

Small nucleolar RNA networks are up-regulated during human anaphylaxis

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Abstract

Background: Anaphylaxis is a severe, potentially life-threatening allergic reaction driven primarily by the activation of mast cells. We still fail to understand factors underlying reaction severity. Furthermore, there is currently no reliable diagnostic test to confirm anaphylaxis in the emergency department (ED).

Objective: This study sought to explore gene expression changes associated with anaphylaxis severity in peripheral blood leucocytes and evaluate biomarker potential.

Methods: Microarray analysis (total RNA) was performed using peripheral blood samples from ED patients with moderate ($n = 6$) or severe ($n = 12$) anaphylaxis and sepsis ($n = 20$) at presentation (T0) and one hour later (T1). Results were compared between groups and healthy controls ($n = 10$ and $n = 11$ matched to anaphylaxis and sepsis patients, respectively). Changes in gene expression were determined using R programming language, and pathway analysis applied to explore biological processes and pathways associated with genes. Differentially expressed genes were validated in an independent cohort of anaphylaxis ($n = 30$) and sepsis ($n = 20$) patients, and healthy controls ($n = 10$), using quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

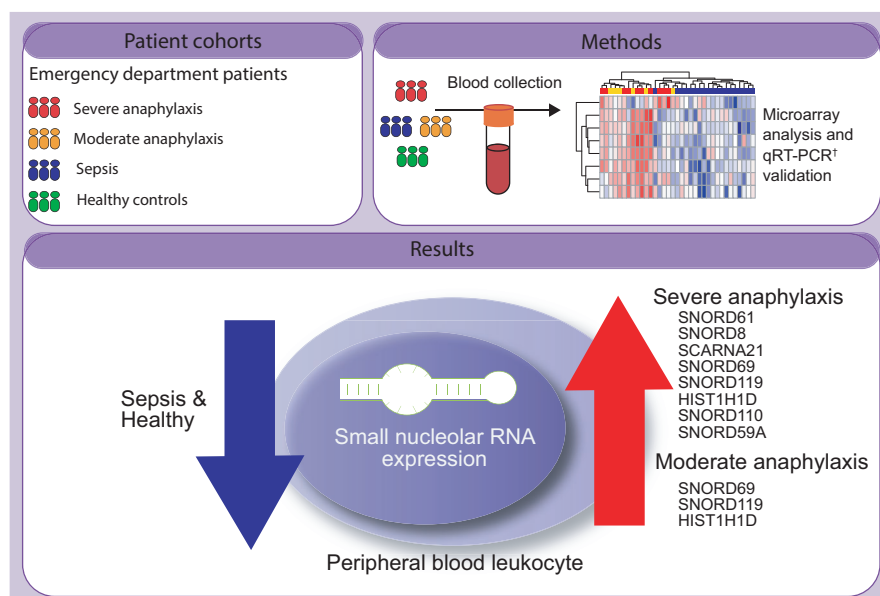
Results: Significant up-regulation of small nucleolar RNAs (snoRNAs) was demonstrated in anaphylaxis compared to sepsis patients in the microarray cohort, at T0 and T1. qRT-PCR analysis of the validation cohort showed five genes: *SNORD61*, *SNORD8*, *SNORD69*, *SNORD119* and *HIST1H1D* to be significantly up-regulated (adjusted $p < 0.05$) in severe anaphylaxis compared to sepsis. Seven genes (*SNORD61*, *SNORD8*, *SCARNA21*, *SNORD69*, *SNORD110*, *SNORD119* and *SNORD59A*) were significantly up-regulated (adjusted $p < 0.05$) in severe anaphylaxis compared to healthy controls.

Conclusion: This study demonstrates for the first time the unique involvement of snoRNAs in the pathogenesis of anaphylaxis and suggests they are not a general feature of systemic inflammation. Further investigation of snoRNA expression in anaphylaxis could provide insights into disease pathogenesis.

Clinical relevance: SnoRNAs are up-regulated during acute anaphylaxis in humans and could potentially be used as biomarkers of severe anaphylaxis.

KEYWORDS

allergy, anaphylaxis, biomarker, differential diagnosis, sepsis, small nucleolar RNA, snoRNA



GRAPHICAL ABSTRACT

Small nucleolar RNA (snoRNA) upregulation is a unique feature of acute anaphylaxis in humans, not a general feature of systemic inflammation. SnoRNA upregulation may be associated with anaphylaxis reaction severity. Further investigation is needed to determine the diagnostic significance of snoRNAs. [†]qRT-PCR (quantitative reverse transcription-polymerase chain reaction).

1 | INTRODUCTION

Anaphylaxis is a severe, potentially life-threatening allergic reaction caused primarily by the activation of mast cells triggering a peripheral blood cell response that affects multiple organs.¹ Anaphylaxis is diagnosed in the emergency department (ED) based on clinical presentation. Cases that lack conventional symptoms, or where a trigger cannot be identified, can therefore mimic other types of distributive shock.²⁻⁴ Both anaphylaxis and sepsis are types of distributive shock that develop through inflammatory mediator release and vasodilation.^{5,6} In severe cases, both conditions present with hypotension, and excluding either possibility can be difficult.^{4,6} Laboratory tests, including measurements of soluble protein mediators, have been developed to improve diagnosis of anaphylaxis; however, inadequate sensitivity and specificity limit their use.⁷ The development of more reliable diagnostic testing is an active area of anaphylaxis research. Identifying a biomarker of anaphylaxis would provide diagnostic confirmation, improving patient management and providing retrospective verification for research purposes.

In addition, while studies have demonstrated that multiple cell types and effector molecules induce anaphylaxis, several questions remain. These include how allergic reactions become rapidly generalized, why only some sensitized individuals develop anaphylaxis, and why severity varies between individuals.⁸ To address these issues, we developed an unbiased, genomics-based strategy to further our understanding of human anaphylaxis and

Key Messages

- We studied peripheral blood leucocyte gene expression in anaphylaxis and sepsis patients attending emergency departments.
- A subset of small nucleolar RNAs was up-regulated in severe anaphylaxis but not in sepsis.
- Several snoRNA genes were confirmed as specifically up-regulated in severe anaphylaxis in a second cohort.

highlight potential diagnostic markers. Our approach is rationalized by the concept that anaphylaxis is initiated and amplified by a mast cell-leucocyte cytokine cascade.⁹ Our approach entails comparative profiling of gene expression patterns in peripheral blood leucocytes (PBLs) from patients presenting to hospital with acute anaphylaxis or from age- and sex-matched healthy controls. Employing this approach, we previously demonstrated that anaphylaxis was characterized by up-regulation of innate inflammatory gene networks, and tumour necrosis factor was identified as a candidate driver of these networks.¹⁰ In the present study, we build on these findings by exploring differences in gene expression between severe and moderate anaphylactic reactions and draw comparisons between anaphylaxis and sepsis as examples of distributive shock.

2 | METHODS

2.1 | Study population

This study assesses two independent cohorts. The microarray cohort consists of 18 anaphylaxis patients, 20 sepsis patients, and 10 and 11 healthy controls age-sex matched to the anaphylaxis and sepsis patients, respectively. The validation cohort consists of 30 anaphylaxis patients, 20 sepsis patients, and 10 healthy controls age-sex matched to the anaphylaxis patients. All patients from both cohorts, and healthy controls, were recruited through our Critical Illness and Shock Study (CISS), as is described below.

Adults with features of acute anaphylaxis were recruited in four Australian EDs between July 2010 and December 2014 through CISS.¹¹ Because need for emergency care took priority, waiver of initial consent was approved. Once treatment was started, fully informed written consent was obtained, and patients were given the option of declining further involvement and having all research samples destroyed. Ethics approval, including waiver of initial consent, was obtained from the Human Research Ethics Committees of each participating site.

Adults (≥ 16 years) with features of acute anaphylaxis (National Institute of Allergy and Infectious Diseases and the Food Allergy and Anaphylaxis Network criteria¹²) were recruited. All patients received treatment with epinephrine, and if hypotension was present, intravenous fluid resuscitation. Severe anaphylaxis was defined by presence of hypotension (systolic blood pressure < 90 mmHg), hypoxia (cyanosis or $\text{SpO}_2 \leq 92\%$) or neurological compromise (confusion, collapse or loss of consciousness) as per the Brown severity grading system.¹³ The remainder of cases were classified as moderate anaphylaxis. By definition, severe patients were characterized by the presence of significant cardiovascular symptoms, while moderate cases lacked such symptoms.

Patients presenting to EDs with sepsis were also recruited through CISS. Inclusion criteria for these patients have been described previously.^{11,14}

2.2 | Sample collection and storage

Venous blood samples were collected from patients upon presentation to the ED (T0) and one hour later (T1). Samples were also collected from healthy controls. RNA was stabilized in PAXgene tubes (PreAnalytiX GmbH) and stored in accordance with manufacturer's recommendations.

2.3 | Gene expression profiling

Total RNA was extracted employing PAXgene Blood RNA Extraction Kits (PreAnalytiX GmbH) using an automated extraction protocol (Qiacube, Qiagen, Australia), following manufacturer's recommendations. RNA sample quality was assessed on a Bioanalyzer instrument

(Agilent), with all samples having an RNA integrity number (RIN) > 7.5 . Total RNA samples were hybridized to Affymetrix Human Gene 2.1 ST microarrays (Ramaciotti Centre for Gene Function Analysis, UNSW). Raw microarray data are available from the Gene Expression Omnibus (accession: GSE69063).

2.4 | Microarray analysis

Microarray data were analysed using R programming language (version 3.6.2). Data underwent robust multi-array expression measure (RMA) normalization (background correction, normalization and summarization) using the *oligo* Bioconductor package. The *limma* Bioconductor package was employed for statistical analysis and identification of differentially expressed genes (DEGs; false discovery rate (FDR) adjusted p -value < 0.05 and a $\log_2$ fold change ≥ 0.6 , or ≤ -0.6). Probe sets were mapped to genes using hugene21sttranscriptcluster (version 8.7.0). Known batch effects were adjusted for using the Combat function (*sva* package). Gene Ontology (GO) analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource (version 6.8) was used to assess biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of DEGs. DAVID pathways were considered significant if they had a Benjamini-Hochberg FDR $q < 0.50$ and p -value < 0.01 . Gene Set Enrichment Analysis (GSEA) was performed on RMA normalized samples to identify gene signatures distinguishing cellular pathways. GSEA pathways were considered enriched if they had a nominal p -value < 0.05 , and a FDR $q < 0.25$.

2.5 | Validation: RNA extraction and quality control

Samples taken at T0 from an independent cohort of 30 anaphylaxis patients (12 severe), 20 sepsis patients and 10 healthy controls (age and sex matched to anaphylaxis patients) were selected to validate microarray findings. Automated RNA extraction from PAXgene tubes (BD Biosciences) was performed as previously described. RNA quantity was measured using the Qubit Flex Fluorometer 4 Extended Range (XR) assay (Thermo Fisher Scientific), and RIN was measured using Bioanalyzer (Agilent). Median RNA quantity was 463 ng/ μl (IQR 381–519 ng/ μl). Median RIN was 8.8 (IQR 8.3–9.0).

2.6 | qRT-PCR analysis

Total RNA (1.5 μg) was converted to 20 μl cDNA (SuperScript IV VILO Master Mix Kit Thermo Fisher Scientific) for qRT-PCR analysis using a TaqMan-based approach. Housekeeper genes (*PSMC4*, *ELF1*, *POP4* and *ATP6*) were selected following the analysis of 11 randomly selected samples from all groups, using TaqMan™ Array Human Endogenous Control plates. geNorm (GenEx) was used to select genes with minimal variation between samples, reflected by

an M-value. Triplicate qRT-PCR reactions were performed using TaqMan gene expression assay probes, on a ViiA™7 Real-Time PCR System (Thermo Fisher Scientific). No template controls, no reverse transcription controls and water blanks were run for each target. Expression data were normalized to housekeeper genes using GenEx software and the $2^{-\Delta\Delta CT}$ method applied. One-way ANOVA with Tukey honesty significance difference (HSD) adjustment was used to determine differences between groups. Receiver operating characteristic (ROC) curves were generated (*p*ROC function) for genes, and area under the ROC curve (AUC) was calculated. The *co-ords* function was used to determine sensitivity and specificity of genes in differentiating between severe anaphylaxis and sepsis.

3 | RESULTS

Initial results for this study were generated by comparing differences in gene expression between groups in the microarray cohort (18 anaphylaxis patients, 20 sepsis patients and 10 and 11 healthy controls age-sex matched to the anaphylaxis and sepsis patients, respectively). These results were subsequently validated in an independent validation cohort (30 anaphylaxis patients, 12 sepsis patients and 10 healthy controls age-sex matched to the anaphylaxis patients).

3.1 | Microarray cohort characteristics

A complete description of the clinical characteristics of the microarray anaphylaxis and sepsis cohort is presented in Table 1. The 18 anaphylaxis patients were graded as severe ($n = 12$) or moderate ($n = 6$) following the Brown severity scale.¹³ The majority of severe anaphylactic reactions were caused by beta-lactam antibiotics ($n = 7$, 58%) and non-steroidal anti-inflammatory drugs (NSAID) ($n = 3$, 25%) (Table 1). Route of exposure for all drug-triggered reactions ($n = 10$) was oral. All moderate severity reactions were caused by food ($n = 6$). Timing between anaphylaxis onset and blood sampling ranged from 15 min to 2 h and 22 min (mean 67 min).

Of the 20 sepsis patients, 12 (60%) had severe sepsis with organ failure (defined by a sequential organ failure assessment (SOFA) score >2).¹⁴

3.2 | Severe anaphylaxis patients display a unique gene expression profile

To assess differences in gene expression in anaphylaxis and sepsis patients compared to healthy controls, microarray analysis (Affymetrix Human Gene 2.1 ST) was performed using RNA isolated from whole blood (PBL) samples. The differentially expressed genes (DEGs) were determined following comparisons between groups (moderate anaphylaxis, severe anaphylaxis and sepsis) and matched healthy controls at T0 and T1.

We found 67, 306, and 930 up-regulated genes and 13, 262, and 2434 down-regulated genes for moderate anaphylaxis, severe anaphylaxis and sepsis, respectively, when compared to healthy controls at T0 (Figure 1A). Heatmaps and volcano plots of all DEGs at T0 can be found in (Figures S1-S4). Venn diagram analysis demonstrated that 250 up-regulated genes out of 306 (82%) and 212 down-regulated genes out of 262 (81%) were unique to severe anaphylaxis (Figure 1C-D). Thirty-nine up-regulated genes out of 67 (58%) and 5 down-regulated genes out of 13 (38%) were unique to moderate anaphylaxis (Figure 1C-D). Nine hundred and one up-regulated genes out of 930 (97%) and 2388 down-regulated genes out of 2434 (98%) were unique to sepsis (Figure 1C-D).

We found 31, 643 and 1006 up-regulated genes and 12, 460 and 2448 down-regulated genes for moderate anaphylaxis, severe anaphylaxis and sepsis, respectively, at T1 (Figure 1B). Heatmaps and volcano plots of all DEGs at T1 can be found in (Figures S5-S8). Venn diagram analysis showed 305 up-regulated genes out of 643 (47%) and 270 down-regulated genes out of 460 (59%) were unique to severe anaphylaxis (Figure 1E-F). Three up-regulated genes out of 31 (10%) were unique to moderate anaphylaxis, and no genes were uniquely down-regulated (Figure 1E-F). Six hundred and eighty-four up-regulated genes out of 1006 (68%) and 2264 down-regulated genes out of 2448 (92%) were unique to sepsis (Figure 1E-F).

3.3 | Biological processes and pathways associated with severe anaphylactic reactions are not associated with sepsis.

DAVID analysis was performed to associate genes uniquely up-regulated in patients compared to healthy controls with biological processes and pathways (Figure 1C-E). Significant terms associated with the 250 genes unique to severe anaphylaxis at T0 included 'T-cell receptor signalling pathway' (GO:0050852 ($p = 1.81 \times 10^{-4}$)) and KEGG pathway hsa04660 ($p = 1.78 \times 10^{-3}$), and 'Natural killer (NK) cell-mediated cytotoxicity' (KEGG pathway hsa04650, $p = 2.49 \times 10^{-5}$) (Figure 2A). At T1, the 305 DEGs unique to severe anaphylaxis were associated with 'NK cell-mediated cytotoxicity' ($p = 3.84 \times 10^{-3}$, KEGG pathway hsa04650). There were not enough unique DEGs in moderate anaphylaxis for meaningful pathway analysis at either time-point. 'Innate immune response' (GO:0045087), and 'inflammatory response' (GO:0006954) were top pathways up-regulated in sepsis at T0 ($p = 2.41 \times 10^{-13}$ and $p = 2.88 \times 10^{-10}$, respectively) and T1 ($p = 6.75 \times 10^{-5}$ and $p = 3.75 \times 10^{-4}$, respectively) (Figure).

Analysis of patients compared to healthy controls was repeated using GSEA focussing on KEGG pathways shown up-regulated in DAVID analysis. GSEA results showed the pathways of 'NK cell-mediated cytotoxicity' and 'T-cell receptor signalling' were positively correlated with severe anaphylaxis at T0 (nominal $p = 1.23 \times 10^{-2}$ and nominal $p = 1.0 \times 10^{-2}$, respectively, Figure 2E-F). These pathways were not enriched in moderate anaphylaxis or sepsis. GSEA also showed 'NK cell-mediated cytotoxicity' was positively correlated with severe anaphylaxis at T1 (nominal $p = 4.09 \times 10^{-2}$, Figure 2G).

TABLE 1 Clinical reaction features of anaphylaxis and sepsis presentations assessed in the microarray and validation cohorts

Anaphylaxis patients				
	Microarray cohort		Validation cohort	
	Severe	Moderate	Severe	Moderate
<i>n</i>	12	6	12	18
Age, mean (SD)	45.8 (10.8)	50.6 (19.0)	37.2 (17.5)	35.7 (16.4)
Male sex, <i>n</i> (%)	3 (25.0)	1 (16.7)	7 (58.3)	10 (55.6)
Suspected cause				
Food, <i>n</i> (%)	2 (16.7)	6 (100.0)	2 (16.7)	7 (38.9)
Non-steroidal anti-inflammatory drugs, <i>n</i> (%)	3 (25.0)	-	3 (25)	3 (16.7)
Beta-lactam antibiotics, <i>n</i> (%)	7 (58.3)	-	-	2 (11.1)
Drug other/uncertain, <i>n</i> (%)	-	-	3 (25)	-
Venoms, <i>n</i> (%)	-	-	3 (25)	3 (16.7)
Physical, <i>n</i> (%)	-	-	1 (8.3)	-
Pollen, <i>n</i> (%)	-	-	-	1 (5.6)
Unknown, <i>n</i> (%)	-	-	-	2 (11.1)
Onset to first sample, median (min) (IQR)	62.5 (63)	63.5 (33.8)	57.5 (35.8)	71 (31.8)
Any skin features, <i>n</i> (%)	11 (91.7)	5 (83.3)	12 (100.0)	18 (100.0)
Urticaria, <i>n</i> (%)	6 (50.0)	1 (16.7)	9 (75.0)	9 (50.0)
Erythema, <i>n</i> (%)	9 (75.0)	2 (33.3)	7 (58.3)	13 (72.2)
Angioedema, <i>n</i> (%)	5 (41.7)	3 (50.0)	4 (33.3)	5 (27.8)
Periorbital oedema, <i>n</i> (%)	4 (33)	1 (16.7)	2 (16.7)	4 (22.2)
Any gastrointestinal features, <i>n</i> (%)	5 (41.7)	4 (66.7)	4 (33.3)	5 (27.8)
Nausea, <i>n</i> (%)	5 (41.7)	3 (50.0)	4 (22.2)	4 (22.2)
Vomiting, <i>n</i> (%)	2 (16.7)	1 (16.7)	2 (16.7)	3 (16.7)
Abdominal/pelvic pain, <i>n</i> (%)	2 (16.7)	1 (16.7)	-	2 (11.1)
Any respiratory features, <i>n</i> (%)	10 (83.3)	6 (100.0)	12 (100.0)	18 (100.0)
Chest/throat tightness, <i>n</i> (%)	7 (58.3)	5 (83.3)	7 (58.3)	13 (72.2)
Dyspnoea, <i>n</i> (%)	7 (58.3)	4 (66.7)	6 (50)	8 (44.4)
Stridor, <i>n</i> (%)	1 (8.3)	1 (16.7)	1 (8.3)	2 (11.1)
Wheeze, <i>n</i> (%)	4 (33.3)	3 (50.0)	4 (33.3)	8 (44.4)
Any cardiovascular features, <i>n</i> (%)	12 (100.0)	1 (16.7)	12 (100.0)	3 (16.7)
Dizziness, <i>n</i> (%)	-	-	4 (33.3)	3 (16.7)
Diaphoresis, <i>n</i> (%)	4 (33.3)	1 (16.7)	1 (8.3)	-
Hypotension (systolic BP <90 mmHg), <i>n</i> (%)	10 (83.3)	-	7 (58.3)	-
Confusion, <i>n</i> (%)	3 (25.0)	-	1 (8.3)	-
Collapse, <i>n</i> (%)	4 (33.3)	-	7 (58.3)	-
Loss of consciousness, <i>n</i> (%)	2 (16.7)	-	1 (8.3)	-
Cyanosis, <i>n</i> (%)	3 (25.0)	-	1 (8.3)	-
Treated with adrenaline prior to ED, <i>n</i> (%)	5 (41.7)	1 (16.7)	5 (41.7)	4 (22.2)
Length of stay, median (IQR) (days)	0.55 (0.53)	0.25 (0.33)	0.55 (0.65)	0.2 (0.35)
Death, <i>n</i> (%)	-	-	-	-
Sepsis patients				
	Microarray cohort		Validation cohort	
<i>n</i>	20		20	
Age, mean (SD)	58.20 (16.14)		59.25 (19.60)\	

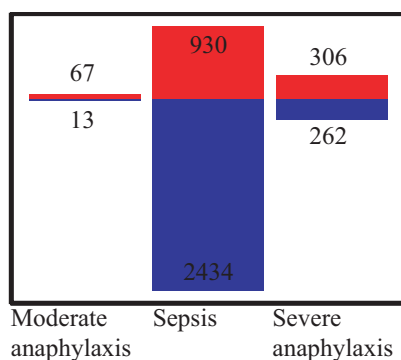
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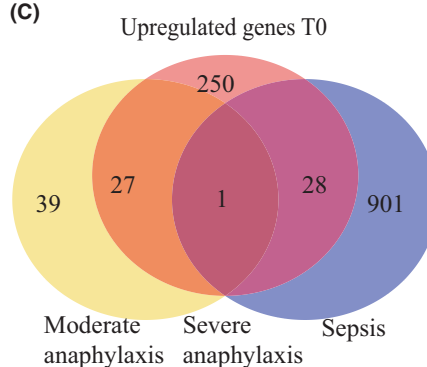
Sepsis patients		
	Microarray cohort	Validation cohort
Male sex, <i>n</i> (%)	10 (50)	13 (65)
Source of infection		
Respiratory <i>n</i> (%)	6 (30)	4 (20)
Urinary tract, <i>n</i> (%)	3 (15)	7 (35)
Other, <i>n</i> (%)	11 (55)	9 (45)
MAP (mmHg) (median) (IQR)	88.50 (32)	56.50 (16.25)
WCC ($\times 10^9$ /L) (median) (IQR)	10.6 (7.14)	10.70 (5.83)
Lactate (mmol/L) (median) (IQR)	2.55 (2.43)	1.75 (1.20)
SOFA score (median) (IQR)	4 (7)	7 (3)
CCI (median) (IQR)	1 (2.5)	2.50 (3)
Length of stay (days) (median) (IQR)	7.7 (11.35)	7.50 (11.55)
Death within 30 days, <i>n</i> (%)	4 (20)	4 (20)

Abbreviations: CCI, Charlson Comorbidity Index; IQR, Interquartile range; MAP, mean arterial pressure; SD, Standard deviation; SOFA, sequential organ failure assessment; WCC, total White Blood Cell Count.

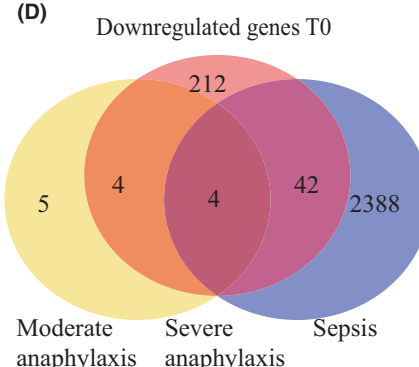
(A) Differentially expressed genes at T0



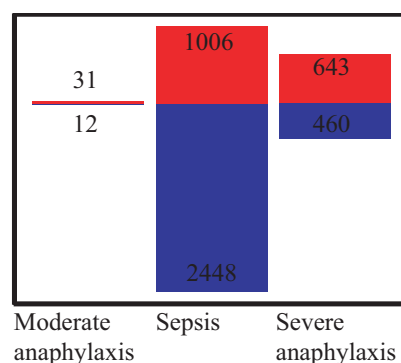
(C)



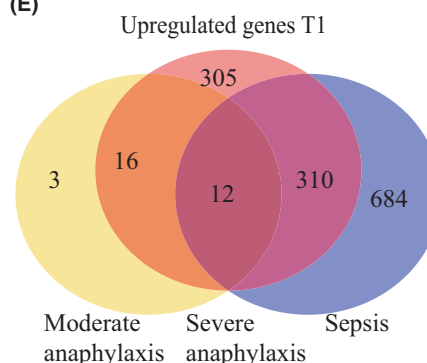
(D)



(B) Differentially expressed genes at T1



(E)



(F)

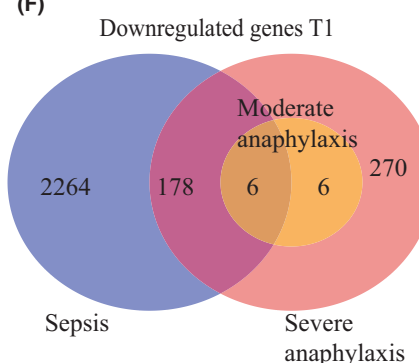


FIGURE 1 Genes found differentially expressed between patient groups and controls. Number of up-regulated/down-regulated genes (adjusted *p*-value < 0.05, log fold changes ≤ -0.6 or ≥ 0.6) in patient groups at (A) T0 and (B) T1. Venn diagrams showing unique and common genes up-regulated between groups at (C) T0 and (E) T1 and down-regulated between groups at (D) T0, and (F) T1

3.4 | SnoRNA networks are up-regulated in anaphylaxis compared to sepsis

In order to identify biomarker candidates, differential gene expression comparisons were made between moderate and severe

anaphylaxis groups to sepsis. At T0, we found 8 genes up-regulated in anaphylaxis groups compared to sepsis (5 genes in severe and 8 in moderate anaphylaxis) (Figure 3A). At T1, we found 12 up-regulated genes in anaphylaxis compared to sepsis (9 genes in severe and 12 in moderate anaphylaxis) (Figure 3B). All genes up-regulated in severe

anaphylaxis were also up-regulated in moderate anaphylaxis at both time-points. In addition, the 8 genes seen up-regulated at T0 were also up-regulated at T1. There were no down-regulated genes at either time-point. Of the 8 genes up-regulated in anaphylaxis at T0, 7 were snoRNAs (Figure 3A). Of the 12 up-regulated genes in anaphylaxis at T1, 11 were snoRNAs (Figure 3B).

3.5 | Signalling pathways associated with snoRNAs are enriched in anaphylaxis

Specific snoRNAs are associated with two pathways involved in mast cell degranulation and anaphylaxis, tumour protein 53 (p53) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signalling.^{15,16} Following this association, GSEA was performed on microarray data comparing anaphylaxis patients and healthy controls to determine if these pathways were enriched. The 'Hallmark p53 pathway' was enriched in severe and moderate anaphylaxis at T0 (nominal $p = 3.43 \times 10^{-2}$ and nominal $p = 3.19 \times 10^{-2}$, respectively) and T1 (nominal $p = 6.01 \times 10^{-3}$ and nominal $p = 3.36 \times 10^{-2}$, respectively) (Figure 3C-F), and the 'Hallmark PI3K_AKT_MTOR signalling pathway' was enriched in severe anaphylaxis at T1 (nominal $p = 3.75 \times 10^{-2}$, Figure 3G).

3.6 | A gene signature for severe anaphylaxis

Following observations of snoRNA up-regulation in severe anaphylaxis, we investigated the possibility of a genetic signature for severe anaphylaxis. To define this signature, a subset of genes was selected including genes significantly up-regulated in the microarray comparison of severe anaphylaxis with sepsis (mainly snoRNAs, $p < 0.05$) and genes with 'core enrichment' in p53 and PI3K Hallmark pathways in the comparison of severe anaphylaxis to healthy controls (GSEA analysis). This subset included 159 genes (Table S1) and was confirmed to be enriched in severe anaphylaxis compared to sepsis by GSEA (nominal $p = 0.00 \times 10^{-0}$, Figure S9A). Further analysis of these 159 genes by GSEA found 46 showed 'core enrichment' in severe anaphylaxis compared to sepsis (nominal $p = 0.00 \times 10^{-0}$, Figure S9B).

3.7 | Validation cohort characteristics

Clinical data characterizing the 30 anaphylaxis (12 severe) and 20 sepsis patients in the independent validation cohort are described

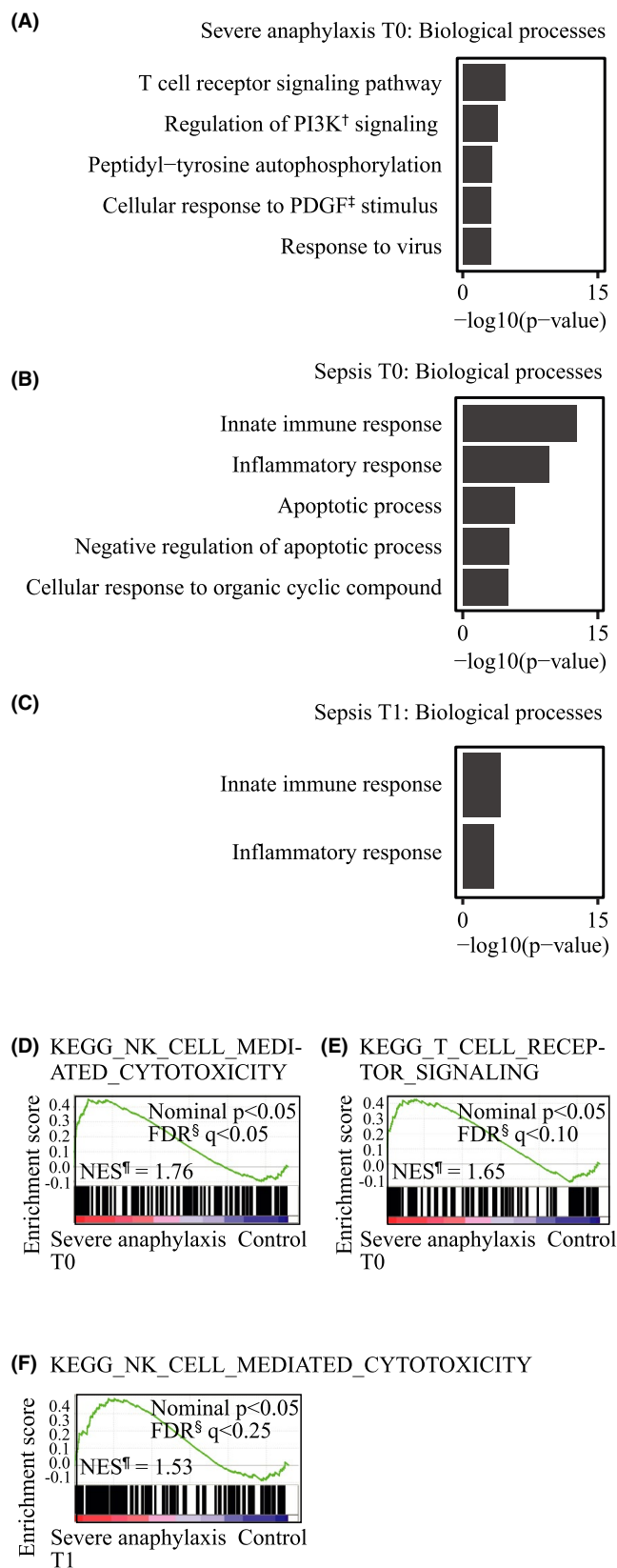


FIGURE 2 Pathways associated with unique gene expression. Biological processes associated with uniquely up-regulated genes (adjusted p -value < 0.05 , log fold change ≥ 0.6) in (A) severe anaphylaxis at T0 and (B) sepsis at T0 and (C) sepsis at T1. Gene set enrichment analysis at (D-E) T0, and (F) T1. [†]PI3K (Phosphoinositide 3-kinases), [‡]PDGF (platelet-derived growth factor), [§]FDR (false discovery rate), [¶]NES (normalized enrichment score)

in Table 1. Half of severe anaphylactic reactions were caused by drugs ($n = 6$; 50%), with food, *Hymenoptera* venom and physical triggers causing the remaining reactions. Moderate reactions were caused by foods, drugs, *Hymenoptera* venom, unknown and pollen triggers in descending order of frequency. Of all the drug-triggered

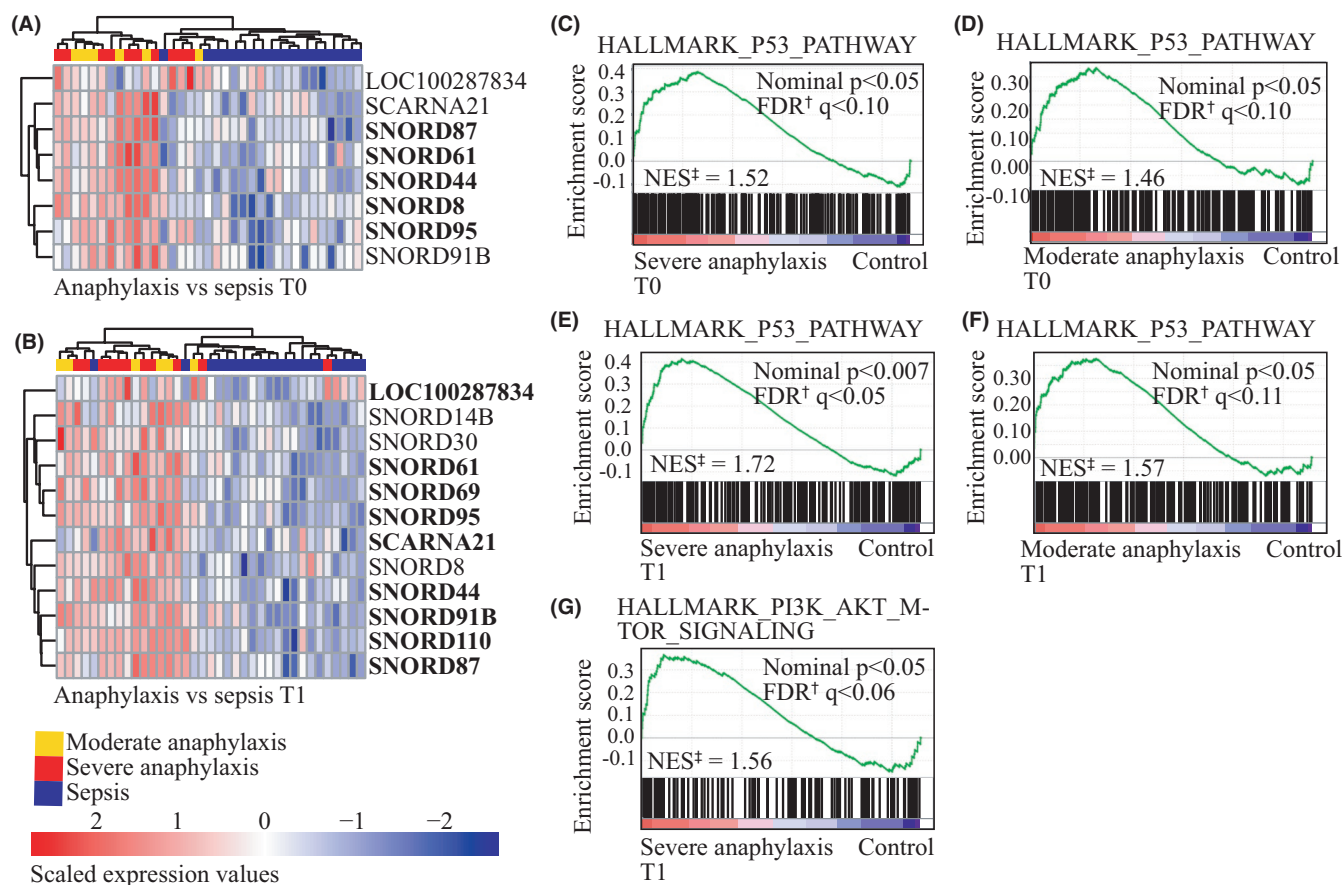


FIGURE 3 Genes found differentially expressed in moderate and severe anaphylaxis compared to sepsis. Heatmap of genes found significantly up-regulated (adjusted p -value < 0.05 , log fold change ≥ 0.6) in anaphylaxis vs sepsis at (A) T0, and (B) T1, with genes up-regulated in severe groups in bold. (C-G) Gene set enrichment analysis using expression from severe/moderate anaphylaxis, sepsis and controls. [†]FDR (false discovery rate), [‡]NES (normalized enrichment score)

reactions ($n = 12$), most ($n = 10$; 83%) were administered orally, with the remaining 2 administered intravenously. Both cases of intravenous exposure were severe reactions. Timing between illness onset and blood sampling ranged from 17 min to 2 h and 30 min (mean = 65min).

All sepsis patients had a SOFA score > 2 .¹⁴ Fourteen patients (70%) met the criteria for shock (MAP < 65 mmHg).¹⁴

3.8 | SnoRNAs are up-regulated in severe anaphylaxis compared to sepsis

In order to validate up-regulation of snoRNAs seen in the microarray analysis comparison of anaphylaxis and sepsis, we selected an independent cohort of ED patients (anaphylaxis and sepsis) and healthy controls, and performed qRT-PCR. This validation focussed on the up-regulation of genes, mostly snoRNAs, seen in anaphylaxis compared to sepsis. A group of 10 genes, *SNORD61*, *SNORD95*, *SNORD8*, *SNORD110*, *SNORD69*, *RNU4ATAC*, *SCARNA21*, *HIST1H1D*, *SNORD119*, and *SNORD59A*, were chosen for validation and will subsequently be referred to as the 'validation panel' (Table 2). These genes were chosen based on p -values ($p < 0.05$) in the comparison

TABLE 2 P-values of genes in validation panel

Gene name	Severe anaphylaxis ^a	Moderate anaphylaxis ^a
SNORD61	2.94E-09	5.32E-08
SNORD95	6.81E-09	3.49E-06
SNORD8	9.52E-07	1.49E-06
SNORD110	1.48E-04	1.59E-03
SNORD69	3.09E-04	7.79E-04
RNU4ATAC	5.03E-04	5.81E-03
SCARNA21	1.74E-03	2.49E-06
HIST1H1D	3.53E-03	4.27E-04
SNORD119	2.45E-02	8.78E-04
SNORD59A	4.93E-02	4.01E-02

^aGenes chosen for validation based on the microarray analysis comparison of moderate and severe anaphylaxis groups to sepsis ($p < 0.05$).

of anaphylaxis groups compared to sepsis (microarray analysis). Pearson's correlation analysis found no correlation between gene expression patterns of these 10 genes at ED arrival and time lag from

onset of symptoms to blood collection (Figure S10). All genes in the validation panel were included in the 46 genes shown to have core enrichment in severe anaphylaxis compared to sepsis through GSEA.

Five genes in the validation panel (*SNORD61* (adjusted $p = 2.52 \times 10^{-4}$), *SNORD8* (adjusted $p = 1.28 \times 10^{-3}$), *SNORD69* (adjusted $p = 1.41 \times 10^{-2}$), *SNORD119* (adjusted $p = 0.00 \times 10^{-0}$) and *HIST1H1D* (adjusted $p = 4.52 \times 10^{-3}$)) were significantly up-regulated in severe anaphylaxis compared to sepsis (Figure 4). Seven genes (*SNORD61* (adjusted $p = 6.79 \times 10^{-4}$), *SNORD8* (adjusted $p = 1.12 \times 10^{-4}$), *SCARNA21* (adjusted $p = 1.80 \times 10^{-2}$), *SNORD69* (adjusted $p = 2.22 \times 10^{-2}$), *SNORD110* (adjusted $p = 2.84 \times 10^{-2}$), *SNORD119* (adjusted $p = 3.12 \times 10^{-4}$) and *SNORD59A* (adjusted $p = 1.36 \times 10^{-2}$)) were significantly up-regulated in severe anaphylaxis compared to healthy controls (Figure 4). Three genes (*SNORD69* (adjusted $p = 4.28 \times 10^{-2}$), *SNORD119* (adjusted $p = 3.00 \times 10^{-7}$) and *HIST1H1D* (adjusted $p = 1.28 \times 10^{-3}$)) were significantly up-regulated in moderate anaphylaxis compared to sepsis (Figure 4). Two genes (*SNORD119* (adjusted $p = 9.35 \times 10^{-3}$) and *HIST1H1D* (adjusted $p = 4.03 \times 10^{-2}$)) were significantly up-regulated in moderate anaphylaxis compared to healthy controls (Figure 4). Two genes (*SNORD61* (adjusted $p = 8.71 \times 10^{-3}$) and *SNORD8* (adjusted $p = 1.53 \times 10^{-3}$)) were significantly up-regulated in severe anaphylaxis compared to moderate anaphylaxis (Figure 4). No genes were significantly up-regulated or down-regulated in sepsis compared to healthy controls.

3.9 | AUC analysis suggests

SNORD119 and *HIST1H1D* can differentiate between severe anaphylaxis and sepsis

To test the validation panel's potential in the differential diagnosis of severe anaphylaxis and sepsis, AUC analysis was performed using the validation cohort of severe anaphylaxis ($n = 12$) and sepsis ($n = 20$). Analysis showed *SNORD119* and *HIST1H1D* to have high diagnostic potential. At a threshold of 1.14, *SNORD119* had a specificity and sensitivity of differentiating between severe anaphylaxis and sepsis of 1 and 0.95, respectively (Figure 5A). At a threshold of 1.43, *HIST1H1D* had a specificity and sensitivity of 0.83 and 0.85, respectively (Figure 5B). Combining *SNORD119* and *HIST1H1D* resulted in a specificity and sensitivity of 0.95 and 1, respectively, at a threshold of 0.61 (Figure 5C).

4 | DISCUSSION

Anaphylaxis is an immediate-type generalized hypersensitivity reaction thought to be caused by the activation of mast cells and rapid mediator release. Differentiation of anaphylaxis from other forms of distributive shock in the ED is largely clinical. In addition, mechanisms that determine how local allergic reactions become rapidly generalized are unknown, and it is unclear why patients experience remarkable variations in reaction severity. To address these issues,

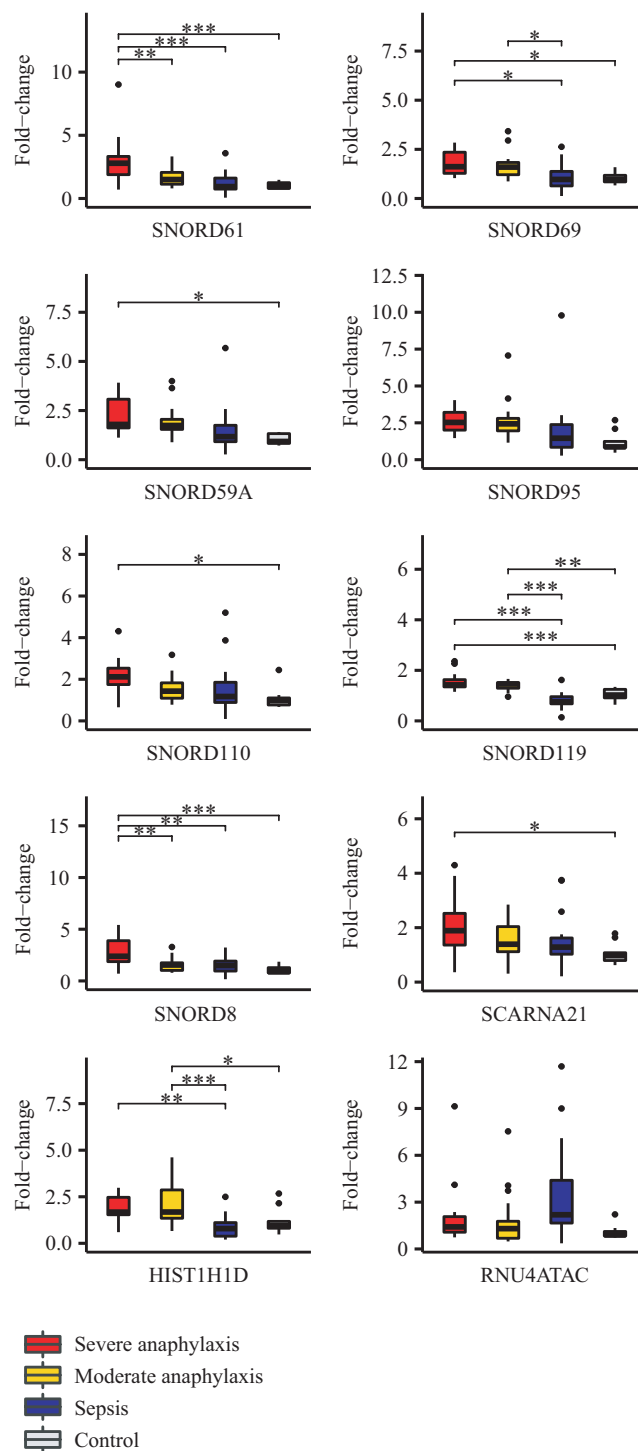


FIGURE 4 qRT-PCR validation of differential gene expression for selected snoRNA genes. Fold changes were determined using the $2^{-\Delta\Delta CT}$ method where groups were normalized to healthy controls. Comparison groups included severe anaphylaxis ($n = 12$), moderate anaphylaxis ($n = 18$), sepsis ($n = 20$) and healthy controls ($n = 10$). *ANOVA p -value < 0.05 , **ANOVA p -value < 0.01 , ***ANOVA p -value < 0.001

we hypothesized that a mast cell-leucocyte cascade underpins variations in reaction severity, and accordingly, we employed genomic profiling of PBLs to investigate underlying mechanisms. Our findings showed that at T0, severe cases of anaphylaxis were associated with

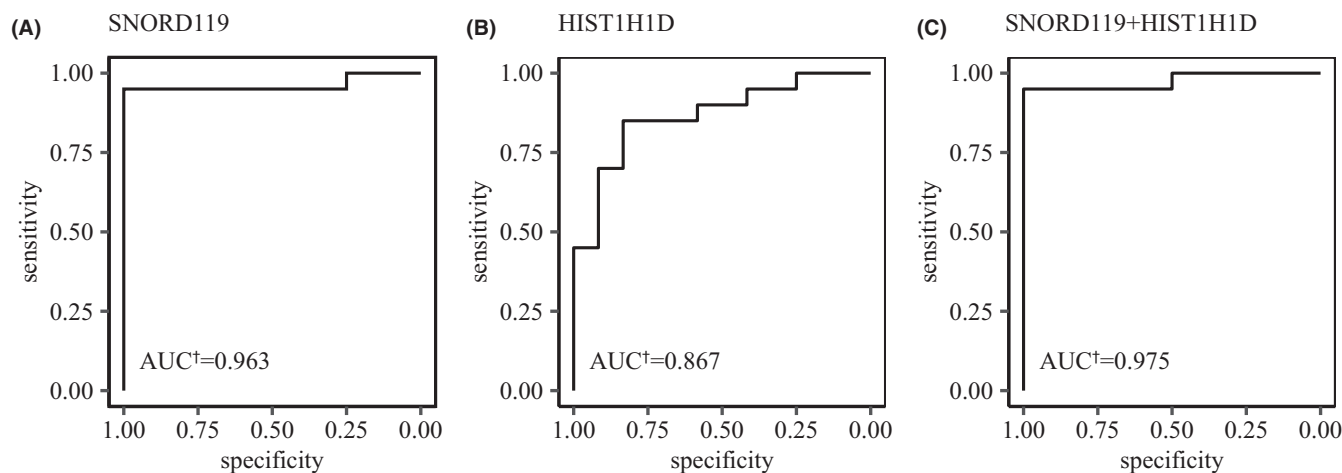


FIGURE 5 SNORD119 and HIST1H1D show high sensitivity and specificity for severe anaphylaxis. Receiver operating characteristic (ROC) curve analysis of (A) SNORD119, (B) HIST1H1D, (C) SNORD119+HIST1H1D. The analysis cohort included 12 severe anaphylaxis patients and 20 sepsis patients. † AUC (area under the curve)

up-regulation of a subset of snoRNAs, and genes involved in NK cell cytotoxicity and T-cell receptor signalling. At T1, snoRNA and NK cell cytotoxicity up-regulation persisted. Overall, our findings suggest that up-regulation of snoRNAs could be a driver of reaction severity in anaphylaxis. Additionally, expression of snoRNAs in severe anaphylaxis, but not sepsis, suggests snoRNA up-regulation is a unique feature of the pathogenesis of anaphylaxis, and not a general feature of systemic inflammation.

SnoRNAs are short (60–300 nucleotides) non-coding RNAs located in the nucleolus, which are involved in post-transcriptional modification of cellular RNAs.¹⁷ SnoRNAs fall into one of two groups: C/D box snoRNAs (involved in methylation) and H/ACA box snoRNAs (involved in pseudouridylation).¹⁷ Dysregulation of snoRNAs in allergy has been reported, with H/ACA box snoRNAs being up-regulated in patients with severe allergic reactions compared to mild reactions.¹⁸ Interestingly, samples were taken prior to allergen challenge, suggesting that snoRNAs may be up-regulated in severely allergic individuals at baseline, not as a response to allergen exposure. Similarly, our results showed that snoRNAs were up-regulated both at T0 and T1, which could suggest they are continuously expressed in anaphylactic persons. While there is no literature surrounding the potential roles of snoRNAs in allergy, snoRNAs have been demonstrated to play a role in gene expression regulation, stress responses, and oncogenic functions in different types of cancer.^{17,19–22} Specifically, there is evidence that snoRNAs are associated with p53, and PI3K/AKT signalling pathways, which our results showed to be enriched during anaphylaxis.^{15,16,22–24} P53 is a well-known tumour suppressor, which is activated by cellular stress and responds by regulating genes involved in cell cycle arrest, apoptosis and DNA repair.²⁵ In addition to its role in cancer, p53 has been shown to act as a negative regulator in mast cells in models of IgE-mediated anaphylaxis.²⁶ Additionally, we previously proposed that p53 is an upstream regulator driving anaphylaxis.¹⁰ SnoRNAs have also been implicated in PI3K/AKT signalling in cancer. For example, SNORD126 can activate the PI3K/AKT pathway and promote cancer

cell growth.¹⁶ PI3K pathways, as with p53, are essential for mast cell degranulation and anaphylaxis.²⁷ Whilst the function of snoRNAs in anaphylaxis will require investigation in follow-up studies, their up-regulation highlights an intriguing role for snoRNAs that has not been previously reported during acute anaphylaxis. Specifically, the potential of snoRNAs to affect pathways associated with mast cell degranulation and drive reaction severity in anaphylaxis needs to be explored.

Microarray analysis identified a panel of 46 genes, mostly snoRNAs, which could act as a potential gene signature of severe anaphylaxis, although validation with independent data is needed. Validation of 10 genes from this panel identified five genes (SNORD61, SNORD8, SNORD69, SNORD119 and HIST1H1D) that are significantly up-regulated in severe anaphylaxis compared to sepsis. Specifically, the combination of SNORD119 and HIST1H1D had high specificity (0.95) and sensitivity (1.00) in differentiating severe anaphylaxis from sepsis, albeit in a very small cohort. These preliminary findings highlight the potential of a panel of snoRNAs to differentiate severe anaphylaxis from sepsis; however, further larger studies are needed to confirm results. In addition, two snoRNAs (SNORD61 and SNORD8) were up-regulated in severe anaphylaxis compared to moderate anaphylaxis, suggesting these snoRNAs may be drivers of reaction severity.

This study has limitations. The genomic profiling studies were performed on whole blood leucocytes; thus, the cellular source(s) of the gene expression signals is unclear. Due to the nature of human anaphylaxis, the timing between symptom onset and ED presentation was variable. However, we did try to minimize this potential confounder by only selecting patients that had initial sampling performed within 2.5 h of symptom onset. In our microarray, severe reactions were triggered primarily by NSAID and beta-lactam antibiotics, whereas moderate reactions were induced by foods. We have previously demonstrated that medications are more likely to cause severe anaphylaxis; however, the dichotomy between drugs and foods in the microarray cohort was unusual.²⁸ These limitations

were partially addressed in the validation cohort, with 50% of severe reactions being caused by drugs. In addition, while in the microarray cohort moderate reactions were all caused by foods, only 39% were caused by foods in the validation cohort. The validation cohort also included individuals with alternate triggers, primarily bee stings. As the vast majority of drug-triggered reactions were orally administered, both in severe and moderate cases, we do not believe the route of exposure has affected results. The Human Gene 2.1 ST Array, while the most advanced technique available when these samples were analysed, is limited by the analysis of well-annotated genes. The application of RNA sequencing in future studies will have the advantage of identifying novel uncharacterized genes and may advance the discovery in this field. Notwithstanding these limitations, the findings from our study shed a unique level of insight into the pathogenesis of anaphylaxis and highlight snoRNA networks as pathways contributing to reaction severity and as potential biomarkers.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

All authors were involved in editing, review and approval of the final manuscript. FM contributed towards writing of the original manuscript draft, performed laboratory experiments, data analysis and data interpretation. AF contributed towards study concept and design, data curation and data interpretation. DF contributed towards study design and concept. SM contributed towards data curation and investigation. GA contributed towards study design, data curation and investigation. DF, SM and GA contributed towards participant recruitment. AB contributed towards study concept and methodology. AW contributed towards data analysis and interpretation. EB contributed towards study concept, data curation, study design and interpretation of data.

ETHICAL STATEMENT

Ethics approval was obtained from the Human Research Ethics Committees of each participating site (Royal Perth Hospital, Fremantle Hospital, 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 could

subsequently choose to provide delayed informed consent or withdraw from the study.

DATA AVAILABILITY STATEMENT

Microarray data from which the findings of this study were generated are available in the Gene Expression Omnibus at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wpenmkucldxatcr&acc=GSE69063>, reference number GSE69063. Other data pertaining to this study can be made available upon request.

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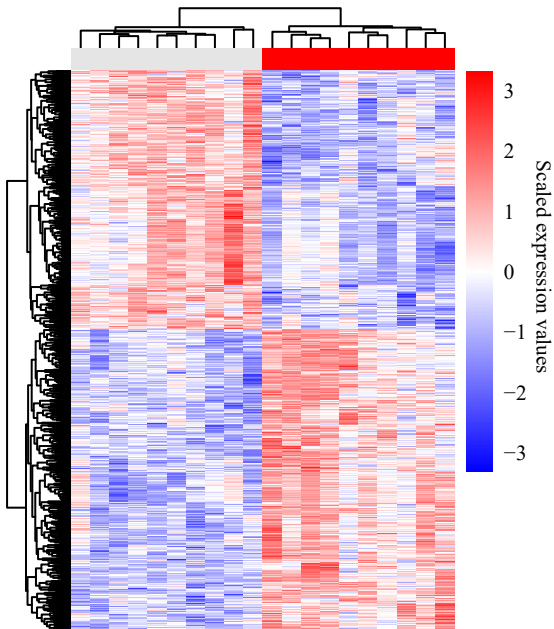
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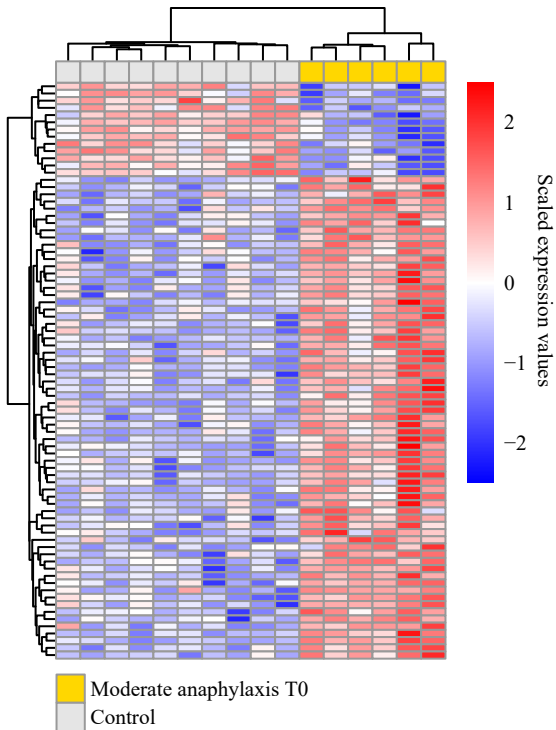
SUPPORTING INFORMATION

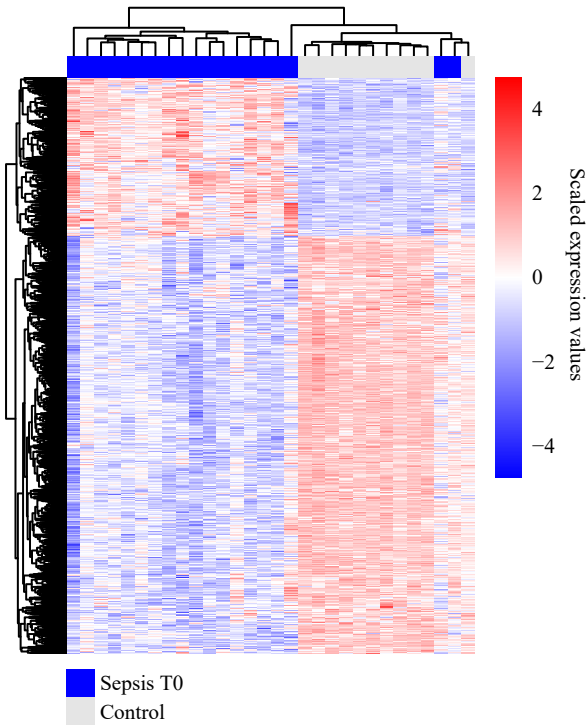
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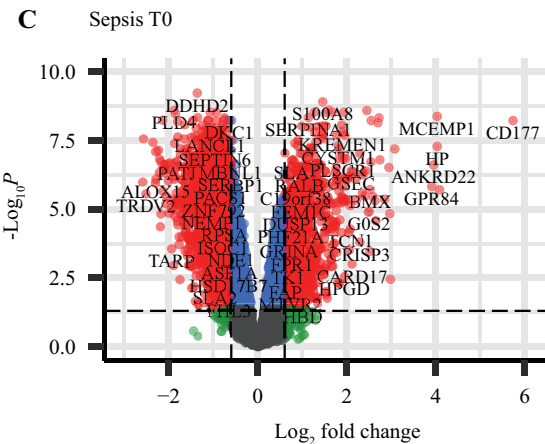
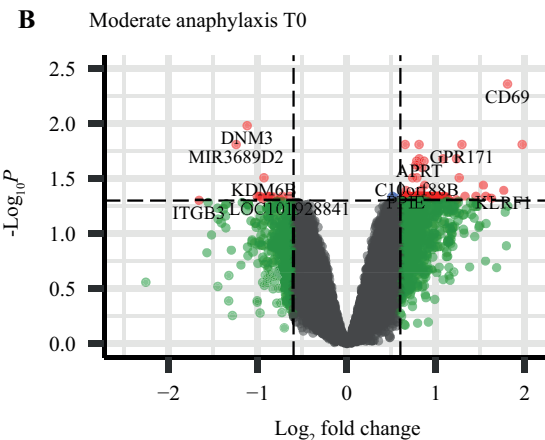
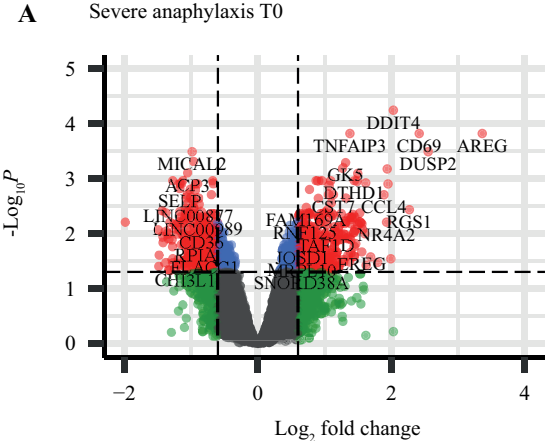
How to cite this article: McGrath FM, Francis A, Fatovich DM, et al. Small nucleolar RNA networks are up-regulated during human anaphylaxis. *Clin Exp Allergy*. 2021;00:1-12. <https://doi.org/10.1111/cea.13982>



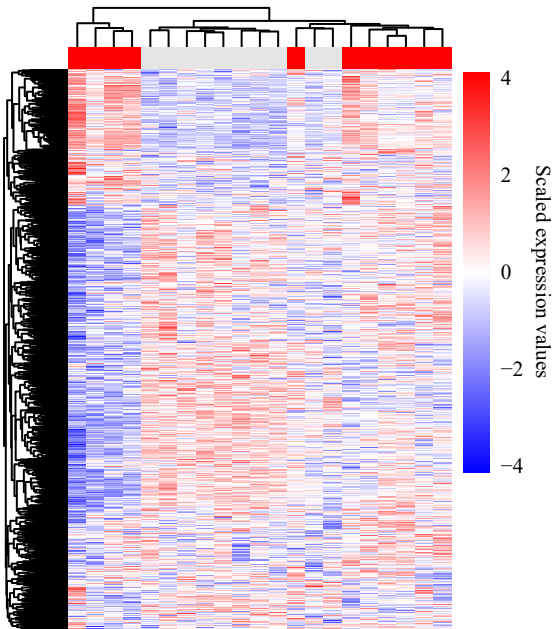
Severe anaphylaxis T0
Control



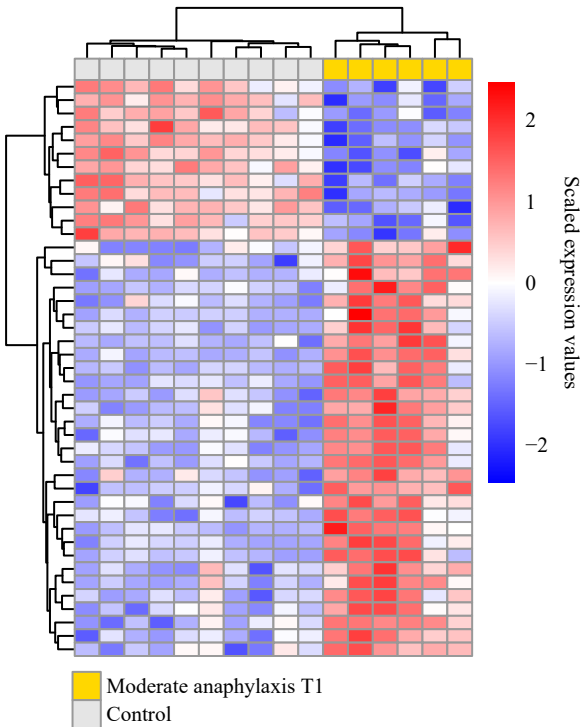


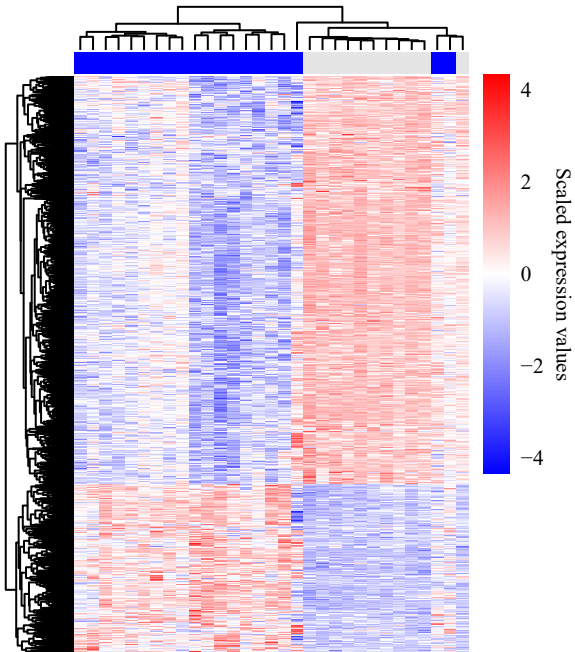


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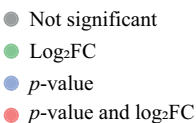
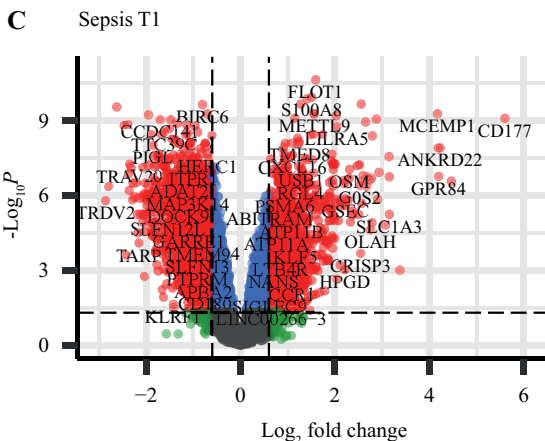
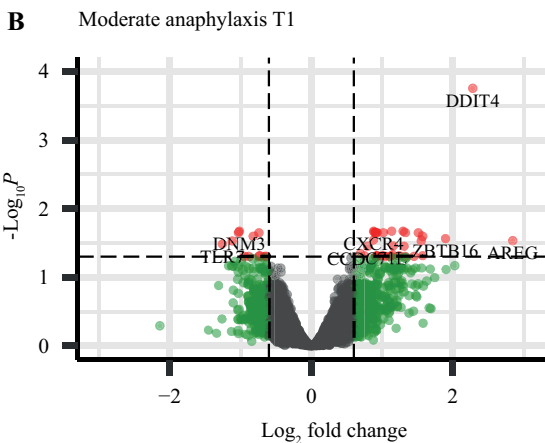
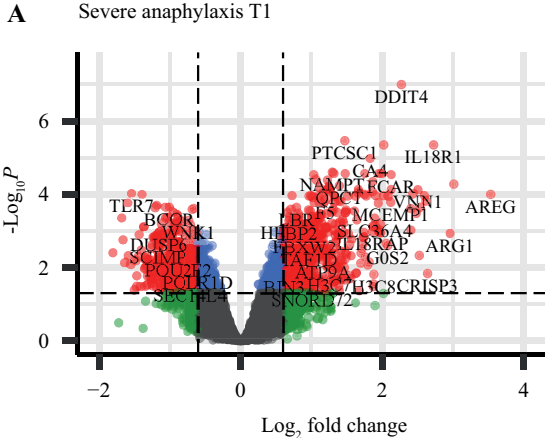


Severe anaphylaxis T1
Control

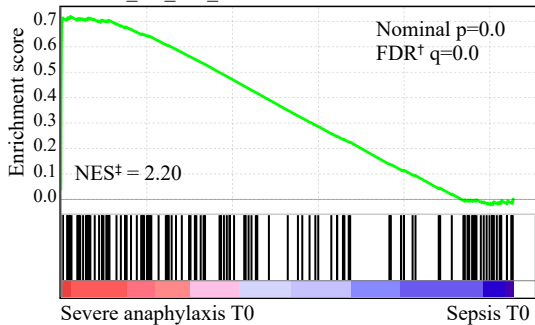




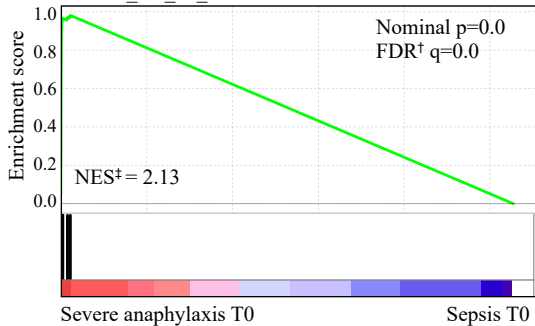
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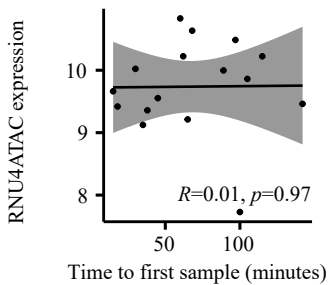
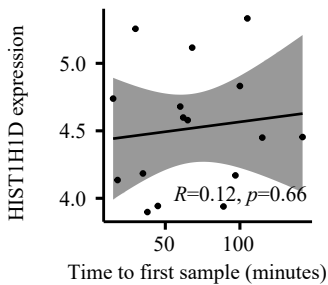
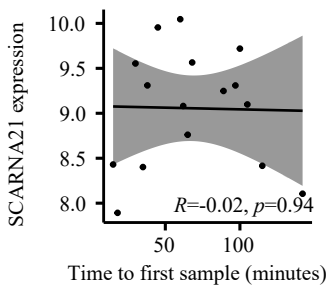
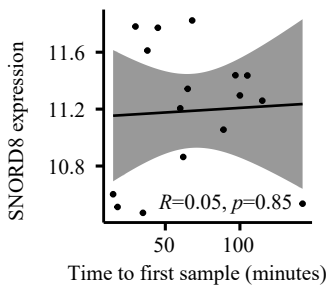
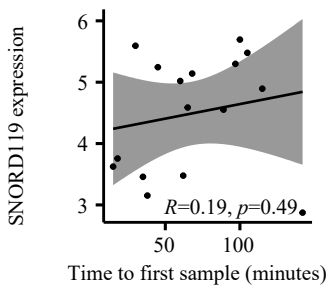
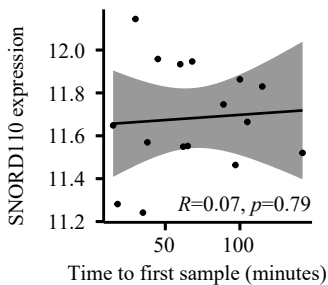
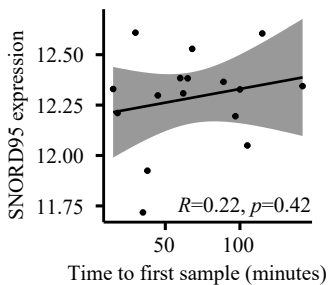
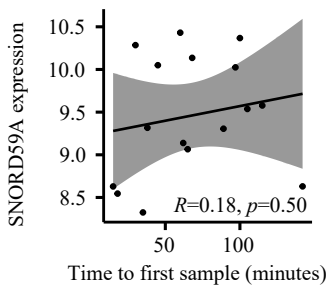
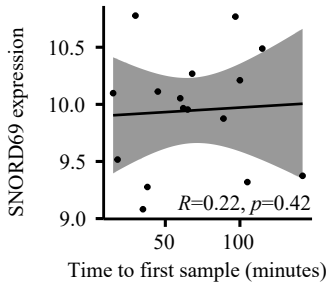
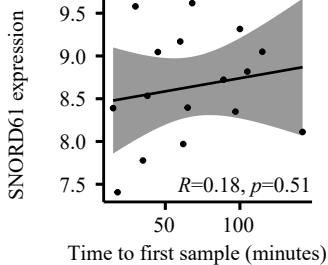


A PANEL_OF_159_GENES



B PANEL_OF_46_GENES





Supporting Information

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Tables

Table S1: Severe anaphylaxis gene signature subset (159 genes). Genes selected include those significantly upregulated ($p < 0.05$) in microarray analysis of severe anaphylaxis compared to sepsis, or shown to have ‘core enrichment’ through GSEA analysis of hallmark ‘P53_PATHWAY’ and ‘PI3K_AKT_MTOR_SIGNALING,’ comparing severe anaphylaxis and healthy controls. The set of 159 genes were then assessed by GSEA to define an anaphylaxis gene signature with ‘core enrichment’ in severe anaphylaxis compared to sepsis, revealing 46 genes (highlighted in red).

Microarray severe anaphylaxis vs sepsis		Core enrichment PI3K_AKT_MTOR_SIGNALING	Core enrichment P53_PATHWAY		
SNORD61	RPS27A	CXCR4	DDIT4	RAD51C	RRAD
SNORD95	ZMYND11	FASLG	SOCS1	CCP110	GADD45A
SNORD44	ATP5F1A	CDK1	BTG1	VAMP8	PVT1
SNORD8	SNORD3D	PIK3R3	TOB1	INHBB	DCXR
SNORD87	SNORD59B	TRIB3	PCNA	CGRRF1	NUDT15
SNORD110	FKBP2	CDKN1B	FOS	CDK5R1	WWP1
LOC100287834	TAOK3	LCK	ADA	TAP1	EI24
SNORD14B	ANTXRLP1	TBK1	F2R	CYFIP2	TRIAP1
SNORD42A	SNORD59A	SLA	DNTTIP2	PROCR	WRAP73
SNORD69	DDIT4	RPTOR	RPL36	PLK3	TP63
RNU4ATAC		NCK1	RCHY1	BAX	S100A10
SNORD91B		IRAK4	TSPYL2	AEN	GLS2
SCARNA21		PRKAG1	SESN1	IRAK1	
SNORA33		PIN1	HBEGF	FDXR	
SNORD15B		PRKAR2A	RPS27L	RRP8	
SNORD30		MAP2K6	ANKRA2	IER3	
HIST1H1D		VAV3	PITPNC1	CCND3	
HNRNPA1P10		THEM4	TRIB3	RAP2B	
RPS2		EIF4E	CCNG1	IL1A	
SNORD81		CAMK4	SAT1	TNFSF9	
SNRPD3		CDK2	TXNIP	DDIT3	
HNRNPR		ACACA	BTG2	PRMT2	
SNORD46		DDIT3	CDKN2AIP	RB1	
SNORD28		HSP90B1	DDB2	STOM	
SNORD60		IL2RG	ZBTB16	CD81	
NCL		NOD1	HINT1	SLC19A2	
HNRNPM		MAPK8	PLK2	CSRNP2	
PNN		PPP2R1B	UPP1	RHBDF2	
SNORD119		CALR	RALGDS	PERP	
SNHG6		UBE2N	ERCC5	RACK1	
SNORD21		RIPK1	BAK1	SEC61A1	
C11orf1		ACTR3	SLC35D1	SLC3A2	
SNORD63		NFKBIB	ATF3	CTSF	
FAM98B		MAP3K7	CCND2	NOL8	
CEP128		YWHAB	RPL18	JUN	

Figure Legends

Figure S1: Heatmap of the 568 differentially expressed genes identified in microarray analysis of severe anaphylaxis compared to controls at T0. Heatmap was generated in R, with scaled expression values represented as a color spectrum from low (blue) to high (red). Relationships between genes and patients are represented along the top (samples) and left (genes) borders.

Figure S2: Heatmap of the 80 differentially expressed genes identified in microarray analysis of severe anaphylaxis compared to controls at T0. Heatmap was generated in R, with scaled expression values represented as a color spectrum from low (blue) to high (red). Relationships between genes and patients are represented along the top (samples) and left (genes) borders.

Figure S3: Heatmap of the 3364 differentially expressed genes identified in microarray analysis of sepsis compared to controls at T0. Heatmap was generated in R, with scaled expression values represented as a color spectrum from low (blue) to high (red). Relationships between genes and patients are represented along the top (samples) and left (genes) borders.

Figure S4: Volcano plots highlighting significant genes, identified through microarray analysis, at T0 in A severe anaphylaxis, B moderate anaphylaxis, and C sepsis, compared to controls. Volcano plots generated in R. Genes were identified as: not significant (grey circles), significant Log₂FC (blue circles), significant adjusted *p*-value (green circles), or significant adjusted *p*-value and Log₂FC (red circles).

Figure S5: Heatmap of the 1103 differentially expressed genes identified in microarray analysis of severe anaphylaxis compared to controls at T1. Heatmap was generated in R, with scaled expression values represented as a color spectrum from low (blue) to high (red).

Relationships between genes and patients are represented along the top (samples) and left (genes) borders.

Figure S6: Heatmap of the 43 differentially expressed genes identified in microarray analysis of moderate anaphylaxis compared to controls at T1. Heatmap was generated in R, with scaled expression values represented as a color spectrum from low (blue) to high (red). Relationships between genes and patients are represented along the top (samples) and left (genes) borders.

Figure S7: Heatmap of the 3454 differentially expressed genes identified in microarray analysis of sepsis compared to controls at T1. Heatmap was generated in R, with scaled expression values represented as a color spectrum from low (blue) to high (red). Relationships between genes and patients are represented along the top (samples) and left (genes) borders.

Figure S8: Volcano plots highlighting significant genes, identified through microarray analysis, at T1 in A severe anaphylaxis, B moderate anaphylaxis, and C sepsis, compared to controls. Volcano plots generated in R. Genes were identified as: not significant (grey circles), significant Log₂FC (blue circles), significant adjusted *p*-value (green circles), or significant adjusted *p*-value and Log₂FC (red circles).

Figure S9: GSEA of 159 genes identified as a signature of severe anaphylaxis using gene expression data from severe anaphylaxis (T0) and sepsis patients. A Subset of 159 genes including those upregulated in severe anaphylaxis compared to sepsis in microarray analysis ($p < 0.05$), and genes with ‘core enrichment’ in p53 and PI3K Hallmark pathways comparing severe anaphylaxis (T0) and healthy (GSEA). **B** GSEA of the 46 genes with ‘core enrichment’ from panel A. [†]FDR (false discovery rate), [‡]NES (normalized enrichment score).

Figure S10: Correlation of gene expression patterns at ED arrival with the time lag from onset of symptoms to blood collection. All 16 anaphylaxis patients from the microarray cohort with T0 samples were used in this analysis. Only the 10 small nucleolar RNAs chosen for validation were assessed. Microarray signal intensity was used to determine gene expression levels. Grey area represents 95% confidence intervals. R is the correlation coefficient (scale of 0-1) where a value of 0.4 or less shows weak or no correlation.