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ORIGINAL ARTICLE

Correlation of Adrenomedullin gene expression in peripheral blood leukocytes with severity of ischemic stroke

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Human adrenomedullin (ADM), a 52-amino acid peptide, belongs to the calcitonin/calcitonin gene-related peptide (CGRP)/amylin peptide family. ADM acts as a multifunctional regulatory peptide and is upregulated in response to hypoxia. Previous microarray studies have found increased ADM gene (*ADM*) expression in peripheral blood cells of patients with stroke, however, it is unknown if an increased *ADM* level is correlated with severity of human ischemic stroke. This study investigated *ADM* expression in peripheral blood leukocytes (PBL) of healthy controls and subjects at day 1, week 1 and week 3 postacute ischemic stroke using rtPCR methodology. We found that *ADM* expression was significantly upregulated on the first day of stroke compared to the healthy subjects and the disease controls; the levels remained elevated for up to week 3. Further, *ADM* expression at day 1 was correlated with stroke severity measured by the National Institute of Healthy Stroke Scale (NIHSS), the modified Barthel Index (mBI) and the modified Rankin Scale (mRS). This could indicate that *ADM* expression level is related to the severity of tissue damage. We suggest that increased *ADM* expression in PBL after acute ischemic stroke is most likely to indicate that these cells have been subjected to hypoxia and that the magnitude of expression is likely to be related to the volume of hypoxic tissue. Hypoxia can affect lymphocytes function and could affect the immune response to stroke. The correlation of *ADM* expression level with the measures of stroke severity implicates *ADM* – a potential blood bio-marker in studies of ischemic stroke.

KEYWORDS: gene expression, rtPCR, microarray, NIHSS, mBI, mRS

Introduction

Acute ischemic stroke is resultant from a sudden loss of blood supply to focal areas of the brain. This leads to cell death and local inflammation. Systemic responses induced by ischemic stroke include modulation of the immune system and alteration of gene expression in peripheral blood cells [1–3].

Adrenomedullin (ADM) is a 52 amino acid peptide, belongs to the calcitonin/CGRP (calcitonin gene-related peptide)/amylin peptide family. The ADM gene (*ADM*) is located on chromosome 11 with a single locus [4]. ADM was originally isolated from human pheochromocytomas [5,6]. The *ADM* product exists in plasma and a variety of tissues including blood vessels, heart and lungs [7], however, measurement of the ADM protein presents difficult due to a short half-life [8,9]. The biological activities of ADM has been investigated intensively and include vasodilation, diuresis and natriuresis, positive inotropic effect, inhibition of endothelial cell apoptosis, induction of angiogenesis, inhibition of cardiomyocyte apoptosis, suppression of aldosterone production, anti-inflammatory activity and antioxidant activity [7].

ADM has been shown to be protective in ischemic stroke. In a rat model, postischemic infusion of ADM has been shown to protect against ischemic stroke by inhibiting apoptosis and promoting angiogenesis [10]. ADM also plays important autocrine and paracrine roles by regulating circulation and endothelial blood-brain barrier functions [11]. Evidence from cell culture and animal model studies have shown that ADM has

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protective effects against vascular injury, including the effects of oxidative stress [7]. Increased expression of both *ADM* and a component of the ADM receptor, Calcitonis-receptor-like receptor (CRLR), have been found in cultured endothelial cells under hypoxic conditions [7,11]. Treatment with ADM has been thought to limit infarct size through mitochondrial K_{Ca} in PKA signaling pathways [12].

A study using the microarray technique examined gene expression profile in peripheral blood mononuclear cells of human acute ischemic stroke and showed that *ADM* level was increased [13]. Further studies have shown that *ADM* level is regulated by hypoxia-inducible factor-1 in other diseases or tissues [14–16].

It is unknown if endogenous *ADM* levels are correlated with stroke severity postischemia. Our study aimed to investigate *ADM* expression in peripheral blood leukocytes (PBL) in patients at day 1, week 1 and week 3 after acute ischemic stroke using rtPCR, and correlate this with stroke severity, as measured by the most common stroke severity scales the National Institute of Healthy Stroke Scale (NIHSS) (http://www.ninds.nih.gov/doctors/NIH_Stroke_Scale.pdf) [17] and with disability using the modified Barthel Index (mBI) and the modified Rankin Scale (mRS) [18–21]. The NIHSS measures the impairment of a patient's ability caused by stroke using 42 scores, with a low score correlating to lesser symptoms and a high score correlating with more severe stroke symptoms. The mBI is a modification of the original Barthel Index initially developed in 1965 [18,19]. The mBI measures the daily performance of patients in a range from 0–100, with high score showing better performance. The mRS measures patient's independence, not performance, in 6 grades, from 0 to 5, with low score corresponding to better symptoms.

Our results indicated that the *ADM* RNA level at the initial stroke period is correlated with severity as compared to healthy controls and the subjects with motor neurone disease (MND), and suggests that *ADM* expression level at day 1 poststroke may be a potential blood bio-marker in studies of ischemic stroke.

Material and methods

Participants

The study design and protocol was approved by the Human Research Ethics Committees of the Royal Brisbane and Women Hospital, the Wesley Hospital and the Medical Research Ethics Committees of the University of Queensland. All participants provided informed consent. Detail of patients and controls are summarized in Table 1.

Stroke patients were recruited from the Royal Brisbane and Women Hospital and Wesley Hospital, Brisbane, Australia, between August 2008 and December 2011. Ischemic stroke was diagnosed on clinical history, presence of focal neurological signs and symptoms, and by MRI or CT brain scans. The strokes were classified according to the Oxfordshire Stroke Classification [22] into total anterior circulation infarcts (TACI), partial anterior circulation infarcts (PACI), posterior circulation infarcts (POCI) and lacunar infarcts (LACI). Patient stroke severity was evaluated on day 1, week 1 and week 3 by NIHSS, mBI and mRS. Demographic data was collected from the patient or their family members.

Blood was collected from stroke subjects at three time points: day 1, 7–10 d (referred as Week 1 in the text) and 3–6 weeks (referred as Week 3 in the text) after stroke. We also recruited age and sex matched healthy subjects and the patients with MND, recruited from the MND clinic, as a disease control. Blood from these subjects was collected only once. In total, samples from 44 patients with acute ischemic stroke, 19 healthy subjects and 27 patients with MND were studied (Table 1).

Table 1. General condition of participants.

	Acute ischemic stroke			Total stroke patients	Healthy controls	MND Disease patients
	day 1	week 1	week 3			
Number						
Total	24	37	34	37	19	28
Female	10	13	12	13	11	10
Male	14	24	22	24	8	18
Median age in years (range)	67.5 (32–85)	65 (27–87)	67.5 (32–88)	66.7 (27–88)	60.5 (24–95)	62.8 (36–76)
Hypertension N (%)	17 (71%)	23 (62%)	21 (62%)	23 (62%)	4 (21%)	N.A.
Hyperlipidemia N (%)	12 (50%)	15 (41%)	14 (41%)	15 (41%)	2 (11%)	N.A.
Diabetes N (%)	1(4.2%)	3 (8%)	3 (8%)	3 (8%)	0 (0%)	N.A.
Smoking N (%)	15 (63%)	20 (54%)	20 (59%)	2 (59%)	2 (11%)	N.A.
NIHSS median (range)	6.5 (0–24)	4.5 (0–18)	3 (0–19)	4.7 (0–24)	N.A.	N.A.
mBI median (range)	26.5 (0–97)	80.5 (0–100)	95 (0–100)	81 (0–100)	N.A.	N.A.
mRS median (range)	4 (2–5)	3 (1–5)	2 (1–5)	3 (1–5)	N.A.	N.A.

N.A. = not applicable

Not all stroke patients provided blood samples at all 3 time points: 24 patients provided blood samples on day 1 after onset, 37 patients at week 1 and 34 patients at week 3.

RNA extraction and first strand cDNA synthesis

Peripheral blood (2.5 ml) was collected into PAXgeneTM Blood RNA tubes (Qiagen, Australia). The blood RNA tubes were left at room temperature for 8 h and then kept in -20°C until RNA extraction (up to 36 months). RNA was extracted using a PAXgeneTM Blood RNA Extraction Kit (PreAnalytiX, Qiagen, Australia). RNA concentration was determined by the Nanodrop and RNA quality was determined using the ExperionTM Automated Electrophoresis System (Bio-Rad, Australia). All RNA samples used in this study had a RNA quality index >7.

First strand cDNA was synthesized using an RT2 Easy first strand synthesis kit (PreAnalytiX, Qiagen, Australia). The final concentration of extracted RNA samples was adjusted to 125 ng/μl. First strand cDNA synthesis reactions were conducted with 8 μl of 125 ng/μl RNA samples, according to the manufacture's protocol (SABiosciences, Qiagen, Australia). Briefly the samples were incubated at 42°C for 5 min, followed by incubation at 42°C for 15 min and 95°C for 5 min for the reverse transcription.

qPCR array

Customized qPCT plate and the primers for ADM gene were prepared by SABiosciences (Qiagen, Australia). SYBER green master mix including Taq polymerase was activated at 95°C for 10 min. Thermal cycling was done using an iQ5 qPCR machine (Bio-Rad. Australia) with 40 cycles at 95°C for 15 s and 60°C for 1 min. β-actin was used to normalize the sample loading. The threshold value of each reaction and the quality of controls were analyzed using Web-based software, supplied by SABiosciences.

Quantification of ADM serum level

A commercial enzyme linked immune-sorbent assay kit (ADM EIA kit, Phoenix pharmaceuticals, Inc, California, USA) was used to detect the serum level of ADM. The sequence of the kit detected was Tyr – Arg – Gln – Ser – Met – Asn – Asn – Phe – Gln – Gly – Leu – Arg – Ser – Phe – Gly – Cys – Arg – Phe – Gly – Thr – Cys – Thr – Val – Gln – Lys – Leu – Ala – His – Gln – Ile – Tyr – Gln – Phe – Thr – Asp – Lys – Asp – Lys – Asp – Asn – Val – Ala – Pro – Arg – Ser – Lys – Ile – Ser – Pro – Gln – Gly – Tyr – NH2. The serum samples were tested in a serial dilution in preliminary experiments. As the level of ADM was low, we decided to use undiluted serum in the experiment. A standard curve and negative controls were included in each plate. a 50 μl of undiluted serum from each patient and healthy control was added in triplicate. The assay was carried out according to manufacturer's instruction.

Flow cytometry of PBL

Cell populations (T cells, B cells and memory and naïve cells) of PBL were analyzed by flow cytometry according to our previously published methods [3].

Statistical analysis

Statistical analysis was performed using InStat version 4 (GraphPad Software, San Diego, California, USA). Normality tests were performed to determine whether data normally distributed. If data were normally distributed, then mean values $\pm$ SE are shown, pairs of data were compared using student's *t*-test, and ANOVA was used to compare more than three groups. If analysis of the group as a whole showed a significant difference ($p < 0.05$), then the Bonferroni was used as a posttest to compare pairs of group. If the data were not normally distributed, data is presented as median $\pm$ interquartile range, pairs of data were compared used the Mann–Whitney *u*-test and three or more groups were compared by Kruskal–Wallis test. Spearman's rank correlation coefficient test, which gives a Spearman *r* value, was performed to assess the degree of correlation between *ADM* expression fold-changes with age and gender, and severity of stroke patients at each time point following stroke. Correlation test and nonlinear regression (curve fit) were used for analysis of the association between *ADM* expression level with NIHSS, mBI and mRS scores. The slope of the curves of male and female was compared to determine if there was significant difference in different gender, or between total stroke and gender.

Results

Increased ADM gene expression in PBL of stroke patients compared to controls

ADM expression was examined in the samples collected from patients after stroke, patients with MND and healthy controls. Since the data were not normally distributed across all groups, the median fold-changes were compared between the groups. There was a highly statistically significant elevation in *ADM* mRNA levels at day 1 after stroke compared to healthy controls and

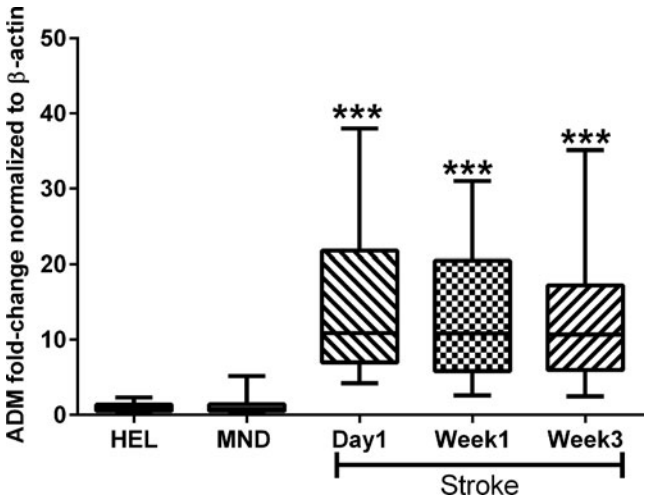

Figure 1. Fold-change in expression of the *ADM* gene. Box and Whisker plots showing fold-change in gene expression of *ADM*. Box extends from 25% to 75% percentile, with horizontal line at median (50% percentile). Whiskers extend down to smallest value and up to largest value. Stroke samples at all time points showed significant fold-change compared to healthy controls (HEL) or patients with MND. $^{***}p < 0.001$.

patients with MND and this was maintained at week 1 and week 3 (Figure 1). The elevated *ADM* expression level in stroke was not affected by gender or age (data not shown).

ADM expression levels in different stroke types

Stroke patients were classified according to the Oxfordshire Stroke Classification (OSSC) as TACI, PACI, LACI and POCI [22]. The patients with LACI were excluded due to small sample size. *ADM* expression levels in each type of stroke was all significantly different from healthy controls at three time points; patients with TACI, the most severe clinical stroke group showed the greatest increase in *ADM* expression levels at week 1 and week 3, and these were significantly different to healthy controls ($p < 0.01$) (Figure 2).

Correlation of ADM expression with measures of stroke severity and disability

The severity of stroke was assessed using the NIHSS, the mBI and the mRS for the stroke subjects. Figure 3 shows that patients with more severe disease and/or greater disability had significantly high *ADM* fold-changes at day 1 after stroke ($p < 0.006$ for NIHSS, $r = 0.5553$; $p = 0.004$ for mBI, $r = 0.5719$ and $p < 0.001$ for mRS, $r = 0.7023$), and remained high at week 1 ($p = 0.006$ for NIHSS, $r = 0.4665$; $p < 0.027$ for mBI, $r = 0.3861$ and $p = 0.035$ for mRS, $r = 0.6657$). The correlations of stroke severity

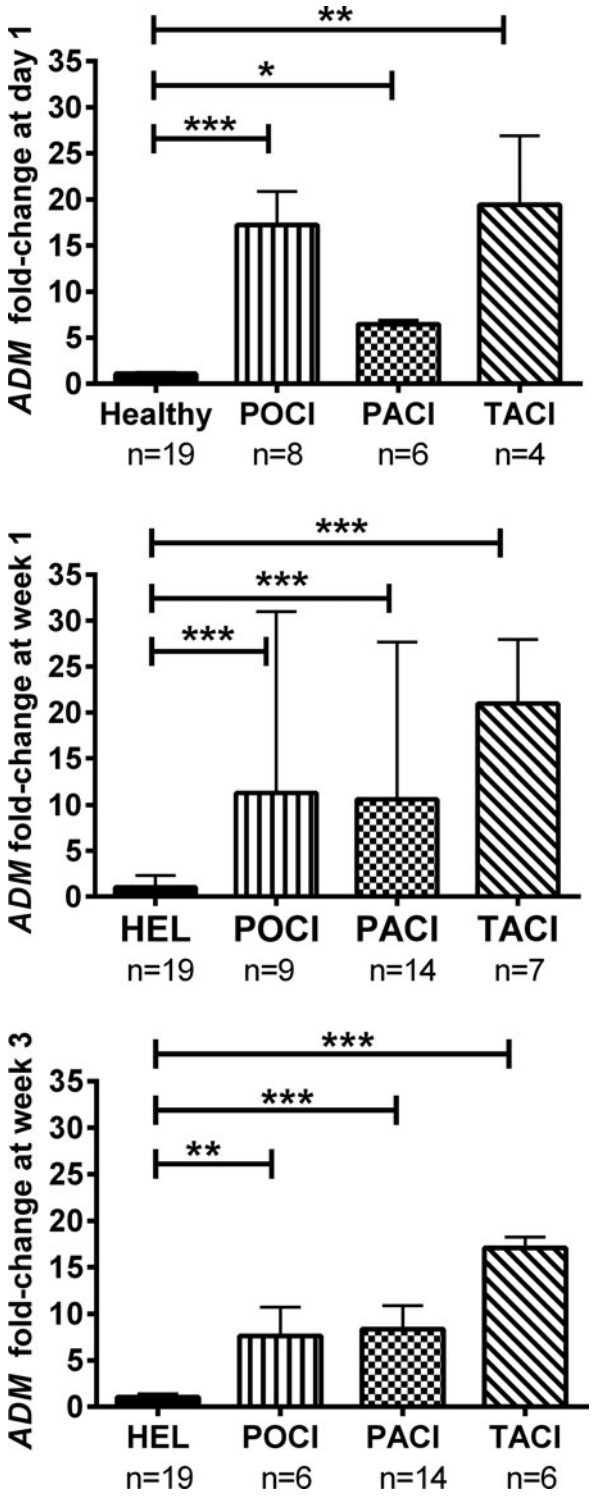

Figure 2. ADM gene expression in subjects classed with OSSC. *ADM* expression levels in stroke patients at day 1, week 1 and week 3 (grouped according to OSSC) compared to healthy controls (HEL). Bars represent median ± interquartile range. Significant differences were found between stroke groups and healthy controls for each time point. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. There were no significant difference when compared within the stroke groups, for example, between TACI and POCI.

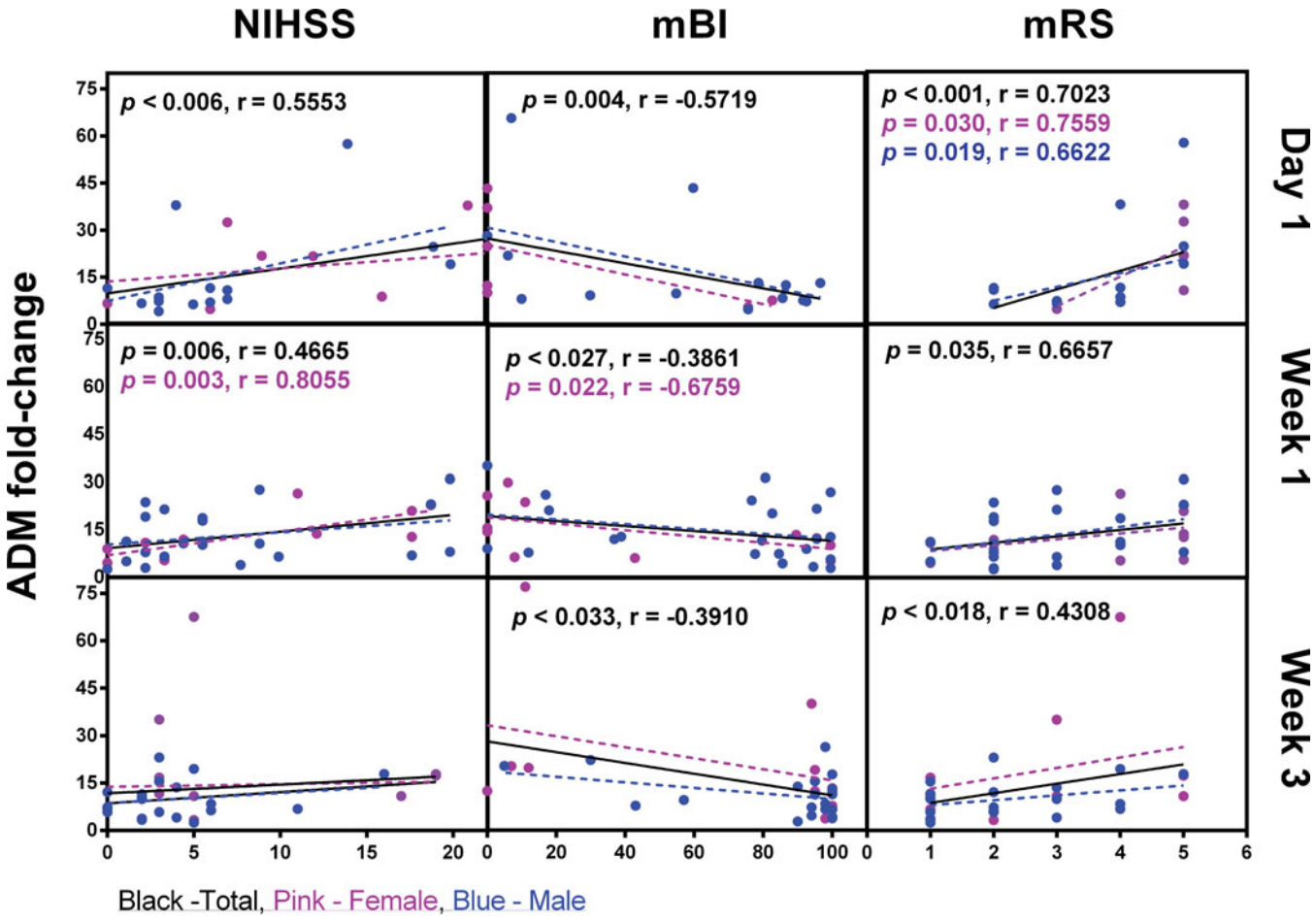

Figure 3. Correlation of ADM gene expression with NIHSS, mBI and mRS at each time point poststroke. *ADM* expression levels versus NIHSS scores, mBI and mRS was significant in stroke day 1 samples and week 1 but not week 3. Statistically significant of the correlations (the *p* values) were calculated by Spearman's rank correlation in GraphPad Prism. The lines, which indicating the trend of the correlation, were done by nonlinear regression in GraphPad Prism.

with NIHSS, mBI and mRS were also analyzed against gender (Figures 3 and 4; Tables 2 and 3). The correlations between *ADM* fold-change and NIHSS, mBI or mRS in week 3 patients had reduced significance (Table 2).

Possibility of ADM as a prognosis marker of stroke

In order to assess if ADM can be a prognostic biomarker, we correlated *ADM* expression on day 1 with the clinical severity score measured on week 1 and 3. Figure 4 and Table 3 show that *ADM* expression on day 1 stroke had significant positive correlation with NIHSS and mRS week 1 and 3 scores, and negative correlation with mBI. These results indicated severe stroke insult induces high *ADM* expression at the earliest stage of stroke. Alternatively, early *ADM* expression levels are correlated to stroke severity and appear prognostic of longer term disability.

ADM peptide levels

We measured the levels of ADM peptide in the blood of 15 healthy subjects and 21 subjects sampled at day 1, day 7 and week 3 after stroke. There was no significant difference between the levels of healthy subjects and patients with stroke (data not shown).

Flow cytometry analysis

To exclude the possibility that the changes in gene expression are due to changes in the circulating cell populations, we measured the total $CD3^+$ T cells, $CD20^+$ B cells, $CD45RO^+$, $CD45RA^+$ memory and naïve cell populations (Figure 5). There were no significant differences between stroke subjects at day 1, week 1 and week

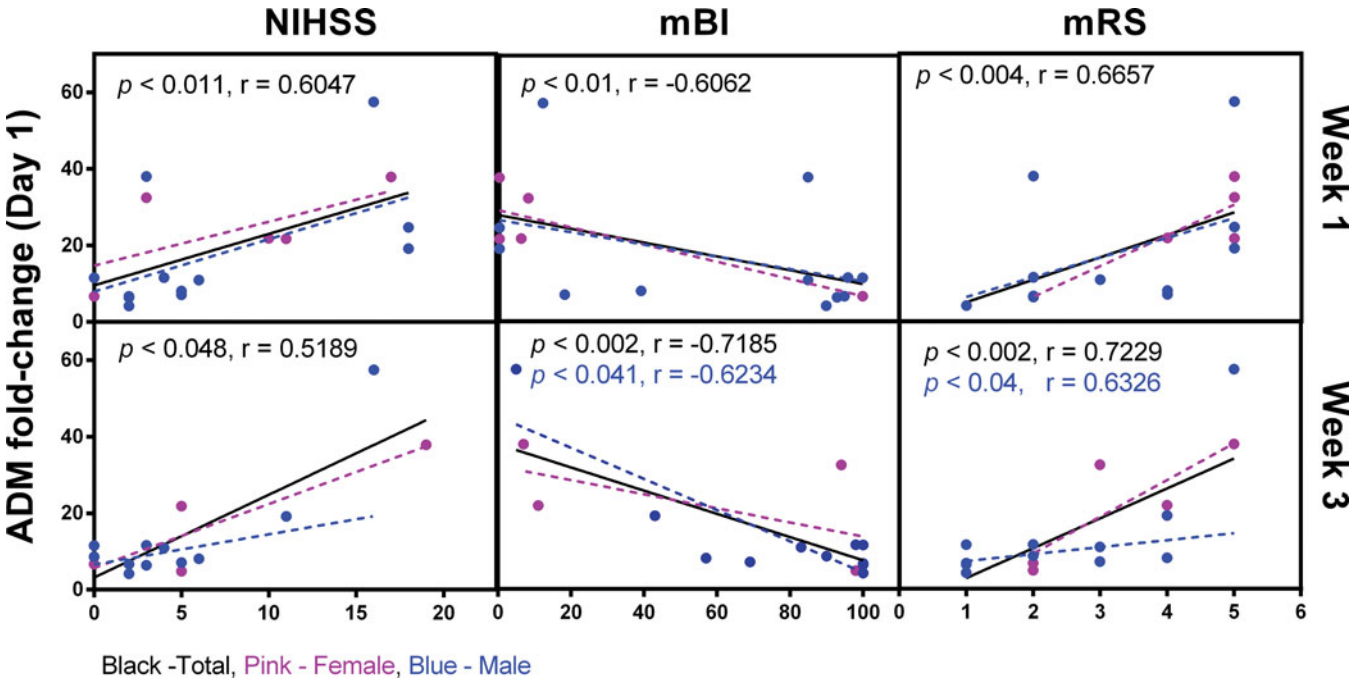

Figure 4. Correlation of ADM gene expression on day 1 with stroke severity scores on week 1 and week 3. The scores of NIHSS, mBI and mRS measured at week 1 and week 3 versus ADM expression levels on day 1. Statistically significant of the correlations (the *p* values) were calculated by Spearman's rank correlation in GraphPad Prism. The lines, which indicating the trend of the correlation, were done by nonlinear regression in GraphPad Prism.

3 stroke compared to healthy controls. There were no significant differences in DC and NK cell populations between stroke and healthy controls (data not shown).

Discussion

In this study, we have investigated expression of *ADM* in PBLs of subjects with acute ischemic stroke. We studied the levels of ADM expression over time and also correlated the expression of *ADM* with severity of stroke. In accordance with previously published microarray data [13], we observed an increased *ADM* gene expression in PBL of stroke patients using an rtPCR technique. Examining *ADM* expression over a 3-week period allowed the observation of the temporal profile of *ADM* expression. Notably, levels remained significantly increased from day 1 for 3 weeks after stroke, suggesting that *ADM* expression occurs as an early event postinsult, and participate in the responses induced by stroke over at least the next three weeks.

It is known that gender and age can influence gene expression within blood [23]. In our study, these confounding factors have been excluded through the

Table 2. ADM level versus stroke severity measured on each time point.

	Day 1			Week 1			Week 3		
	Total	Female	Male	Total	Female	Male	Total	Female	Male
NIHSS									
Spearman R	0.555	0.343	0.511	0.467	0.806	0.280	0.253	0.224	0.112
(*p* value)	(0.006)	(ns)	(ns)	(0.006)	(0.003)	(ns)	(ns)	(ns)	(ns)
mBI									
Spearman R	−0.572	−0.708	−0.477	−0.386	−0.676	−0.299	−0.391	−0.567	−0.179
(*p* value)	(0.004)	(0.033)	(ns)	(0.027)	(0.022)	(ns)	(0.033)	(ns)	(ns)
mRS									
Spearman R	0.702	0.756	0.662	0.371	0.504	0.344	0.431	0.430	0.337
(*p* value)	(0.001)	(0.03)	(0.019)	(0.037)	(ns)	(ns)	(0.018)	(ns)	(ns)

ns = not significant

Table 3. ADM expression level on day 1 versus stroke severity measured on week 1 and week 3.

	Day 1/week 1			Day 1/week 3		
	Total	Female	Male	Total	Female	Male
NIHSS						
Spearman R	0.555	0.343	0.510	0.519	0.633	0.426
(*p* value)	(0.006)	(ns)	(0.06)	(0.048)	(ns)	(ns)
mBI						
Spearman R	−0.606	−0.462	−0.442	−0.719	−0.800	−0.623
(*p* value)	(0.004)	(0.033)	(ns)	(0.002)	(ns)	(0.04)
mRS						
Spearman R	0.666	0.671	0.567	0.723	0.872	0.633
(*p* value)	(0.004)	(ns)	(ns)	(0.002)	(ns)	(0.04)

ns = not significant

correlation of *ADM* expression levels with the gender and age of stroke subjects. There was no significant difference between females and males or any differences among different age groups of patients.

We have also correlated *ADM* levels with stroke severity, measured as NIHSS and patient disability measured as the mBI and mRS scores. One limitation of the study is that we chose to analyze the correlation in consider of stroke severity scores as continuous variables, although these are ordinal variables. There was a significant positive correlation on day 1 and week 1 with NIHSS and mRS, and negative correlation at the same time points with mBI (Figure 3 and Table 2) potentially indicating that *ADM* expression occurs as an adaptive response to hypoxic tissue injury. Our results found that the *ADM* fold-change was influenced by the type

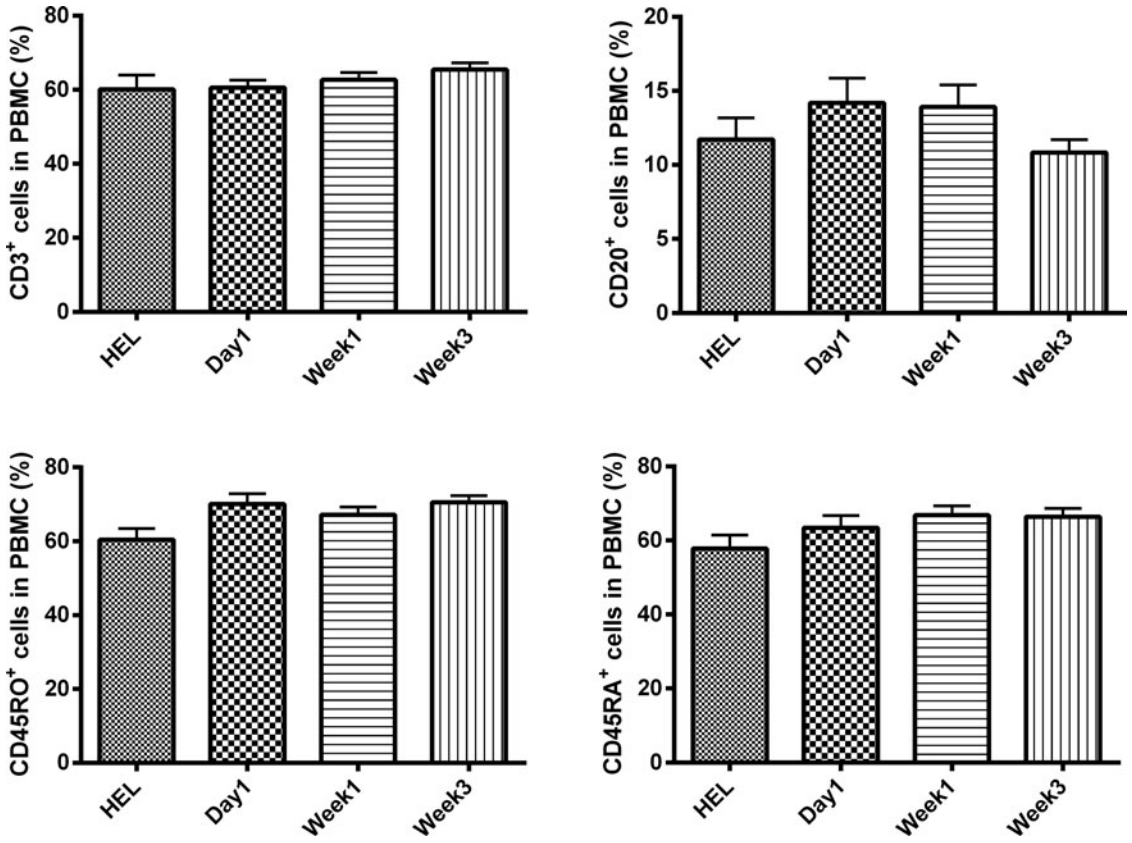

Figure 5 . PBL were labeled with antibodies against CD3, CD20, CD45RO and CD45RA. Each cell population was tested for their changes using flow cytometry. There were no significant variation in these cell populations at day 1, week 1 and week 3 strokes.

of ischemic stroke (Figure 2). It was observed that the most severe stroke subtype TACI has the highest expression level at week 1 and week 3 points following stroke (Figure 2). These results suggested that *ADM* expression could be related to stroke severity. Furthermore, the *r* values for the ADM levels and stroke severity scores measured at week 1 and week 3 indicated a strong correlation of ADM level to the stroke severity and disability.

It is possible that the changes in gene expression in PBL are due to exposure of these cells to hypoxic environments in the region of the stroke. It has been reported that *ADM* expression is regulated by hypoxia in rat ventricular cardiac myocytes [15], implying tissue ischemia and inflammation response related activity. Although we have reported that there are increased levels of activated T cells in subjects with stroke [3,24], there was no difference in the overall percentages of cell populations in stroke compared to healthy controls. This suggests that increased *ADM* gene expression was not affected by variation of cell populations. We do not think that death of neurons alone is the cause of increased ADM expression because there was no significant difference between the subjects with MND, another severely disabling neurological disease and the healthy controls.

This increased *ADM* expression could possibly be a neuro-protective mechanism in line with current literature [25,26]. A key function of the *ADM* product is to produce substantial vasodilation of arterioles [5, 27]. In an animal model, ADM-induced vasodilation has been suggested to occur through the binding of the CGRP receptor and ADM-R [28,29]. ADM has also been shown to inhibit reactive oxygen species (ROS) in ADM knockout heterozygous mice [26]. Additionally, in a rabbit model, the neuroprotective mechanisms of ADM has been suggested to function through the cAMP/PKA-mediated priming of mitochondrial Calcium activated Potassium (mitoKca) channels [12,26].

There is a need for a biomarker of stroke. The optimal blood bio-marker should be rapidly detected, be sensitive and specific. Thus, far there are over 58 proteins and 7 panels of proteins which have been described to possess the features to be potential biomarkers for the diagnosis and prognosis of ischemic stroke [30–33]. However, several studies have described the possibility of using peripheral blood gene expression in the diagnosis in stroke [34–36], and the advantages of using a RNA biomarker in stroke have also been considered. Gene transcription is affected by stroke rapidly; it can occur within minutes, whereas protein translation occurs later than gene transcription. Another reason for considering marker of gene expression is that, circulating proteins present detection and accuracy issues [36]. RNA expression in possible ischemic stroke patients has the potential to yield a sensitive and specific marker for measurement.

The clinical observation of an early response *ADM* expression in our study, has suggested that it could be a potential bio-marker of ischemic stroke. The correlation of the *ADM* expression levels on day 1 with the stroke severity scores measured on week 1 and week 3 was to examine if early stroke-induced *ADM* expression levels can predict disease severity. The results have shown that high *ADM* level on day 1 was correlated with worse stroke (Figure 4 and Table 3).

ADM protein expression has previously been recommended as a biomarker for the other disease [37,38]. However, despite expression levels, we found no significant difference in levels of ADM peptide in the serum of subjects with stroke and healthy controls. This could potentially be due to the rapid degradation or utilization of ADM in the circulation. Another possibility is that there are many sources of ADM and increased *ADM* produced by hypoxic leukocytes might not be sufficient to change the overall systemic levels. ADM is released from pro-ADM, and methods for ADM or pro-ADM serum levels using radioimmunoassay or an immunoluminometric assay have been described [8,9]. It is possible that pro-ADM measurements may differ between stroke patients and controls. It would be worth investigation in the future.

Conclusion

The novel contribution of our results to the study of *ADM* in ischemic stroke includes the time-course of *ADM* expression and correlation with clinical features. The potential of *ADM* as a clinical blood prognostic marker for ischemic stroke was also explored. In summary, elevated expression of *ADM* postischemic stroke was observed, and this was correlated with stroke severity and disability. Further, there is potential for consideration of *ADM* expression as a useful clinical biomarker. Future investigations aim to examine whether hypoxia is the causative factor in increased *ADM* expression in leukocytes postischemic stroke.

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Declaration of Interest

No conflict of interest declared. The authors alone are responsible for the content and writing of this paper.

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