Distinct inflammatory responses differentiate cerebral infarct from transient ischaemic attack


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Abstract: We previously reported on a 26 year-old patient who presented early during a large and eventually fatal cerebral infarct. Microarray analysis of blood samples from this patient demonstrated initially up-regulated and subsequently down-regulated Granzyme B (GzmB) expression, along with progressive up-regulation of genes for S100 calcium binding protein A12 (S100A12) and Matrix metalloproteinase 9 (MMP-9). To confirm these findings, we investigated these parameters in patients with suspected stroke presenting within 6 hours of symptom onset to a single centre. Blood samples were taken at enrolment, then 1 hour, 3 hours and 24 hours post-enrolment for the examination of cellular, protein and genetic changes. Patients with subsequently confirmed ischaemic (n=18) or haemorrhagic stroke (n=11) showed increased intracellular concentrations of GzmB in all cell populations investigated (CD8+, CD8- and Natural Killer (NK) cells). Infarct patients however, demonstrated significantly reduced GzmB gene expression and increased circulating MMP-9 and S100A12 levels in contrast to TIA patients or healthy controls. Furthermore, a pronounced neutrophilia was noted in the infarct and haemorrhage groups, while TIA patients (n=9) reflected healthy controls (n=10). These findings suggest a spectrum of immune response during stroke. TIA showed few immunological changes in comparison to infarct and haemorrhage, which demonstrated inhibition of GzmB production and a rise in neutrophil numbers and neutrophil-associated mediators. This implies a greater role of the innate immune system. These markers may provide novel targets for inhibition and reduction of secondary injury.
DISTINCT INFLAMMATORY RESPONSES DIFFERENTIATE CEREBRAL INFARCT FROM TRANSIENT ISCHAEMIC ATTACK

Article category: Laboratory study

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Highlights

- Infarct and haemorrhage show neutrophilia, transient ischaemic attack (TIA) does not.
- In infarct, Granzyme B (GzmB) expression is downregulated while matrix metalloproteinase 9 (MMP-9) and S100A12 increase.
- Infarct shows increases in circulating MMP-9 and S100A12, while TIA does not.
- GzmB accumulates intracellularly during stroke and is not released.
- Different mechanisms underlie TIA and infarct.
Laboratory studies

Distinct inflammatory responses differentiate cerebral infarct from transient ischaemic attack

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Conflicts of Interest/Disclosures

The authors declare that they have no financial or other conflicts of interest in relation to this research and its publication.
Abstract

We previously reported on a 26-year-old patient who presented early during a large and eventually fatal cerebral infarct. Microarray analysis of blood samples from this patient demonstrated initially up-regulated and subsequently down-regulated Granzyme B (GzmB) expression, along with progressive up-regulation of genes for S100 calcium binding protein A12 (S100A12) and matrix metalloproteinase 9 (MMP-9). To confirm these findings, we investigated these parameters in patients with suspected stroke presenting within 6 hours of symptom onset to a single centre. Blood samples were taken at enrolment, then 1 hour, 3 hours and 24 hours, post-enrolment for the examination of cellular, protein and genetic changes. Patients with subsequently confirmed ischaemic (n = 18) or haemorrhagic stroke (n = 11) showed increased intracellular concentrations of GzmB in all cell populations investigated (CD8+, CD8- and Natural Killer [NK] cells). Infarct patients however, demonstrated significantly reduced GzmB gene expression and increased circulating MMP-9 and S100A12 levels in contrast to transient ischaemic attack (TIA) patients or healthy controls. Furthermore, a pronounced neutrophilia was noted in the infarct and haemorrhage groups, while TIA patients (n = 9) reflected healthy controls (n = 10). These findings suggest a spectrum of immune response during stroke. TIA showed few immunological changes in comparison to infarct and haemorrhage, which demonstrated inhibition of GzmB production and a rise in neutrophil numbers and neutrophil-associated mediators. This implies a greater role of the innate immune system. These markers may provide novel targets for inhibition and reduction of secondary injury.
Keywords: Granzyme B; Inflammatory markers; MMP-9; Neutrophils; S100A12; Stroke.

1. Introduction

Neuronal injury during ischaemic stroke and subsequent neurologic deficit result from a combination of primary and secondary factors, the former being dependent on the location, volume[1] and duration of ischaemia. Following the initial ischaemic insult, multiple pro-inflammatory enzymes and signaling pathways are activated. This results in recruitment and subsequent migration of peripheral leucocytes into the area of infarction where they potentiate further inflammation and cell apoptosis within the ischaemic core and penumbra [2].

We previously reported on a 26-year-old woman who presented very early after the onset of a large and subsequently fatal middle cerebral artery infarct[3] and who was enrolled in our Critical Illness and Shock study [4]. Blood samples collected from this patient underwent gene expression analysis by microarray, which were compared to the patient’s healthy identical twin and other healthy controls. In the first sample collected, this microarray analysis showed marked upregulation of Granzyme B (GzmB), a serine protease most commonly found in cytotoxic T cells (CTLs) and natural killer cells (NK cells), known to activate intracellular caspases to induce apoptosis [5]. This early GzmB upregulation then declined over time, whilst a progressive upregulation of other inflammatory and immunomodulatory genes, including S100 calcium binding protein A12 (S100A12, also known as calgranulin
C), matrix metalloproteinase 9 (MMP-9) and interleukin 1 receptors, was demonstrated.

In this study we aimed to further investigate these findings to ascertain if they were reproducible in a larger patient group. Given the similar pathophysiology of transient ischaemic attack (TIA) and stroke, we also aimed to explore any differences in the inflammatory and immune responses between the two conditions. Differences between these markers may be useful in the differentiation of ischaemic stroke from TIA or stroke mimics, and present a potential target for inhibition and therefore reduction of secondary injury.

2. Methods

2.1. Study design

This single centre, prospective convenience study was conducted at the Royal Perth Hospital Emergency Department. Ethics approval was obtained from the Royal Perth Hospital Research Ethics Committee, approval number HREC: 2011-142. Patients over 18 years of age, presenting with suspected stroke, were screened by research staff to ensure adherence to inclusion criteria and were enrolled with informed consent. Patients suitable for inclusion presented within 6 hours of symptom onset, or within 1 hour of waking with symptoms. Blood sampling was performed at enrolment (Emergency Department [ED] arrival, T0), 1 hour (T1), 3 hours (T3) and 24 hours (T24) post-enrolment. EDTA-treated whole blood was collected for full blood count, (Royal Perth Hospital pathology service [PathWest]), and flow cytometry. Serum was
obtained for inflammatory marker analyses and mRNA isolation from whole blood was completed following blood collection using the PAXgene system (PreAnalytiX GmbH, Hombrechtikon, Switzerland) at each time point. Diagnostic grouping for the purpose of our analysis was based on discharge diagnosis: haemorrhage and infarct groups having evidence of such on either initial or follow up CT scan/MRI, and TIA patients having non-diagnostic imaging and resolution of symptoms by 24 hours. A consort diagram defining the samples available for study is provided in supplementary Figure 1. Healthy controls were recruited via a targeted blood drive with consent by default, following approval by the Royal Perth Hospital Human Research Ethics Committee. Ten of these healthy controls were then sex and age-matched to the final patient cohort and used as comparators for all subsequent analyses.

2.2. Extraction and quality control of RNA

RNA was extracted using PAXgene Blood RNA Extraction Kits (PreAnalytiX GmbH) by automation with the Qiacube instrument (Qiagen, Chadstone Centre, VIC, Australia). RNA purity was assessed by NanoDrop (Thermo Scientific, Thermo Fisher Scientific, Scoresby, VIC Australia). Samples with total RNA <1 μg were excluded.

2.3. Synthesis of complementary DNA

Complementary DNA (cDNA) was synthesised using 1 μg RNA, 200 ng random primers and 10 mM dNTPs (Invitrogen Life Technologies, Australia), incubated at
65°C for 5 min. Superscript III reverse transcriptase (200 units), first-strand buffer, 40 units of RNase inhibitor and 100 mM dithiothreitol (Invitrogen Life Technologies, Thermo Fisher Scientific) were added and incubated at 25°C for 5 min then at 50°C for 50 min. This was followed by heat inactivation at 70°C for 15 min. RNase H (1 unit; New England BioLabs, Ipswich, Massachusetts, USA) was added and incubated for 20 min at 37°C. Storage of cDNA was at -20°C.

2.4. Quantitative PCR (qPCR)

The measurement of mRNA levels of target genes was performed following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines [6]. PCR reactions were performed in a total volume of 10 µl, comprising 37.5 ng of each primer (Supplementary Table 1), 0.5 µl of ResoLight Dye (Roche Diagnostics, Dee Why, NSW, Australia), 1 µl of 10x PCR buffer, 5 mM MgCl₂, 0.2 mM dNTPs, 0.33 Units Platinum Taq DNA Polymerase (Invitrogen Life Technologies, Thermo Fisher Scientific) and 2 µl of 1/10 cDNA. Cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, annealing temp (Supplementary Table 1) for 15 sec and 72°C for 15 sec. A dissociation curve was established as follows: Samples were ramped from 60°C to 95°C stepwise at 0.05°C per second. Reactions were performed in triplicate and were optimised for temperature and magnesium concentration (Rotorgene 6000; Viia 7, Applied Biosystems, Thermo Fisher Scientific). Dissociation profiles were used to check for single product amplification.
2.5. Template cloning for standard curve preparation

RNA extracted from peripheral blood leukocytes stimulated overnight with phorbol myristate acetate was used to prepare cDNA. Amplification of targets of interest was carried out using the same primers used for qPCR and products were ligated into pGEM-T Easy (Promega, Alexandria, NSW, Australia). JM109 Competent cells (Promega) 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 by Sanger sequencing. Plasmids were linearised with AatII (New England Biolabs) and standard curves were prepared immediately prior to each run.

2.6. Analysis using qBase plus

Viia7 software determined Cq values using the Baseline Threshold algorithm. Two reference genes; Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Hypoxanthine Guanine Phosphoribosyltransferase (HPRT), were determined as appropriate to normalise Cq data using qBase plus software, v. 2.6 (Biogazelle, Gent, Belgium). Replicates that varied by greater than 0.8 Cq were excluded.

2.7. Measuring serum protein levels

Serum concentrations of MMP-9, S100A12 and GzmB were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (MMP-9: DuoSet R&D Systems Inc. Minneapolis, MN, USA; S100A12: CircuLex, MBL Life Science,
Naka-ku, Nagoya, Japan; GzmB: MAbTech, Sweden) according to the manufacturer's instructions. Intra and interassay coefficients of variation (CVs) were 5.50% and 5.87% for the MMP-9 ELISA, 8.71% and 15.31% for the GzmB ELISA and 6.37% and 16.4% for the S100A12 ELISA.

2.8. Flow cytometry

Erythrocytes were lysed from 200µl EDTA-treated whole blood by gentle rocking for 10 min at room temperature following the addition of 1ml NH₄Cl lysis solution (0.15M NH₄Cl, 0.01M K₂CO₃ and 0.08mM tetrasodium EDTA). At the completion of the incubation, samples were diluted with 9 ml fluorescence activated cell sorting (FACS) buffer (1% bovine serum albumen [BSA] in phosphate buffered saline [PBS]) and centrifuged for 10 min at 1500 rpm. Pellets were resuspended in FACS buffer prior to staining for 15 min, in the dark, with anti CD3-PE-Cy7, anti CD8-APC, CD56-AF488 and anti CD16-APC-H7 antibodies (all antibodies from BD Pharmingen, BD Biosciences, San Diego, CA, USA), together with propidiium iodide in a final staining volume of 50 µl. Samples were washed twice in FACS buffer prior to fixation for 10 min at 4°C in 50 µl cytofix solution (BD Biosciences). Following incubation, 1 ml of 1 times cytoperm/wash buffer (BD Biosciences) was added to each sample prior to centrifugation. Pellets were resuspended in 50 µl anti GzmB-PE antibody (BD Pharmingen, BD Biosciences) diluted in cytoperm/wash buffer, and incubated for 30 min at 4°C, protected from light. Samples were washed twice in cytoperm/wash buffer, prior to resuspension in FACS buffer and analysis using a FACSARia flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA).
2.9. Statistical analysis

Our primary analysis was descriptive, looking at changes in mRNA expression relative to infarct onset, CTL and NK cell numbers and the serum concentrations of GzmB and related inflammatory cytokines and chemokines. This exploratory approach enabled us to assess the likely pathophysiological processes involved.

Without information on laboratory peaks and timing, between-subject variability and the impact of factors such as stroke size and location, it was difficult to perform a sensible power analysis. However, on the basis of our previous inflammatory mediator studies into sepsis and anaphylaxis [7, 8], we estimated that a convenience sample of 60 patients, of which about half present very early (<2 hours), would be sufficient to provide useful information on the timing and peaks of important (i.e. frequently elevated) inflammatory mediators. Comparisons were made with 10 healthy age and sex-matched controls.

Given the small sample numbers available, data were analysed using non-parametric approaches. Data are presented as median (interquartile range [IQR]). In order to compare the medians of three or more groups, the Kruskal–Wallis test was used first. Following demonstration of significance, pairwise comparisons were then performed using the Mann–Whitney U (Wilcoxon rank-sum) test. When results were available from two groups only, the Mann–Whitney U test was performed directly. The Skillings–Mack test was used to test for differences over time within each patient group. Correction for multiple comparisons was not performed due to the exploratory nature of this study. Statistical analysis was performed with Stata version 13 (StataCorp, College Station, TX, USA).
3. Results

3.1. Overview of patients recruited

Among the 53 patients enrolled from April 2013 to September 2014, 27 (17 male, median age 70.0 [IQR 59–83] years) met the inclusion criteria of CT scan or MRI evidence of brain infarction (n = 18) or a clinical diagnosis of TIA (n = 9) without any evidence of infarction on CT scan only (n = 5), or CT scan and MRI (n = 4). Of the remaining enrolled patients, 11 had an intra-cerebral haemorrhage (four male, median age 79 [IQR 66–84] years); eight were lost to analysis from insufficient samples; three patients with a persistent neurologic deficit diagnosed as stroke were excluded because the diagnosis was not confirmed by neuroimaging; three were excluded following subsequent non-infarct/TIA diagnosis (cardiac syncope, unspecified weakness and possible migraine); and one patient withdrew consent. There were seven deaths. Patient clinical characteristics are summarised in Supplementary Table 2.

All patients underwent examination to ascertain their National Institutes of Health Stroke Score (NIHSS) with a median score of 6 (IQR 4–19) in the infarct group, 1.5 (IQR 0–3) for TIA patients and 13 (IQR 4–19) in the haemorrhage group. Comparisons were made against 10 healthy controls (six males, median age 72 [IQR 60–9] years).
The median time between symptom onset and T0 blood sampling for all patients in the study was 2.7 (IQR 1.5–3.4) hrs. Subsequent median times post-onset were: T1: 4.1 (IQR 2.9–4.6) hours; T3: 5.8 (IQR 4.8–6.5) hours and T24: 26.1 (IQR 24.7–8.1) hrs.

3.2. High intracellular Granzyme B observed early during stroke

Flow cytometric analysis demonstrated small differences in population percentages in some of the treatment groups when compared to those in healthy controls (data not shown). However, there was a clear indication of elevated intracellular GzmB content, as indicated by high mean fluorescence intensities (MFI) in these populations (Fig. 1).

The increases in MFI were noted in all three of the populations investigated (CD8+ T cells, CD8−T cells and NK cells). While these changes were not significant for infarct patients when compared to healthy controls, significant increases were observed in TIA patients in two of the three populations investigated (GzmB+CD8+ T cells; T1: p = 0.0147, vs. healthy controls) (GzmB+NK cells; T0: p = 0.033, T1: p = 0.0147, T3: p = 0.0147). Of interest, haemorrhage patients also showed significantly elevated GzmB MFI values versus healthy controls (GzmB+CD8+ T cells; T1: p = 0.0126) (GzmB+ CD8− T cells; T1: p = 0.0067) (GzmB+ NK cells; T0: p = 0.0034, T1: p = 0.0034, T3: p = 0.05). In all patients, the trends suggest increased intracellular concentrations of GzmB in the cell populations investigated, during the progression of stroke.

3.3. S100A12 and MMP-9 serum levels may distinguish infarct from TIA
Despite high intracellular levels of GzmB, circulating levels of the protein were low and did not change in any of the patient cohorts (data not shown). In contrast, circulating levels of S100A12 and MMP-9 proteins were different between the study groups (Fig. 2, Supp. Table 3). Significant differences were observed between infarct and TIA groups at T1 (p = 0.027) and T3 (p = 0.0105) for S100A12, while MMP-9 levels were significantly different between infarct and both TIA (p = 0.027, p = 0.0206) and healthy controls (p = 0.0030, p = 0.0049) at T3 and T24 respectively.

3.4. Neutrophilia is a feature of infarction and haemorrhage but not TIA

Neutrophil counts were significantly increased at all time points in infarct patients (p<0.017 compared to healthy controls for all time points). Alterations to this parameter in the haemorrhage group was even more pronounced, again demonstrating statistically significant increases in neutrophil count at all time points investigated (p<0.018 vs. healthy controls).

Eosinophil counts were significantly reduced at T1 (p = 0.0055, p = 0.0429), T3 (p = 0.0022, p = 0.0260) and T24 (p = 0.0154, p = 0.0086) in both infarct and haemorrhage respectively. In contrast, neutrophil and eosinophil counts in the TIA patients closely resembled the values observed in healthy controls (Fig. 3).

3.5. Early and rapid alteration in gene expression observed following infarct

A contrast in gene expression values and patterns of expression over time was observed between the two patient groups (Fig. 4). While gene expression levels for
TIA resembled the expression patterns of healthy controls, gene expression in the
infarct patients was different, and essentially in direct opposition to that observed in
the other groups.

Most notably, GzmB expression was lower in infarct patients than the TIA or control
groups at all time points, and significantly lower at T1 (p = 0.0145), T3 (p = 0.0027)
and T24 (p = 0.0016). GzmB expression showed a significant progressive reduction
over the study period (p = 0.0040, Skillings–Mack). This response suggests that
GzmB gene expression is being actively suppressed in the infarct patients, in contrast
to patients presenting with a TIA, who did not have significant alterations in gene
expression.

Both MMP-9 and S100A12 expression were significantly increased in infarct patients
with the largest increases observed at T1 and T3 (MMP-9: T0: p = 0.0165, T1: p =
0.0165, T3: p = 0.0153, infarct vs. healthy controls) (S100A12: T1: p = 0.0054, T3: p
= 0.0037, infarct vs. healthy controls). As previously observed for GzmB, gene
expression levels in TIA patients resembled that in healthy controls. For both of these
genes, infarct patient expression levels approached those in healthy controls by T24.

4. Discussion

Our original patient report detailed an early rise in GzmB expression (1 hour after
onset of symptoms) followed by significant downregulation of this gene. This study
was designed to explore this phenomenon in a pilot cohort of stroke patients, together
with other candidate inflammatory markers in order to determine the significance of such a response following cerebral infarct.

The progressive downregulation of GzmB expression seen in the initial patient was replicated in the infarct patients in this study. Whilst the early rise in GzmB expression was not observed, there was a substantial difference in elapsed time between time of onset and T0 in the patient report (one hour) and this study (2.7 hours). GzmB is an important component of the apoptotic pathway predominantly mediated by CTL and NK cells, but is also important during immune activation processes [5, 9]. It is also believed to be an important mediator of ischaemic brain injury following stroke [10]. It is possible that the downregulation of GzmB gene expression observed is an innate protective mechanism, attempting to minimise damage to neural tissue following the onset of stroke by dampening down pro-apoptotic signals. In support of this, we found that intracellular GzmB content increased in the cytotoxic cell populations investigated in all patient groups, but was not released into the circulation, suggesting that GzmB secretion may be inhibited in these cells. Together with down-regulation of gene expression, it would appear that mechanisms to avoid GzmB production and release are triggered during the onset of stroke.

While these adaptive responses appear to be downregulated, there was evidence of strong innate responses during cerebral infarct. This was demonstrated by the presence of neutrophilia in the infarct and haemorrhage groups, increases in early MMP-9 and S100A12 gene expression, as well as increases in the circulating levels of MMP-9 and S100A12 proteins. While these changes occurred during cerebral infarct,
no such changes were seen in the TIA group, which reflected healthy controls. These proteins have been shown to be functionally important in ischaemic stroke and a potential predictor of poor outcome [11, 12]. MMP-9 is a mediator of cell migration (especially neutrophils) across basement membranes and is thought to play a detrimental role in ischaemic stroke, being associated with increased breakdown of the blood-brain barrier [13]. S100A12 is also a product of granulocytes (i.e. neutrophils) and a component of the innate immune response. High levels of S100A12 (also known as the ligand for the receptor for advanced glycation end products [EN-RAGE]) are associated with a number of inflammatory disorders [14].

Our results suggest that the pathology of ischaemic stroke may have more to do with the innate immune response through neutrophil-mediated damage, as shown by the MMP-9 and S100A12 responses; and less to do with an adaptive immune response via NK and CTLs, as suggested by the down-regulation of GzmB gene expression and apparent reduced GzmB secretion. Of interest, the alterations seen in the adaptive immune response in all groups contrasted with the significant change in innate responses only seen in patients with established infarct or haemorrhage. Given our current understanding of TIAs being caused by either a temporary interruption in circulation or a small, compensated area of infarct, the differences between the innate inflammatory responses observed in TIA, infarct and haemorrhage may be a graded response to the volume and duration of tissue injury.

The results of this study provide evidence of increasing immune response severity between cerebral infarct and TIA and add further weight to the body of evidence suggesting the use of MMP-9 and S100A12 as potential biomarkers or therapeutic
targets. This may have important implications given the difficulties in differentiating ischemic stroke and TIA early following onset, and the absence of reliable biomarkers for the diagnosis of ischaemic stroke [15-18]. A larger study is needed however to clarify the mechanisms and significance behind the decreased expression of GzmB in the setting of acute stroke, along with the role of neutrophils in the secondary inflammatory process.

4.1. Limitations

This was a single centre exploratory convenience study that is hypothesis generating. The small sample size cannot address the heterogeneity of stroke and stroke mimics nor the influence of treatment or co-morbidity. A larger study is required to assess these factors.

5. Conclusion

Our findings suggest that neutrophils play a significant role in the evolution of cellular injury after stroke. We found progressive downregulation of GzmB gene expression over time and sustained increased intracellular concentrations in CTL and NK cells. We also observed significant upregulation of neutrophil-associated markers S100A12 and MMP-9 in the infarct group, whereas TIA had similar levels to healthy controls, indicating a potential damage-response relationship. These markers may be useful in the differentiation of ischaemic stroke from TIA or stroke mimics, and present a potential target for inhibition and therefore reduction of secondary brain injury.
Acknowledgements

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References


**Figure legends**

Fig. 1. Time course assessment of Granzyme B (GrzB) expression on lymphocyte subsets in stroke patient groups. Granzyme B expression was determined by flow cytometric analysis of whole blood collected at defined time points post-presentation to the Emergency Department (ED). Granzyme B expression levels were defined by measurements of mean fluorescence intensity (MFI) in lymphocyte populations of interest. Data describe median and interquartile ranges for the respective group MFI values.

T0 = ED arrival and 0 hours post enrolment, T1 = 1 hour post enrolment, T3 = 3 hours post enrolment, T24 = 24 hours post-enrolment.

- - - Median value of Healthy Control group.

NK cells = natural killer cells, TIA = Transient ischaemic attack.

Fig. 2. Differences in serum S100 calcium binding protein A12 (S100A12) and Matrix metalloproteinase 9 (MMP-9) levels in stroke patient groups. S100A12 and MMP-9 levels were determined by enzyme-linked immunosorbent assay (ELISA) in patient serum samples collected at enrolment (T0), 1hr (T1), 3 hrs (T3) and 24 hrs
(T24) post-presentation to the Emergency Department following the onset of stroke. Data represent the median and interquartile range values of concentrations in each group. A detailed data summary including outlier information is available in Supplementary Table 3.

- - - Median value of Healthy Control group.

TIA = transient ischaemic attack.

Fig. 3. Comparison of neutrophil and eosinophil numbers present in stroke patient groups. Absolute cell counts were determined from samples of whole blood collected at defined time points post-presentation to the Emergency Department following the onset of stroke. Median cell counts per group at each time point are reported together with group interquartile ranges.

T0 = ED arrival and 0 hours post-enrolment, T1 = 1 hour post-enrolment, T3 = 3 hours post-enrolment, T24 = 24 hours post-enrolment.

- - - Median value of Healthy Control group.

TIA = transient ischaemic attack.

Fig. 4. Changes in the gene expression profile of Granzyme B (GzmB), Matrix metalloproteinase 9 (MMP-9) and S100 calcium binding protein A12 (S100A12) in stroke patient groups over time. RNA was extracted from whole blood collected from patients during the course of stroke. Gene expression was determined by quantitative polymerase chain reaction (qPCR) and Calibrated Normalized Relative Quantity (CNRQ) calculated using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and
Hypoxanthine Guanine Phosphoribosyltransferase (HPRT) as calibrators in samples run in triplicate. Data represent the median and interquartile range (IQR) of the CNRQ values for each group IQR.

- - - Median value of Healthy Control group.

TIA = transient ischaemic attack.
Figure 3
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