.007**	28 (38.9)
Steroids, n (%)	20 (57.1)	28 (75.7)	0.095	48 (66.7)
Fluids, n (%)	16 (45.7)	28 (75.7)	**0.009**	44 (61.1)
Positive histamine* (>1.2ng/mL), n (%)	12 (52.2)	21 (65.6)	0.315	33 (60.0)
Positive MCT (>11.4 ng/mL, or ΔMCT >2ng/mL), n (%)**	15 (48.4)	32 (88.9)	**<0.001**	47 (70.2)

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 = dyspnea, stridor, wheeze, chest/throat tightness, hypoxemia, cyanosis

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

^ = adrenaline administered at any stage

Note, 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