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Circulating miRNA profiles provide a biomarker for severity of stroke outcomes associated with age and sex in a rat model

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Abstract

Small non-coding RNA [miRNA (microRNA)] found in the circulation have been used successfully as biomarkers and mechanistic targets for chronic and acute disease. The present study investigated the impact of age and sex on miRNA expression following ischaemic stroke in an animal model. Adult (6 month) and middle-aged (11–12 months) female and male rats were subject to MCAo (middle cerebral artery occlusion) using ET-1 (endothelin-1). Circulating miRNAs were analysed in blood samples at 2 and 5 days post-stroke, and brain miRNAs were analysed at 5 days post-stroke. Although stroke-associated infarction was observed in all groups, infarct volume and sensory-motor deficits were significantly reduced in adult females compared with middle-aged females, adult males or middle-aged males. At 2 days post-stroke, 21 circulating miRNAs were differentially regulated and PCA (principal component analysis) confirmed that most of the variance was due to age. At 5 days post-stroke, 78 circulating miRNAs exhibited significantly different regulation, and most of the variance was associated with sex. A small cohort (five) of miRNAs, *miR-15a*, *miR-19b*, *miR-32*, *miR-136* and *miR-199a-3p*, were found to be highly expressed exclusively in adult females compared with middle-aged females, adult males and middle-aged males. Predicted gene targets for these five miRNAs analysed for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways revealed that the top ten KEGG pathways were related to growth factor signalling, cell structure and PI3K (phosphoinositide 3-kinase)/Akt and mTOR (mammalian target of rapamycin) signalling. Overall, the pattern of circulating miRNA expression suggests an early influence of age in stroke pathology, with a later emergence of sex as a factor for stroke severity.

Keywords

biomarker; microRNA; middle cerebral artery occlusion; principal component analysis; small non-coding RNA; sex difference; stroke

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Author Contribution: Amutha Selvamani performed the stroke surgeries, behavioural assays and miRNA analyses, and was involved with writing the paper; Madison Williams assisted with miRNA analyses; Rajesh Miranda was involved in statistical analyses and writing the paper; and Farida Sohrabji conceived the study, and was involved with statistical analyses and writing the paper.

Introduction

Stroke is the third leading cause of death and a leading cause of long-term disability. The risk for stroke and poor outcomes due to stroke can be affected by several factors, including age and sex. Age is the greatest risk factor for stroke [1], and stroke rates double every decade after the age of 55 years [2]. Sex differences are also observed in the epidemiology of stroke [3]. Clinically, paediatric stroke appears to be more common in boys irrespective of age [4]. In the elderly, women have a higher incidence of stroke compared with age-matched men and also suffer poorer outcomes and higher mortality [5–7]. Since age and sex are important risk factors for stroke severity, biomarkers associated with these populations provide a unique opportunity to develop diagnostic profiles for stroke severity and, importantly, uncover potential mechanisms that influence stroke severity.

microRNAs (miRNAs) are small non-coding RNAs of ~25 nucleotides long that regulate gene expression post-transcriptionally by binding to complementary sequences in the 3'-UTR of multiple target mRNAs [8]. miRNAs are abundantly present in all human cells, target ~60 % of all genes, and are each able to repress hundreds of targets [9]. miRNAs exhibit functional dysregulation in almost all aspects of human pathology, including cancer, cardiovascular diseases, metabolic disorders and neurodegenerative diseases. They form tissue-specific molecular profiles that further define significant pathological features. Although initially thought to be exclusively intracellular, miRNAs are now found in virtually all body fluids and are virtually indestructible in blood [10].

Recent studies reported distinct miRNA expression patterns in the stroke pathogenic process, including hyperlipidaemia, hypertension and plaque rupture [11], and atherosclerosis [12]. Specific stroke-induced miRNA expression profiles have been reported in the blood and brain in both experimental models and patients as a function of different reperfusion times [9,13,14]. Altered inflammation-related miRNA profiles following intracerebral haemorrhage have been reported in plasma [15]. In addition, miRNA expression patterns have been used to predict subtypes of stroke [14]. However, to date, no studies have examined the effects of age and sex on miRNA expression patterns post-stroke, either using brain or circulating miRNA.

The present study has investigated the impact of age and sex on miRNA expression following ischaemic stroke. We report that the expression profiles of circulating miRNAs during the post-stroke period reflect both age and sex differences, with a unique temporal pattern, such that age differences are observed earlier than sex differences. Moreover, the expression profiles of brain miRNAs at 5 days post-stroke are dissimilar to circulating miRNAs at 5 days post-stroke. A small cohort of five miRNAs were significantly up-regulated in adult females compared with middle-aged females, adult males and middle-aged males. As the adult female group had the smallest infarct volume and the least amount of sensory-motor deficit, this cohort of miRNAs may represent a neuroprotective profile.

Materials and Methods

Animals

All animals were purchased from Harlan Laboratories. Females were purchased as proven adults (6–7 months, 230–320 g, $n = 12$) and middle-aged (10–12 months, 280–360g, $n = 12$), while adult males ($n = 12$) and middle-aged males ($n=12$) were age-matched to females. All animals were maintained in a constant 12-h dark/12-h light cycle in AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care)-accredited vivarium facilities. Food and water were available *ad libitum*. Within each age and sex, animals were assigned randomly to the stroke and intact groups

Middle cerebral artery occlusion

Intact animals ($n = 6$ in each group) used in the study were not subject to surgery. All other animals were subjected to stereotaxic surgery to occlude the left MCA (middle cerebral artery) as reported previously [16–18]. Briefly, MCAo (MCA occlusion) was induced by microinjecting 3 μ l of ET-1 (endothelin-1) (0.5 ml in 1 ml of PBS; American Peptide Company). Animals were randomly assigned to treatment groups. Rats were maintained at 37 °C throughout surgery. All animals were killed on day 5 post-MCAo. At termination, the brain was rapidly removed and processed for TTC (triphenyl tetrazolium chloride) staining to assess infarct volume. For molecular analyses, brain tissue was dissected and stored at – 80 °C.

Infarct volume

Infarct volume estimation was performed on six rats in each experimental group. Brain slices (2-mm-thick) between – 2.00 mm and +4.00 mm from Bregma were incubated in a 2% TTC solution at 37 °C for 20 min and later photographed using a Nikon E950 digital camera attached to a dissecting microscope. Infarct volume was determined from digitized images using the Quantity One software package (Bio-Rad Laboratories). Three such slices were used for analysis, since the infarct was restricted to only three slices in all of the animals analysed. Only the superior face of each slice, which was clearly stained by TTC, was analysed. The area of the infarct was measured in all slices, as well as the total area of the contralateral hemisphere. In each case, the infarct area of two adjacent slices was averaged and then multiplied by the thickness of the slice, and values across all slices were added to derive the volume of the infarct. A similar approach was used to determine the volume of the non-occluded hemisphere. The volume of the infarct was then expressed as a percentage of the contralateral (non-occluded) hemisphere [16]. To ensure a reliable, consistent and unbiased estimation of the infarct zone, all images were first coded. Images were digitally converted into black and white and magnified, and all traces were performed by one investigator, who was blinded to the codes. Application of the volume algorithm and statistical analysis was performed by a separate investigator, also blind to experimental conditions. In a subset of animals (eight out of 24), the infarct zone and contralateral hemisphere was traced by another investigator (also blinded to the experimental groups). The correlation between the infarct volume estimated by the two independent traces was +0.96.

Behavioural assays

Motor impairment following MCAo was assessed using the vibrissae-evoked forelimb placement task, as well as the Sticky-tape test. The vibrissae-elicited forelimb placement test was used both before and after the MCAo surgery. Animals were subject to same-side placing trials and cross-midline placing trials elicited by stimulating the ipsi- and contra-lesional vibrissae. During the same-side forelimb placing trials, the animal was gently held such that all four limbs were free to move. The animal's ipsilesional vibrissae were brushed against the edge of a table to elicit a forelimb placing response, which typically consisted of the forelimb ipsi-lateral to the stimulated vibrissae. Ten trials were performed before the same was repeated for the contralesional vibrissae. In the cross-midline placing trials, the animal was held gently by the upper body such that the ipsilesional vibrissae lie perpendicular to the table top and the forelimb on that side is gently restrained as the vibrissae was brushed on the top of the table to evoke a response from the contralateral limb and vice versa. Between each trial the animal was allowed to rest all four limbs briefly on the table top to help relax its muscles. Trials in which the animal seemed to struggle or make premature forelimb movements were not counted [16].

The adhesive tape test was performed both before and after surgery. Two pieces of adhesive-backed foam tape (2.5 cm × 1.3 cm) were used as bilateral tactile stimuli attached to the palmar surface of the paw of each forelimb. For each forelimb, the time it took to remove each stimulus (tape) from the forelimbs was recorded during three trials per day for each forepaw. Animals were allowed to rest for 1 min between sessions, and each test session had a maximum time limit of 120 s.

Sample collection and miRNA analyses

miRNAs were analysed in plasma and brain samples, $n = 6$ in each experimental group for 2 day serum, 5 day serum and 5 day brain. A saphenous blood draw was obtained at 2 days post-stroke and trunk blood was collected at termination on day 5 post-stroke. Blood was centrifuged at 1300 g for 30 min to obtain serum. Brain tissue (cortex and striatum from ipsilateral hemisphere) was obtained from animals killed at 5 days post-stroke.

RNA extraction—To each tube, containing either 200 μ l of serum or 175 mg of brain tissue, 750 μ l of QIAzol master mix [800 μ l of QIAzol and 1.25 μ l of 0.8 μ g/ μ l MS2 (carrier) RNA per sample] was added. Following a 5-min incubation at room temperature (21–22°C), 200 μ l of chloroform was added to each sample. Following a 2-min incubation at room temperature, samples were centrifuged at 12000 g for 15 min at 4 °C. The aqueous phase was then transferred to a fresh tube and mixed with ethanol (1.5 vol). The sample was then loaded on to an RNeasy Mini Spin Column and centrifuged at 13000 g for 30 s at room temperature. After sequential washes in RWT and RPE buffers, the columns were transferred to a fresh tube and RNA was eluted with 50 μ l of DNase/RNase-free water. Sample purity was assessed by Nan-drop technology and a ratio of 1.8 was considered acceptable. Samples were stored at –20 °C until use.

PCR amplification—Template RNA (25 ng of total RNA per sample) was incubated with reverse transcriptase for 60 min at 42°C, followed by heat-inactivation of the enzyme (5 min

at 95 °C) and was used immediately. cDNA was diluted 80-fold and then incubated with SYBR® Green master mix. A portion (10 µl) was dispensed to each well of the 384-well PCR plate. Plates were centrifuged at 192 g for 1 min at 25 °C before insertion into the thermocycler (ABI Thermal Cycler 7900HT). An activation/denaturation step (95 °C for 10 min) precedes 40 amplification cycles each at 95°C, 10 s, 60°C, 1 min, ramp-rate 1.6°C/s. Each microplate consists of 168 LNA (locked nucleic acid)-miRNA primer sets of serum/plasma relevant human miRNAs and seven reference miRNAs, for use with the ABI 7900HT instrument. miRNA primers in this proprietary panel (Exiqon) were selected from extensive profiling of miRNA from healthy individuals, as well as individuals with diseases including various cancers, neurological disorders, allergies, diabetes and inflammatory disease. All primers are LNA-modified which allows for uniform T_m , and confers greater specificity, allowing for discrimination between miRNA sequences with single nucleotide differences. A subset of samples (three to four) from each group was further subjected to PCR amplification of *U6*. For confirmation, a subset of miRNAs from the 5 day serum samples was subject to qPCR (quantitative real-time PCR) analysis. They were *miR-15a*, *miR-19b*, *miR-32*, *miR-136*, *miR-199a-3p* and *miR-363*, using LNA-miRNA primer sets from Exiqon

Data analysis

Normalization—Cycle thresholds (C_T) were determined for each miRNA in each sample. C_T values for five reference miRNAs in each sample were averaged and then subtracted from each of the 168 miRNAs of interest (C_T). miRNA profiles were obtained from the C_T values.

Internal controls—To determine whether the serum samples were contaminated by haemolysis, we employed the test described by Blondal et al. [19]. For each sample, the C_T values for *miR-451* (enriched in erythrocytes) were subtracted from the C_T values for *miR-23a* (enriched in plasma). Difference values that were less than 5 were considered not contaminated by haemolysis. Additionally, both brain and serum samples were analysed for *U6* as an additional control of cell lysis as a contaminant of circulating miRNA.

miRNA profile analysis— C_T values of target miRNAs were obtained by subtracting each target miRNA C_T value from the average of five reference miRNA controls (*miR-103*, *miR-425*, *miR-423-5p*, *miR-93* and *miR-191*). Reference miRNAs are stably expressed in all groups at relatively high levels. miRNA expression data (C_T) obtained from focus panels were uploaded into the GSEA (Genesifter® Analysis Edition) software program (Geospiza). Differences in miRNA expression were identified using a two-way ANOVA using age and sex as two independent factors, with Benjamini and Hochberg correction for multiple comparisons at a cut-off $\alpha = 0.05$. miRNAs that were significantly regulated at each time point in the blood and brain were graphically represented as heat maps, and Euclidean clustering was used to visualize patterns of the molecular interaction networks of the differentially expressed genes. PCA (principal component analysis) was included to estimate the source of the variance in the data.

Further *in silico* analysis was performed using DIANA-miRPath v2.0 [20], with the micro T-CDS algorithm. Predicted and validated gene targets and the associated KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were identified using a modified Fischer's exact test with an FDR (false discovery rate) (Benjamini and Hochberg)-corrected P value threshold of <0.05 .

Data analysis for infarct volume and behaviour—For infarct volume, a two-way ANOVA (coded for age and sex was used). For behavioural tests, a paired Student's t test was used for each group, comparing the values obtained pre- and post-stroke. Group differences were considered significant at $P < 0.05$ in each case. The statistical package SPSS (version 21, IBM) was used for these analyses.

Results

Effect of age and sex on post-stroke infarct volume

Adult and middle-aged female and male rats were subject to MCAo using ET-1. ET-1 delivered to the MCA causes a cerebrocortical and striatal infarct [16,17,21], typical of other MCAo models, including the filament model [22–24]. TTC-stained brain slices from animals in each group were analysed for infarct volume. Figure 1(A) shows a representative TTC-stained coronal section from each group, with the quantification of the infarct volume normalized to the non-ischemic hemisphere in Figure 1(B). As shown in Figure 1(A), cortical and striatal infarction was observed in all of the groups; however, the extent of cell death was significantly affected by age [$F_{(1,20)}$, 5.37; $P < 0.05$] and sex [$F_{(1,20)}$, 11.94; $P < 0.05$] (Figure 1B). Adult females had significantly smaller infarct volumes compared with middle-aged females and adult and middle-aged males (interaction effect $F_{(3,20)}$, 5.23; $P < 0.05$). At 5 days post-stroke, the extent of stroke-induced infarction in adult females was approximately less than one-third of the infarct volume in middle-aged females and approximately a quarter of that observed in young and middle-aged males (Figure 1). These data confirm the neuroprotection observed in young females compared with older females and age-matched males reported previously [14,16–18,25–28].

Behavioural assays

Sensory motor deficits are commonly observed in MCAo stroke models and the adhesive tape test is a sensitive measure of this deficit [29]. Specifically, a longer latency to remove an adhesive affixed to the paw is indicative of sensory-motor deficits. As expected, there were no significant differences between the groups in the average latency for tape removal from the ipsilesional paw either pre- or post-stroke. However latency for tape removal was significantly affected in the contralesional paw after stroke. As shown in Figure 2, middle-aged males and females, as well as adult males, took 6–8 times longer to remove the tape from the contralesional paw compared with the pre-test duration ($P < 0.05$). However, adult females showed no significant difference in the latency of tape removal pre- and post-stroke, consistent with the smaller infarct volume observed in this group.

Although less effective than the adhesive tape test at discriminating between infarct sizes, the vibrissae-evoked forelimb placement task is a good marker for corticostriatal damage.

Using the ‘same side’ version of this test, males and females both showed significant post-stroke deficits in paw-placement on the contralesional side, but not on the ipsilesional side. On the ‘cross-midline’ test, males and females respectively showed significant post-stroke deficit in paw-placement on both ipsilesional and the contralesional side (Supplementary Figure S1 at <http://www.clinsci.org/cs/127/cs1270077add.htm>).

miRNA regulation in serum

Test for haemolysis—Circulating miRNAs were amplified from serum samples obtained from adult and middle-aged male and female rats using the Exiqon LNA focus panel. This panel consists of 168 miRNAs that are known to be expressed in circulation and in biological fluids, culled from a variety of disease and normal samples. One potential confounding element in the determination of miRNAs in serum samples is contamination from haemolysis. To test for haemolysis, we compared the level of *miR-451a*, which is highly expressed in erythrocytes, with *miR 23a*, a miRNA that is enriched in plasma. The average C_T value is shown in Supplementary Table S1(A) (at <http://www.clinsci.org/cs/127/cs1270077add.htm>). Mean values for all groups were well below 7, which indicates a low probability of haemolytic contamination. We also assessed the expression of *U6*, a well-known reference miRNA for cellular miRNA. As shown in Supplementary Table S1(B), brain *U6* C_T values ranged from 21 to 24, whereas in serum samples the C_T value ranged from 32 to 35, indicating a very low expression of *U6* in serum. Intragroup variation in qPCR analysis was low, ranging from 1.2 to 6.7% of the mean C_T , with an average variation of 4.8% of the mean, indicating reliable miRNA estimates.

Circulating miRNA profiles—To determine circulating miRNA profiles, serum samples from adult and middle-aged females and males were collected at days 2 and 5 post-stroke, and subject to qPCR analysis using a focus panel with 168 LNA-miRNA primer sets of serum/plasma-relevant human miRNAs. Expression of each miRNA of interest was normalized to a set of control miRNA that are enriched in serum. Normalized expression (C_T) was analysed using the GSEA program to determine group differences in miRNA expression, using 2-way ANOVA with the Benjamini–Hochberg correction for false discovery ($\alpha = 0.05$). In each case, the adult female group was considered as the control. Expression patterns of miRNAs that reached statistical significance are represented as heat maps in Figure 3, where fold increases in miRNA expression are colour-coded in green, and decreases are in red.

Circulating miRNAs revealed unique age- and sex-related patterns at day 2 (Figure 3A) and day 5 (Figure 3B) post-stroke respectively. At 2 days post-stroke, 21 miRNAs were differentially regulated (adjusted $P < 0.05$; miRNA names are listed in Supplementary Table S2 at <http://www.clinsci.org/cs/127/cs1270077add.htm>). In each case, there was a main effect of age, indicating a significant difference in miRNA expression between adult and middle-aged animals, irrespective of sex. In contrast, at 5 days post-stroke, the expression of 78 miRNAs was significantly altered (adjusted $P < 0.05$; miRNA names listed in Supplementary Table S2). Moreover, at the 5 day time point, the majority of the significantly regulated circulating miRNA (67 out of 78; 86%) exhibited a main effect of

sex, whereas a smaller cohort (12 out of 78; 15%) showed a main effect of age. Approximately 15% (12 out of 78) exhibited an interaction effect between age and sex (Figure 3B). Overall, the pattern of circulating miRNA expression suggests an early emergence (2 days post-stroke) of the influence of age in stroke pathology, with a later emergence (5 days post-stroke) of sex as a factor for stroke severity.

The ANOVA analysis is supported further by unsupervised hierarchical cluster analysis (Figure 4), where the agglomerative clustering reveals two major clusters defined by age (Figure 4A). Furthermore, PCA was used to visualize the overall response of gene expression, using Eigen values of covariance. The *x*- and *y*-axis values are arbitrary units for Eigen values and specific miRNAs are represented by dots along each component continuum (Figure 5). At 2 days post-stroke, the greatest proportion of the variance, shown as Component 1 (Comp. 1) is attributed to age, whereas little variance was observed in component 2 (sex). PCA for circulating miRNAs at 5 days post-stroke, on the other hand, showed that the greatest proportion of variance (Comp. 1) can be attributed to sex, and a lesser proportion due to age (Comp. 2) (Figure 5B). These results uncover key patterns of change in miRNA expression as a function of time and their modulation by sex and age.

A small subset of 13 miRNAs was found to be differentially regulated at both 2 and 5 days post-stroke (Table 1). Within this cohort, all significantly regulated miRNAs showed a main effect of age at the 2 day time point; however, at 5 days post-stroke, 12 of these 13 miRNAs displayed a main effect of sex, where miRNA expression was significantly elevated in females compared with males. One miRNA (*miR-363*) was regulated by both age and sex at the 5 day time point, and only one (*miR-495*) was regulated by age at both 2 and 5 days post-stroke. In view of the sex and age difference in infarct volume, miRNAs that discriminate along these two variables as a function of time may critically influence stroke outcome.

miRNA regulation in the brain

To determine whether miRNA profiles in the ischaemic brain displayed similar patterns to circulating miRNAs, total RNA isolated from cortex and striatal tissue from adult and middle-aged females and males was also interrogated with the same focus panel. Two-way FDR-corrected ANOVA with age and sex as independent variables revealed that 19 miRNAs were significantly altered by stroke (Figure 6A), of which ten miRNAs were significantly modified by age (main effect of age, $P < 0.05$) and nine miRNA were significantly modified by sex (main effect of sex, $P < 0.05$). Cluster analysis showed that significantly regulated miRNAs were segregated by age (Figure 6B). PCA for brain miRNA expression at 5 days post-stroke also confirmed that middle-aged females and males clustered separately from adult females and males (Figure 6C). Thus the pattern of the brain miRNAs at 5 days post-stroke was similar to the pattern of circulating miRNAs observed at 2 days post-stroke. Interestingly, one miRNA (*miR-424*) was significantly regulated in the brain and at both time points in serum.

miRNAs exhibiting an interaction effect of age and sex

A central goal for the present study was the identification of miRNAs that broadly discriminate the adult female group, which has the smallest infarct volumes and least amount of sensory-motor deficit, from older females, as well as young and older males. The latter groups have significantly worse stroke outcomes than the adult female. To identify miRNAs that would discriminate between these groups, we focused on those miRNAs where there was a significant age $\times$ sex interaction effect. miRNAs with a significant age $\times$ sex interaction effect were only observed in circulating miRNAs at 5 days post-stroke (ANOVA and hierarchical cluster analysis (Figures 3B and 4B). Among the 12 miRNAs that were thus identified, post hoc Student's *t* tests indicated that five of these miRNAs distinguished the adult female group from all of the other groups. As shown in Figure 7, *ACT* values were significantly lower in adult females for *miR-15a*, *miR-19b*, *miR-32*, *miR-136* and *miR-199a-3p*. Raw *C_T* values of these miRNAs are shown in Supplementary Table S3 (at <http://www.clinsci.org/cs/127/cs1270077add.htm>). In every case, these miRNAs were highly expressed in adult females compared with middle-aged females, adult males and middle-aged males, suggesting a pivotal role for these miRNAs in regulating the severity of stroke. This cohort of miRNAs was subject to an additional qPCR analysis, which confirmed the significant age $\times$ sex interaction effect. Fold changes in miRNA expression for the five miRNAs are shown in Figure 7. To ensure that these miRNAs did not simply reflect constitutive differences between adult and middle-aged males and females, a set of non-stroke animals was also analysed for circulating miRNAs. None of the above miRNAs were up-regulated in non-stroke young females compared with the other groups (results not shown).

In silico analysis tools (DIANA-miRPath v2.0, and target database microT-CDS) were utilized to obtain predicted gene targets and associated KEGG pathways of *miR-15a*, *miR-19b*, *miR-32*, *miR-136* and *miR-199a-3p*. The total number of predicted and validated target genes and genes implicated in KEGG pathways are shown in Figure 8(A). In total, 64 KEGG pathways were identified and, from these, a set of ten KEGG pathways are shown in Figure 8(B). The top ten pathways were selected based on the following criteria: smallest *P* values for the union of common pathways targeted by all miRNAs and the largest number of genes represented in those pathways. The top ten KEGG pathway thus included growth factor signalling pathways, such as insulin and neurotrophin, cell survival and cell death pathways, including p53, PI3K (phosphoinositide 3-kinase)/Akt and mTOR (mammalian target of rapamycin) signalling pathways, cell structure-related pathways, such as focal adhesion, actin cytoskeleton and cell function pathways, including ubiquitin proteolysis and endocytosis.

Discussion

The present study reports unique circulating miRNA expression profiles following cerebral ischaemia in adult and middle-aged female and male rats. Of the 168 circulating miRNAs examined, 21 miRNAs were significantly regulated at 2 days post-stroke, whereas at 5 days post-stroke 78 miRNAs were significantly regulated. Furthermore, a small cohort of five miRNAs (*miR-15a*, *miR-19b*, *miR-32*, *miR-136* and *miR-199a-3p*) were found to be highly

expressed exclusively in adult females, a group that, as a whole, exhibited significantly less cortical and striatal damage, and the least amount of sensory-motor deficit. This five miRNA cohort needs to be investigated further as a collective biomarker for stroke outcome. Although sex and age differences in stroke severity have been shown reliably in animal stroke models, to the best of our knowledge this is the first study to demonstrate miRNAs profiles associated with sex and age differences in ischaemic pathophysiology.

The extent of stroke severity, as measured by infarct volume, showed that the corticostriatal infarct was worse in the middle-aged females compared with the adult females, which is consistent with our previous work [16,17] and other reports [14,26–28]. Furthermore, females as a group had smaller infarct volumes compared with males, consistent with other studies where similar sex differences have been reported in infarct volume and cerebral blood flow [25]. The female advantage, observed in young-age demographics, as well as in animal models, may be related to the major ovarian hormone oestrogen. This is supported by evidence that the extent of ischaemic damage is inversely related to circulating levels of oestrogen [30] and that replacement with 17β -oestradiol [31,32] reduces infarct volume in young ovariectomized female animals. Declining levels of oestrogen with age may also underlie the larger infarct volume observed in middle-aged and older female rats [16]. With respect to age, disparity in stroke outcomes has been associated with age-related down-regulation of several proteins including NKCC ($\text{Na}^+ - \text{K}^+ - \text{Cl}^-$ co-transporter) [33], BDNF (brain-derived neurotrophic factor), bFGF (basic fibroblast growth factor) [34] and neuron-specific intermediate filaments, NFs (neurofilaments) [35]. In clinical studies, NO was shown to be reduced in serum of aged stroke patients compared with younger patients, whereas IL-6 (interleukin-6) was elevated in aged patients with a poor outcome [36]. Thus, although biomarkers have been sought for each factor individually, the present study has attempted to integrate markers for both age and sex differences.

Our data show that the complex interaction of age and sex in stroke outcomes is well captured by circulating miRNA profiles. Originally thought to be exclusively intracellular, miRNA are now known to be stably expressed in many body fluids, including blood, saliva and tears [37,38], where their resistance to endonuclease degradation, low/high pH, extreme temperatures, extended storage and freeze–thaw cycles [10,39,40] make them superior biomarkers in comparison with proteins. Hence diseases targeting inaccessible tissue, such as the brain, can now be assessed using surrogate tissues, such as blood. This is a particularly valuable option for stroke, which affects cells in several critical systems, including the brain and its support cells (astrocytes, endothelial cells and microglia), as well as vascular and immune systems.

Circulating miRNAs have been widely investigated as biomarkers to distinguish disease from non-disease cases [10], especially in cardiovascular disease, where specific miRNA signatures have been shown to distinguish stable documented CAD (coronary artery disease) patients [41] or peripheral artery disease patients [42] from controls. Moreover, miRNA profiles are also being used to make more subtle discriminations of disease states as in distinguishing between stable CAD and acute coronary syndrome [43] and grades of severity of myocardial infarction [44]. In the case of stroke, only two studies have examined circulating miRNA expression profiles. One experimental study reported a time course

profile of stroke-induced miRNA at 24 and 48 h post-MCAo [9]. That study focused on adult male rats only, hence few overlapping miRNAs were detected with the present study. A clinical study of young (18–49 years of age) stroke patients reported that specific patterns of circulating miRNAs were associated with stroke outcomes (good or poor), although specific miRNAs associated with stroke severity were not identified [14]. Specific miRNAs have also been associated with stroke outcomes, as in *miR-120*, which is decreased in patients with poor outcomes after 7 and 14 days [45], time points in the progression of stroke further from the ones addressed in the present study.

The present study uncovered a two-step process in miRNA profiles underlying the age/sex difference in stroke outcomes. At 2 days post-stroke, PCA indicated that age was the principal source of variance at this time point. At the 5 day time point, the largest proportion of variance was accounted for by the sex of the animal. Thus the temporal change in the circulating miRNA expression profile is primarily influenced by age at the earlier time point and later by the sex of the animal.

Despite the large differences in brain infarct volume observed among the groups, there were a surprisingly limited number of differentially expressed miRNAs in the ischaemic brain at 5 days post-stroke. It should be noted, however, that this study interrogated ischaemic brain tissue with the circulating miRNA panel, thus it is possible that many brain-specific or brain-enriched miRNAs were not captured by this process. Our goal in using the circulating miRNA panel was to identify commonalities between the target tissue and circulation, and the present study shows, interestingly, that six differentially regulated miRNAs were represented in both the brain and circulation at 5 days post-stroke. This cohort may therefore serve as a proxy marker for brain responses to stroke. Of these, a smaller subset was regulated in the same pattern in both tissues, suggesting that the profiles of brain parenchymal miRNAs are quite different from those in circulation. Such mismatches in miRNA profiles have been reported for other diseases as well, such as cancer, where less than 30 % of tumour miRNAs were captured in the circulation [46]. This discrepancy between brain and circulatory miRNAs underscores the multi-factorial nature of stroke, where central and peripheral responses involving the cardiovascular system and the immune system are simultaneously co-ordinated. These data suggest that peripheral tissues such as blood would be a more global representation of the overall stroke response than local events in the brain. Similarly, only a few miRNAs were regulated in common at the 2 and 5 day time-point in serum (Table 1). However, in most of these cases, miRNAs were regulated by age at one point and sex at the other. Only *miR-363* was regulated by age at 2 days and by age and sex at 5 days post-stroke, and the direction of change is the same at both time points. Fewer overlaps between the two time points may be indicative of the evolving infarct, and the temporal lag between vascular, brain and immune systems, which are the main contributors to the post-stroke miRNA pool.

A related goal of the present study was to identify a miRNA profile that can predict stroke severity. Since infarct volume analysis indicates that adult females had the best outcome in terms of a small infarct volume and virtually no loss of sensory motor performance, we focused on miRNAs that discriminate this group from the others (middle-aged females, adult males and middle-aged males). Our analysis revealed that a small cohort of five miRNA

(*miR-15a*, *miR-19b*, *miR-32*, *miR-136* and *miR-199a-3p*) were significantly up-regulated in adult females compared with all of the other groups, and may represent a signature for stroke severity. These miRNAs showed high-to-modest abundance in serum, as indicated by the raw C_T values shown in Supplementary Table S3. Furthermore, this cohort represents stroke-induced miRNAs and is not constitutively up-regulated in non-stroke adult females. Although, the role of *miR-32*, *miR-136* and *miR-199a-3p* in stroke aetiology is unknown, *miR-15a* and *miR-19b* are reported to be implicated in cerebrovascular protection and cell proliferation respectively. PPAR γ (peroxisome-proliferator-activated receptor γ) and a co-activator of PPAR γ -mediated transcriptional suppression of *miR-15a* are implicated in cerebrovascular protection following MCAo [47]. *miR-19b*, which is part of the *miR17-92* cluster, is known to stimulate stem cell proliferation in the subventricular zone of ischaemic animals [48], as well as promote OPC (oligodendrocyte precursor cell) survival by activating Akt signalling [49].

Since several miRNAs in this cohort have an unknown role in stroke, putative and validated targets of the five miRNAs were subject to KEGG analysis. Among the top ten pathways identified by KEGG analysis were two major signalling pathways that transduce response to stress and injury, namely the PI3K/Akt pathway and the mTOR pathway, both of which are mutually involved with stroke neuroprotection [50,51]. Similarly, miRNAs that target the Wnt/ β -catenin signalling pathway have also been implicated in stroke recovery [52], as well as the inhibition of the endothelial actin cytoskeleton [53]. Finally the insulin signalling pathway, also identified as a target of these miRNAs, is strongly implicated in post-stroke outcomes, with hyperglycaemia contributing to poorer outcomes [54,55].

In conclusion, these studies show that specific miRNA profiles are associated with age and sex differences in stroke-associated infarct volume and sensorimotor deficits. At an early time point, miRNA profiles segregate exclusively based on age, whereas at the later time point miRNA profiles show a preponderant regulation by sex. Thus, although both age and sex eventually influence miRNA expression, each of these variables prevails at different times in the evolution of the serum miRNA response to the infarct. Through this analysis, naturally occurring differences in infarct volume due to age and sex can also be exploited to develop an miRNA profile that is associated with a better outcome. A small cohort of miRNAs identified by this manner aligned with several pathways that are well-associated with stroke, suggesting that these miRNAs may also have significant potential as therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, et al. Heart disease and stroke statistics–2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*. 2008; 117:e25–e146. [PubMed: 18086926]
2. Rojas JI, Zurrú MC, Romano M, Patrucco L, Cristiano E. Acute ischemic stroke and transient ischemic attack in the very old–risk factor profile and stroke subtype between patients older than 80 years and patients aged less than 80 years. *Eur J Neurol*. 2007; 14:895–899. [PubMed: 17662011]
3. Turtzo LC, McCullough LD. Sex-specific responses to stroke. *Future Neurol*. 2010; 5:47–59. [PubMed: 20190872]
4. Cheong JL, Cowan FM. Neonatal arterial ischaemic stroke: obstetric issues. *Semin Fetal Neonatal Med*. 2009; 14:267–271. [PubMed: 19683971]
5. Roquer J, Segura T, Serena J, Castillo J. Endothelial dysfunction, vascular disease and stroke: the ARTICO study. *Cerebrovasc Dis*. 2009; 27:25–37. [PubMed: 19342831]
6. Niewada M, Kobayashi A, Sandercock PA, Kami ski B, Czlonkowska A. Influence of gender on baseline features and clinical outcomes among 17,370 patients with confirmed ischaemic stroke in the international stroke trial. *Neuroepidemiology*. 2005; 24:123–128. [PubMed: 15637449]
7. Fukuda M, Kanda T, Kamide N, Akutsu T, Sakai F. Gender differences in long-term functional outcome after first-ever ischemic stroke. *Intern Med*. 2009; 48:967–973. [PubMed: 19525582]
8. Ambros V. MicroRNAs: tiny regulators with great potential. *Cell*. 2001; 107:823–826. [PubMed: 11779458]
9. Jeyaseelan K, Lim KY, Armugam A. MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke*. 2008; 39:959–966. [PubMed: 18258830]
10. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008; 18:997–1006. [PubMed: 18766170]
11. Rink C, Khanna S. MicroRNA in ischemic stroke etiology and pathology. *Physiol Genomics*. 2011; 43:521–528. [PubMed: 20841499]
12. Cipollone F, Felicioni L, Sarzani R, Uchino S, Spigonardo F, Mandolini C, Malatesta S, Bucci M, Mammarella C, et al. A unique microRNA signature associated with plaque instability in humans. *Stroke*. 2011; 42:2556–2563. [PubMed: 21817153]
13. Dharap A, Bowen K, Place R, Li LC, Vemuganti R. Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome. *J Cereb Blood Flow Metab*. 2009; 29:675–687. [PubMed: 19142192]
14. Tan KS, Armugam A, Sepramaniam S, Lim KY, Setyowati KD, Wang CW, Jeyaseelan K. Expression profile of microRNAs in young stroke patients. *PLoS One*. 2009; 4:e7689. [PubMed: 19888324]
15. Guo D, Liu J, Wang W, Hao F, Sun X, Wu X, Bu P, Zhang Y, Liu Y, Liu F, Zhang Q, Jiang F. Alteration in abundance and compartmentalization of inflammation-related miRNAs in plasma after intracerebral hemorrhage. *Stroke*. 2013; 44:1739–1742. [PubMed: 23559260]
16. Selvamani A, Sathyan P, Miranda RC, Sohrabji F. An antagomir to microRNA Let7f promotes neuroprotection in an ischemic stroke model. *PLoS One*. 2012; 7:e32662. [PubMed: 22393433]
17. Selvamani A, Sohrabji F. The neurotoxic effects of estrogen on ischemic stroke in older female rats is associated with age-dependent loss of insulin-like growth factor-1. *J Neurosci*. 2010; 30:6852–6861. [PubMed: 20484627]
18. Selvamani A, Sohrabji F. Reproductive age modulates the impact of focal ischemia on the forebrain as well as the effects of estrogen treatment in female rats. *Neurobiol Aging*. 2010; 31:1618–1628. [PubMed: 18829137]
19. Blondal T, Jensby Nielsen S, Baker A, Andreassen D, Mouritzen P, Wrang Teilmann M, Dahlsveen IK. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. *Methods*. 2013; 59:S1–S6. [PubMed: 23036329]

20. Vlachos IS, Kostoulas N, Vergoulis T, Georgakilas G, Reczko M, Maragkakis M, Paraskevopoulou MD, Prionidis K, Dalamagas T, Hatzigeorgiou AG. DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Res.* 2012; 40:W498–W504. [PubMed: 22649059]
21. Biernaskie J, Corbett D, Peeling J, Wells J, Lei H. A serial MR study of cerebral blood flow changes and lesion development following endothelin-1-induced ischemia in rats. *Magn Reson Med.* 2001; 46:827–830. [PubMed: 11590661]
22. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke.* 1989; 20:84–91. [PubMed: 2643202]
23. Nagasawa H, Kogure K. Correlation between cerebral blood flow and histologic changes in a new rat model of middle cerebral artery occlusion. *Stroke.* 1989; 20:1037–1043. [PubMed: 2756535]
24. Popp A, Jaenisch N, Witte OW, Frahm C. Identification of ischemic regions in a rat model of stroke. *PLoS One.* 2009; 4:e4764. [PubMed: 19274095]
25. Alkayed NJ, Harukuni I, Kimes AS, London ED, Traystman RJ, Hurn PD. Gender-linked brain injury in experimental stroke. *Stroke.* 1998; 29:159–166. [PubMed: 9445346]
26. DiNapoli VA, Huber JD, Houser K, Li X, Rosen CL. Early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats. *Neurobiol Aging.* 2008; 29:753–764. [PubMed: 17241702]
27. Kelly KA, Li X, Tan Z, VanGilder RL, Rosen CL, Huber JD. NOX2 inhibition with apocynin worsens stroke outcome in aged rats. *Brain Res.* 2009; 1292:165–172. [PubMed: 19635468]
28. Liu F, Akella P, Benashski SE, Xu Y, McCullough LD. Sex differences in caspase activation after stroke. *Stroke.* 2009; 40:1842–1848. [PubMed: 19265047]
29. Martino G, Pluchino S, Bonfanti L, Schwartz M. Brain regeneration in physiology and pathology: the immune signature driving therapeutic plasticity of neural stem cells. *Physiol Rev.* 2011; 91:1281–1304. [PubMed: 22013212]
30. Liao S, Chen W, Kuo J, Chen C. Association of serum estrogen level and ischemic neuroprotection in female rats. *Neurosci Lett.* 2001; 297:159–162. [PubMed: 11137752]
31. Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, Bodor N, Day AL. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *J Neurosurg.* 1997; 87:724–730. [PubMed: 9347981]
32. Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finklestein SP, Rau SW, Wise PM. Estradiol protects against ischemic injury. *J Cereb Blood Flow Metab.* 1998; 18:1253–1258. [PubMed: 9809515]
33. Liu F, Akella P, Benashski SE, Xu Y, McCullough LD. Expression of Na-K-Cl cotransporter and edema formation are age dependent after ischemic stroke. *Exp Neurol.* 2010; 224:356–361. [PubMed: 20406636]
34. Arumugam TV, Phillips TM, Cheng A, Morrell CH, Mattson MP, Wan R. Age and energy intake interact to modify cell stress pathways and stroke outcome. *Ann Neurol.* 2010; 67:41–52. [PubMed: 20186857]
35. Schroeder E, Vogelgesang S, Popa-Wagner A, Kessler C. Neurofilament expression in the rat brain after cerebral infarction: effect of age. *Neurobiol Aging.* 2003; 24:135–145. [PubMed: 12493559]
36. Shakarishvili R, Beridze M. Proinflammatory reactants as determinants of stroke severity in elderly. *Georgian Med News.* 2008; 162:23–27. [PubMed: 18830025]
37. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang K. The microRNA spectrum in 12 body fluids. *Clin Chem.* 2010; 56:1733–1741. [PubMed: 20847327]
38. Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. *Mutat Res.* 2011; 717:85–90. [PubMed: 21402084]
39. Gilad S, Meiri E, Yogeve Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholak H, Melamed N, et al. Serum microRNAs are promising novel biomarkers. *PLoS One.* 2008; 3:e3148. [PubMed: 18773077]
40. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci.* 2010; 101:2087–2092. [PubMed: 20624164]

41. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, Weber M, Hamm CW, Röxe T, Müller-Ardogan M, et al. Circulating microRNAs in patients with coronary artery disease. *Circ Res*. 2010; 107:677–684. [PubMed: 20595655]
42. Li T, Cao H, Zhuang J, Wan J, Guan M, Yu B, Li X, Zhang W. Identification of miR-130a, miR-27b and miR-210 as serum biomarkers for atherosclerosis obliterans. *Clin Chim Acta*. 2011; 14:66–70. [PubMed: 20888330]
43. Diehl P, Olivier C, Bode C, Moser M. Editorial comment: Reduction of oxidative stress: a new indication for acetylsalicylic acid in coronary artery bypass surgery. *Eur J Cardiothorac Surg*. 2013; 43:1163–1164. [PubMed: 23220870]
44. Gidlöf O, Smith JG, Miyazu K, Gilje P, Spencer A, Blomquist S, Erlinge D. Circulating cardio-enriched microRNAs are associated with long-term prognosis following myocardial infarction. *BMC Cardiovasc Disord*. 2013; 13:12. [PubMed: 23448306]
45. Zeng L, Liu J, Wang Y, Wang L, Weng S, Tang Y, Zheng C, Cheng Q, Chen S, Yang GY. MicroRNA-210 as a novel blood biomarker in acute cerebral ischemia. *Front Biosci (Elite Ed)*. 2011; 3:1265–1272. [PubMed: 21622133]
46. Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, Hastings ML, Duelli DM. Selective release of microRNA species from normal and malignant mammary epithelial cells. *PLoS One*. 2010; 5:e13515. [PubMed: 20976003]
47. Yin KJ, Fan Y, Hamblin M, Zhang J, Zhu T, Li S, Hawse JR, Subramaniam M, Song CZ, Urrutia R, et al. KLF11 mediates PPAR γ cerebrovascular protection in ischaemic stroke. *Brain*. 2013; 136:1274–1287. [PubMed: 23408111]
48. Liu XS, Chopp M, Wang XL, Zhang L, Hozeska-Solgot A, Tang T, Kassis H, Zhang RL, Chen C, Xu J, Zhang ZG. MicroRNA-17–92 cluster mediates the proliferation and survival of neural progenitor cells after stroke. *J Biol Chem*. 2013; 288:12478–12488. [PubMed: 23511639]
49. Budde H, Schmitt S, Fitzner D, Opitz L, Salinas-Riester G, Simons M. Control of oligodendroglial cell number by the miR-17–92 cluster. *Development*. 2010; 137:2127–2132. [PubMed: 20504959]
50. Zhang L, Zhao H, Zhang X, Chen L, Zhao X, Bai X, Zhang J. Nobiletin protects against cerebral ischemia via activating the p-Akt, p-CREB, BDNF and Bcl-2 pathway and ameliorating BBB permeability in rat. *Brain Res Bull*. 2013; 96:45–53. [PubMed: 23644141]
51. Xie R, Cheng M, Li M, Xiong X, Daadi M, Sapolsky RM, Zhao H. Akt isoforms differentially protect against stroke-induced neuronal injury by regulating mTOR activities. *J Cereb Blood Flow Metab*. 2013; 33:1875–1885. [PubMed: 23942361]
52. Liu FJ, Lim KY, Kaur P, Sepramaniam S, Armugam A, Wong PT, Jeyaseelan K. MicroRNAs involved in regulating spontaneous recovery in embolic stroke model. *PLoS One*. 2013; 8:e66393. [PubMed: 23823624]
53. Laufs U, Endres M, Stagliano N, Amin-Hanjani S, Chui DS, Yang SX, Simoncini T, Yamada M, Rabkin E, Allen PG, et al. Neuroprotection mediated by changes in the endothelial actin cytoskeleton. *J Clin Invest*. 2000; 106:15–24. [PubMed: 10880044]
54. Weir CJ, Murray GD, Dyker AG, Lees KR. Is hyperglycaemia an independent predictor of poor outcome after acute stroke? Results of a long-term follow up study. *BMJ*. 1997; 314:1303–1306. [PubMed: 9158464]
55. Muir KW, McCormick M, Baird T, Ali M. Prevalence, predictors and prognosis of post-stroke hyperglycaemia in acute stroke trials: individual patient data pooled analysis from the Virtual International Stroke Trials Archive (VISTA). *Cerebrovasc Dis Extra*. 2011; 1:17–27. [PubMed: 22566979]

Abbreviations

CAD	coronary artery disease
FDR	false discovery rate
GSEA	Genesifter [®] Analysis Edition

KEGG	Kyoto Encyclopedia of Genes and Genomes
LNA	locked nucleic acid
MCA	middle cerebral artery
MCAo	MCA occlusion
miRNA (miR)	microRNA
mTOR	mammalian target of rapamycin
PCA	principal component analysis
PI3K	phosphoinositide 3-kinase
PPARγ	peroxisome-proliferator-activated receptor γ
qPCR	quantitative real-time PCR
TTC	triphenyl tetrazolium chloride

Clinical Perspectives

- Clinical and experimental studies indicate that stroke severity is modulated by age and sex.
- The present study shows that circulating miRNA profiles can predict age- and sex-dependent stroke severity in an animal model.
- Furthermore, five miRNA are highly expressed exclusively in adult females, and these miRNA may represent a neuroprotective profile as well as therapeutic targets.

Figure 1. Sex difference in cortical and striatal infarct in adult and middle-aged rats

Adult and middle-aged female and male rats were subject to ET-1-induced MCAo. Infarct volume was assessed using TTC-stained brain sections and quantitative morphometry. **(A)** Adult female rats had significantly smaller cortical and striatal infarct volumes compared with middle-aged females, adult males and middle-aged males. **(B)** Quantification of the infarct size. * $P < 0.05$, $n = 6$ per group. *a*, main effect of age; *b*, main effect of sex; and *c*, sex×age interaction effect.

Figure 2. Test for sensory motor function

Sensory motor function was evaluated by the adhesive tape test by the latency to tape removal from the forepaw. The latency (in s) to tape removal from the palmar surface of the ipsilesional and contralesional paw for each group is shown. **(A)** Post-stroke performance was significantly impaired on the contralesional paw in middle-aged females, but not in adult females compared to pre-stroke performance. **(B)** Post-stroke performance was significantly impaired in adult males and middle-aged males compared with pre-stroke performance. Values are means $\pm$ S.E.M. ($n = 6/\text{group}$), $*P < 0.05$.

Figure 3. miRNA regulation in serum obtained 2 and 5 days post-stroke
miRNA expression was subjected to ANOVA and miRNAs that were significantly regulated (adjusted for FDR) are represented in heat map format. Within each heat map, adult females were used as ‘controls’ and are represented in black, whereas all other groups are depicted in relation to adult females (columns). miRNAs that were up-regulated in comparison with adult females are shown in green, whereas down-regulated miRNAs are shown in red (rows). Main effects of age (a), sex (b) and age×sex interaction (c) is shown for each miRNA. **(A)** Heat map depicts miRNA expression in serum for adult and middle-aged females and males at 2 days post-stroke. **(B)** Heat map depicts miRNA expression in serum for adult and middle-aged females and males at 5 days post-stroke.

Figure 4. Unsupervised hierarchical clustering of differentially expressed serum miRNAs at 2 and 5 days post-stroke

Clustering was based on miRNAs found to be differentially expressed by ANOVA corrected for FDR. **(A)** At 2 days post-stroke, differentially expressed miRNA showed two major clusters based on age (adult compared with middle-aged). **(B)** Serum miRNAs assessed 5 days post-stroke clustered into two major groups, which were segregated by sex (males compared with females). Green indicates up-regulated miRNAs in comparison with adult females, whereas red indicates down-regulation.

Figure 5. PCA of FDR-corrected statistically significant circulating miRNAs from serum at 2 (A) and 5 (B) days post-stroke

PCA of 21 FDR-corrected statistically significant miRNAs at 2 days post-stroke showed that component 1 (Comp. 1) separated miRNAs expressed in serum of adult animals from middle-aged animals, and almost all of the variance was explained by this component. At 5 days post-stroke, PCA of 78 FDR-corrected statistically significant miRNAs showed that component 1 separated miRNAs from males and females, whereas component 2 (Comp. 2) separated miRNAs differentially expressed in adult and middle-aged animals.

Figure 6. Brain miRNA expression profile at 5 days post-stroke

(A) The heat map depicts significantly regulated miRNA expression values for adult and middle-aged males and females in the ischaemic brain at 5 days post-stroke. miRNA expression was considered significantly regulated based on ANOVA with FDR correction. Within the heat map, adult females were used as 'controls' and are represented in black, whereas all other groups are depicted in relation to adult females (columns). miRNAs that are up-regulated in comparison with adult females are shown in green, whereas down-regulated miRNAs are shown in red (rows). Main effects of age (a), sex (b) and age×sex interaction (c) are shown for each miRNA. (B) Hierarchical clustering of differentially expressed brain miRNAs reveal two major clusters: a larger one that discriminates animals by age (adult compared with middle-age; red bar) and the second smaller cluster which discriminates by sex (blue bar). (C) Three-dimensional PCA of differentially expressed genes shows a global view of the 19 significant miRNAs expressed in the brain 5 days post-stroke. Each dot represents the loading of a composite expression profile of one miRNA on to the first and second components. Component 1 (Comp. 1) segregated animals by age, whereas component 2 (Comp. 2) segregated animals by sex.

Figure 7. Group differences in the expression of miRNA that displayed a significant age $\times$ sex interaction at 5 days post-stroke

Group differences in the expression patterns of miRNAs *miR-15a*, *miR-19b*, *miR-32*, *miR-136* and *miR-199a-3p*, which were significantly up-regulated in the adult female group compared with the middle-aged females, adult males and middle-aged males. Values (means + S.E.M.; $n = 6/\text{group}$) are expressed as C_T , where an increased value indicates decreased expression.

Figure 8. KEGG pathways associated with predicted target genes
(A) miRNAs with significant age×sex interaction effects at 5 days post-stroke were subject to *in silico* analysis (DI-ANA/miRPATH, see the Materials and methods section). Predicted gene targets for each miRNA were identified. The Table represents the number of predicted target genes for each miRNA in this cohort. (B) The top 10 KEGG pathways represented by these predicted targets are shown. Each slice represents the number of predicted target genes in the pathway, indicated within the slice. Each pathway is labelled adjacent to the chart. The top ten pathways were selected based on the smallest ‘P’ value and the largest number of target genes in the pathway.

Table 1
Commonalities among circulating miRNAs at 2 and 5 days post-stroke

miRNAs that are significantly regulated (as determined by ANOVA corrected for FDR) at both 2 and 5 days post-stroke are shown. At 2 days post-stroke, all miRNAs were regulated by age, whereas at 5 days post-stroke all of the miRNAs were differentially expressed by sex. Only one miRNA that was regulated by age at 2 days post-stroke was also regulated by age and sex at 5 days post-stroke.

miRNAs common in serum at 2 and 5 days post-stroke	Main effect in serum	
	At 2 days post-stroke	At 5 days post-stroke
<i>miR-127-3p</i>	Age	Sex
<i>miR-335</i>	Age	Sex
<i>miR-543</i>	Age	Sex
<i>miR-139-5p</i>	Age	Sex
<i>miR-33a</i>	Age	Sex
<i>miR-338-3p</i>	Age	Sex
<i>miR-222</i>	Age	Sex
<i>miR-15b-15b*</i>	Age	Sex
<i>miR-92a</i>	Age	Sex
<i>miR-92a</i>	Age	Sex
<i>miR-424</i>	Age	Sex
<i>miR-495</i>	Age	Sex
<i>miR-363</i>	Age	Age and sex