Stroke

CLINICAL AND POPULATION SCIENCES

Circulatory MicroRNAs as Potential Biomarkers for Stroke Risk

The Rotterdam Study

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BACKGROUND AND PURPOSE: MicroRNAs (miRNAs) are post-transcriptionally regulators of gene expression that can be released extracellularly upon pathophysiological processes. By complementary binding of target transcripts, miRNAs can modulate the expression of an abundance of genes. Increasing evidence recognize miRNAs as promising biomarkers for complex traits, including cardiovascular disease and stroke. We conducted a longitudinal study to determine the association between circulatory miRNAs and incident stroke in a population-based setting.

METHODS: Next-generation sequencing was used to measure expression levels of 2083 miRNAs in plasma samples, collected between 2002 and 2005, from 1914 stroke-free participants of the Rotterdam Study. Participants were assessed for incident stroke through continuous monitoring of medical records until January 1, 2016. Cox proportional hazards regression models adjusted for age, sex, and vascular risk factors were used to investigate the association between the levels of 591 miRNAs well-expressed in plasma and incident stroke. Furthermore, stroke subtype analysis was performed to assess the link between identified miRNAs and ischemic, hemorrhagic, and unspecified stroke. Subsequently, post hoc analyses were conducted to gain insight into the association between putative target genes of miRNAs and stroke.

RESULTS: Of 1914 participants (mean age 71.5 years ±7.6; 57.7% women), 138 were diagnosed with incident stroke during a mean follow-up of 9.7±3.2 years. After adjusting for potential confounders, we found plasma levels of 3 miRNAs to be associated with incident stroke (false discovery rate-adjusted $P<0.05$). These include miR-6124 (hazard ratio, 1.66 [95% CI, 1.31–2.09]), miR-5196-5p (hazard ratio, 1.90 [95% CI, 1.39–2.61]), and miR-4292 (hazard ratio, 2.65 [95% CI, 1.62–4.34]). In silico analysis of the putative target genes of these miRNAs showed associations of variants in several target genes with stroke.

CONCLUSIONS: This study indicates that plasma levels of 3 miRNAs are associated with the risk of stroke, proposing them as potential biomarkers for early detection of the disease.

Key Words: biomarkers ■ cardiovascular disease ■ gene expression ■ plasma ■ risk factors

Although environmental and vascular risk factors are well-known to be involved in the pathology of stroke, genetic factors also contribute to the risk of developing ischemic and hemorrhagic stroke.[1–5] It has been previously shown that not only coding genes, in particular loci such as 12q24.2 and *ABO*, account for a proportion of the heritable risk, but also noncoding regions of the genome, such as microRNAs (miRNAs), can play a role in the pathophysiology of stroke.[6] A better understanding of the role of miRNAs in the development of stroke and their potential as biomarkers in the diagnosis of disease could lead to early detection of individuals with high-risk profiles.

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This article was sent to Christoph Kleinschnitz, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.120.031543.

For Sources of Funding and Disclosures, see page 952.

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Nonstandard Abbreviations and Acronyms

FHS	Framingham Heart Study
GWAS	genome-wide association study
HDL	high-density lipoprotein
HR	hazard ratio
miRNA	microRNA
RS	Rotterdam Study
SNP	single-nucleotide polymorphism

miRNAs are small noncoding RNA molecules of ≈22 nucleotides that regulate post-transcriptionally gene expression through complementary binding of target transcripts. They can potentially target hundreds-to thousands genes and are involved in various molecular pathways.[7] In addition to their ability to modulate cellular processes in both disease and nondisease states, miRNAs are released from cells into body fluids, such as plasma.[8] These circulatory miRNAs are very stable in plasma, due to their packaging into membranous vesicles including exosomes and are thereby potentially clinically relevant as biomarkers for diseases.[9] Previous studies have linked changes in plasma miRNA levels with cardiovascular disease and stroke.[10–16] Most of the stroke-associated miRNAs have been identified cross-sectionally in patient cohorts with acute stroke, leaving it uncertain whether the identified miRNAs are related to the risk of developing stroke. Prospective cohorts could overcome this limitation. Indeed, a recent longitudinal study with a modest sample size (n=51 cases) found the association between plasma levels of miR-656-3p and miR-941 and incident stroke with a mean follow-up time of 2.5±1.6 years.[17] However, longitudinal studies with a relatively long follow-up period are limited. In the current study, we determined the association between circulatory miRNAs and the risk of all stroke and its subtypes in a prospective population based study with almost 10 years follow-up. Subsequently, we performed post hoc analyses of the miRNA putative target genes to gain insight into the potential pathways by which the identified miRNAs may play a role in stroke.

METHODS

Data Availability

Requests to access the data set from qualified researchers trained in human subject confidentiality protocols may be sent to Department of Epidemiology, Erasmus MC University Medical Center at f.vanrooij@erasmusmc.nl.

Study Population

This study was conducted within the Rotterdam Study, a large prospective population-based cohort among participants aged ≥45 years in the suburb Ommoord in Rotterdam, the Netherlands. In 1990, 7983 inhabitants aged 55 years or older were recruited to participate in the first cohort of the RS-I (Rotterdam Study I; 78% response rate of 10215 invitees). In 2000, the Rotterdam Study was extended by 3011 participants who moved to Ommoord or turned 55 years old (RS-II). A detailed description of the Rotterdam Study can be found elsewhere.[18] In the current study, miRNA expression profiling (n=2000) was performed in a random subset (n=1000) of the fourth visit of RS-I (RS-I-4) and a random subset (n=1000) of the second visit of RS-II (RS-II-2). These visits of the Rotterdam Study were performed between 2002 and 2005, and with follow-up visits every 4 to 5 years. From 2000 participants with miRNA data, we excluded participants with prevalent stroke (n=83) and individuals with no informed consent for follow-up (n=2). Furthermore, one participant was excluded because of missing data for all miRNAs. For more information regarding the inclusion criteria, see Figure 1.

Informed Consent and Ethics Approval

The Rotterdam Study has been approved by the medical ethics committee at the Erasmus University of Rotterdam and the Ministry of Health, Welfare and Sport of the Netherlands. The study is implemented in the Population Studies Act: Rotterdam Study (Wet Bevolkingsonderzoek ERGO). All participants included in the current study provided written informed consent for participation and for researchers to access medical information from their personal physicians.

Measurements on Circulatory miRNAs

Blood samples were collected in EDTA treated containers and centrifuged. Plasma was then aliquoted and frozen at −80 °C according to standard procedures. Subsequently, plasma miRNA levels were determined using the HTG EdgeSeq miRNA whole transcriptome assay, which measures the expression of 2083 mature human miRNAs (HTG Molecular Diagnostics, Tuscon, AZ) and using the Illumina NextSeq 500 sequencer (Illumina, San Diego, CA). The whole transcriptome assay characterizes miRNA expression patterns and measures the expression of 13 housekeeping genes, allowing flexibility in data normalization and analysis. Quantification of miRNA expression was based on counts per million. Log2 transformation of counts per million was used as standardization and adjusted for total reads within each sample. The lower limit of quantification was used to select well-expressed miRNAs. The lower limit of quantification level was based on a monotonic decreasing spline curve fit between the means and standard deviations of all miRNAs. In our definition, well-expressed miRNA levels in plasma were those with >50% values above lower limit of quantification. This includes a set of 591 miRNAs that were used for association analysis.

Assessment of Stroke

Stroke was defined according to the World Health Organization definition as a syndrome of rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin.[19,20] We assessed the prevalence of stroke at baseline during interview and verified

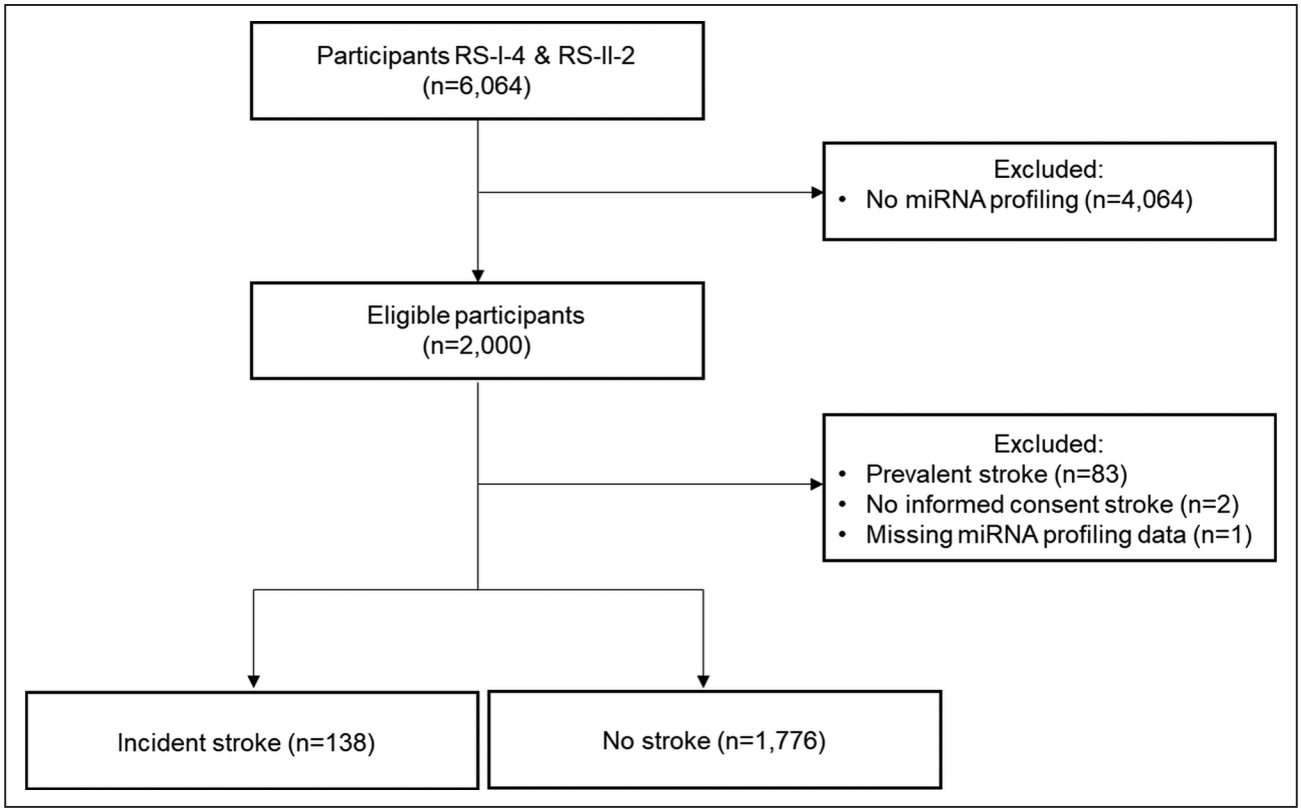

Figure 1. Flowchart of the study participants.
miRNA indicates microRNA; and RS, Rotterdam Study.

it using medical records. After enrollment, participants were continuously monitored for incident stroke by linking the study database to files of general practitioners. Records of nursing homes and records from general practitioners of the participants who moved out of the study district were also checked. Additional information, such as clinical notes and neuroimaging reports, were obtained from hospital records.

Patients with stroke were further classified as ischemic or hemorrhagic based on neuroimaging reports. If neuroimaging was lacking, a stroke was classified as unspecified. This classification corresponds with International Classification of Diseases-10 codes I61, I63, and I64. Potential stroke patients were reviewed by research physicians and verified in a consensus panel led by an experienced stroke neurologist.

The follow-up for incident stroke was conducted until January 1, 2016. Participants were followed from study entry until stroke, death, last health status update when they were known to be stroke-free, or January 1, 2016, whichever came first. Follow-up was complete for 92.9% of potential person-years.

Assessment on Covariates

Information on age, sex, and smoking status (current/former/never) was obtained from questionnaires. Body mass index was calculated based on weight in kilograms divided by the height in meters squared. Blood samples of participants were obtained during the visit to the research center. Using automatic enzymatic method, levels of HDL (high-density lipoprotein) and total cholesterol were measured in serum (mmol/L). Systolic and diastolic blood pressure was measured (mm Hg) in a seating position after a 5 minute rest period on the right upper arm of the participant using a random-zero sphygmomanometer. Prevalent diabetes mellitus type 2 was identified according to the World Health Organization criteria: fasting glucose levels of ≥7.0 mmol/L, nonfasting glucose levels ≥11.1 mmol/L, or the use of glucose-lowering medication. Prevalent coronary heart disease was defined when the participant suffered a myocardial infarction or underwent a coronary artery bypass grafting or percutaneous coronary revascularization procedure. Prevalent atrial fibrillation was assessed using a 10 seconds 12-lead ECG and medical records. Information regarding the use of lipid-lowering medication, blood pressure-lowering medication, and glucose-lowering medication was obtained from pharmacy records and home interviews. All covariates included for statistical analysis were obtained at baseline examination.

Statistical Analysis

We used Cox proportional hazards regression to determine hazard ratios (HR) with 95% CIs for the association between miRNA expression and stroke risk. HRs were reported per each additional unit of log2 counts per million of miRNA expression. Models were adjusted for age, sex, and cohort. Additionally, we adjusted for smoking, body mass index, HDL, total cholesterol, systolic blood pressure, diastolic blood pressure, prevalent diabetes mellitus type 2, prevalent coronary heart disease, prevalent atrial fibrillation, lipid-lowering medication, and blood pressure-lowering medication. To reduce the false-positive results, the Benjamini-Hochberg procedure was

used to adjust the *P* values into false discovery rate.[21] In this study, a false discovery rate corrected $P<0.05$ (5%) was set as significance threshold. Subsequently, we analyzed the association between the expression of the identified miRNAs and risk of ischemic, hemorrhagic, and unspecified stroke separately. To avoid the risk of overfitting, we adjusted models for the association between miRNA expression and hemorrhagic and unspecified stroke only for age, sex, and cohort. To reduce the possible bias induced by missing values, multiple imputation on confounders was performed based on outcome and included covariates for predictors of missing data. Values were imputed with a maximum iteration number of 10 (n=25 imputations) using the Markov Chain Monte Carlo method, R package mice. Areas under the curve for time-dependent receiver operating characteristics at 10 years were calculated for the vascular risk factor model (covariates included in model 2) and the vascular risk factor model complemented by the identified miRNAs. The areas under the curve were calculated using the Kaplan-Meier method, R package *survivalROC*. Furthermore, the identified miRNAs were categorized in high or low expressed based on their median expression values and cumulative hazard graphs were generated. Additionally, we performed a permutation test to check the enrichment of the identified miRNAs in the association with stroke compared with sets of randomly selected miRNAs. Analyses were performed using R version 3.6.1 (The R foundation for Statistical Computing, Vienna, Austria).

Post Hoc Genetic Analyses

To test whether the stroke-associated miRNAs are potentially involved in the pathways underlying disease, we performed in silico analysis on their putative target genes. Three commonly used miRNA target prediction databases, TargetScan (v7.2),[22] miRTarBase,[23] and miRDB,[24] were used to retrieve putative target genes of the stroke-associated miRNAs. Then, we extracted single-nucleotide polymorphisms (SNPs) in these target genes. Moreover, we extracted SNPs located in ±2 kb of the precursor sequences of the associated miRNAs.[25] Subsequently, we performed a look-up to test whether the SNPs located in target genes and in ±2 kb of the miRNA precursor sequences are associated with stroke by using the summary statistics of a previous genome-wide association study (GWAS) on stroke.[3] Target genes were considered significantly associated with stroke based on false discovery rate <0.1 (10%).

Furthermore, we sought to explore whether the identified miRNAs are expressed in the brain, a relevant tissue for stroke. We also retrieved the miRNA host genes as proxy for the identified miRNAs to check their expression in the brain using the Human Protein Atlas (https://www.proteinatlas.org/).[26] The rationale for this analysis is that the genomic location of miRNAs can be discriminated among intergenic and intragenic. The intragenic miRNAs and their host genes may share the same promoter, and these miRNA are likely to be co-expressed with their host genes.[27] The genomic location of the identified miRNAs was obtained using miRIAD.[28]

RESULTS

The baseline characteristics of the 1914 Rotterdam Study participants used for this study are presented in Table 1. The individuals who were diagnosed with incident stroke were on average slightly older compared with noncases (74.6 years versus 71.2 years).

During a mean follow-up of 9.7 years (±3.2), 138 individuals were diagnosed with incident stroke. Of these, 96 were ischemic, 19 were hemorrhagic, and 23 cases were unspecified. The results of the association between miRNA levels in plasma and the incidence of stroke are presented in Table 2. We found significant associations between the expression levels of 3 miRNAs and risk of stroke, miR-6124 (HR, 1.66 [95% CI, 1.31–2.09], $P=2.00\times10^{-5}$), miR-5196-5p (HR, 1.90 [95% CI, 1.39–2.61], $P=6.07\times10^{-5}$), and miR-4292 (HR, 2.65 [95% CI, 1.62–4.34], $P=1.09\times10^{-4}$). In total, 39 miRNAs were at least nominally associated ($P<0.05$) with incident stroke

Table 1. Baseline Characteristics of the Rotterdam Study Participants of This Study

Characteristic	Incident stroke, N=138	No incident stroke, N=1776	*P* value
Age, y	74.6±7.5	71.2±7.5	<0.001
Female, n (%)	76 (55.1%)	1029 (57.9%)	0.51
Body mass index, kg/m^2	27.6±3.4	27.6±4.2	0.87
Systolic blood pressure, mm Hg	156.5±23.0	147.5±20.6	<0.001
Diastolic blood pressure, mm Hg	80.6±12.2	79.4±10.8	0.25
Total serum cholesterol, mmol/L	5.5±1.1	5.7±1.0	0.13
High-density lipoprotein, mmol/L	1.4±0.4	1.5±0.4	0.5
C-reactive protein, mg/L	3.9±6.5	3.0±6.3	0.11
Smoking			0.35
Current, n (%)	20 (14.5%)	260 (14.6%)	
Former, n (%)	83 (60.1%)	966 (54.4%)	
Never, n (%)	35 (25.4%)	550 (31.0%)	
Prevalent diabetes mellitus type 2, n (%)	26 (18.8%)	217 (12.2%)	<0.05
Prevalent coronary heart disease, n (%)	22 (15.9%)	172 (9.7%)	<0.05
Prevalent atrial fibrillation, n (%)	8 (5.8%)	79 (4.4%)	0.46
Prevalent heart failure, n (%)	13 (9.4%)	77 (4.3%)	<0.01
Chronic kidney disease, n (%)	3 (2.2%)	22 (1.2%)	0.34
Any internal carotid artery plaque, n (%)	89 (64.5%)	913 (51.4%)	<0.01
Any internal carotid artery stenosis, n (%)	16 (11.6%)	89 (5.0%)	<0.01
Blood pressure lowering medication, n (%)	77 (55.8%)	737 (41.5%)	<0.01
Lipid lowering medication, n (%)	29 (21.0%)	385 (21.7%)	0.86
Anticoagulant medication, n (%)	50 (36.3%)	348 (19.6%)	<0.001
Antiplatelet medication, n (%)	7 (5.1%)	93 (5.2%)	0.93

Variables are presented as mean±SD, or number (%). Missing values were imputed. Number of missing values for final study population: 8 (0.4%) for systolic blood pressure, 8 (0.4%) for diastolic blood pressure, 12 (0.6%) for blood pressure-lowering medication, 12 (0.6%) for lipid-lowering medication, 26 (1.4%) for coronary heart disease, 28 (1.5%) for smoking, 37 (1.9%) for body mass index, and 75 (3.9%) for diabetes mellitus type 2. Differences between incident stroke cases and noncases were examined by *t* test for continuous variables and χ^2 test for categorical variables.

Table 2. Association Between miRNA Expression and Stroke Incidence

	Any stroke (n=138)				Ischemic stroke (n=96)				Hemorrhagic stroke (n=19)		Unspecified stroke (n=23)	
	Model 1		Model 2		Model 1		Model 2		Model 1		Model 1	
miRNA	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
miR-6124	1.68 (1.35–2.09)	2.49×10^{-6}	1.66 (1.31–2.09)	2.00×10^{-5}	1.62 (1.22–2.15)	8.82×10^{-4}	1.61 (1.17–2.20)	3.03×10^{-3}	2.50 (1.70–3.68)	3.61×10^{-6}	1.38 (0.71–2.68)	3.49×10^{-1}
miR-5196-5p	1.94 (1.44–2.63)	1.71×10^{-5}	1.90 (1.39–2.61)	6.07×10^{-5}	1.85 (1.23–2.78)	2.95×10^{-3}	1.88 (1.21–2.92)	4.98×10^{-3}	3.13 (1.88–5.21)	1.15×10^{-5}	1.38 (0.48–3.91)	5.48×10^{-1}
miR-4292	2.68 (1.63–4.42)	1.03×10^{-4}	2.65 (1.62–4.34)	1.09×10^{-4}	2.61 (1.38–4.97)	3.34×10^{-3}	2.79 (1.43–5.45)	2.64×10^{-3}	4.87 (1.66–14.33)	4.03×10^{-3}	2.31 (0.57–9.33)	2.39×10^{-1}

Model 1: adjusted for age, sex, and cohort. Model 2: model 1+smoking, BMI, HDL, total cholesterol, systolic blood pressure, diastolic blood pressure, prevalent diabetes mellitus type 2, prevalent coronary heart disease, prevalent atrial fibrillation, lipid-lowering medication, and blood pressure-lowering medication. BMI indicates body mass index; FDR, false discovery rate; HR, hazard ratio; and miRNA, microRNA.

(Table I in the Data Supplement). The distribution of plasma expression levels of the 3 identified miRNAs in the study population are illustrated in Figure 2. The areas under the curve for the time-dependent receiver operating characteristics at 10 years was 0.735 for the vascular risk factor model and 0.761 for the model complemented by miR-6124, miR-5196-5p, and miR-4292 (Figure 3). Analyses by stroke subtype showed associations of miR-6124, miR-5196-5p, and miR-4292 with both ischemic and hemorrhagic stroke. However, the number of hemorrhagic cases was small (n=19), which resulted in a wide 95% CI.

To assess the impact of relative expression of the identified miRNAs on 10-year stroke risk, we categorized the expression values based on the median value of each miRNA into 2 categories, including low and high. See Table II in the Data Supplement for the baseline characteristics split by the median expression values of the three miRNAs. We found that high expression levels of miR-6124 (median normalized value >10.01) and miR-5196-5p (median normalized value >8.09) were also significantly associated with higher cumulative hazard of stroke (Figure 4). A combination of the high values of miR-6124, miR-5196-5p, and miR-4292 is represented in 23.7% of the study participants and was associated with a higher cumulative hazard for the risk of stroke ($P=4.20\times10^{-4}$).

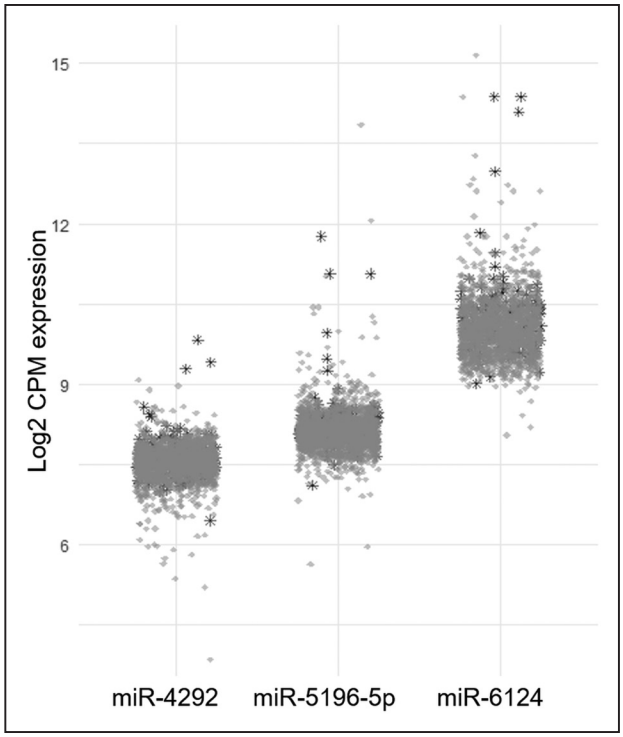

Figure 2. Scatter plots of the distribution of log2 expression values of the 3 identified microRNAs (miRNAs) in the study participants.
Stars indicate cases (incident stroke), and dots indicate noncases (controls). CPM indicates counts per million.

Next, we retrieved the predicted target genes of miR-6124, miR-5196-5p, and miR-4929 from the online miRNA target prediction databases. We focused on the target genes overlapping in at least 2 out of the three databases. This resulted in 1633 target genes for miR-6124, 821 target genes for miR-5196-5p, and 384 target genes for miR-4292. We extracted SNPs in these target genes and tested their associations with risk of stroke using summary statistic from a recent GWAS on stroke.[3] Based on false discovery rate <0.1, we found variants in 10 unique target genes to be associated with stroke (Table III in the Data Supplement). Among these, 9 were putative target genes of miR-6124 (*CASZ1*, *COL15A1*, *HDAC9*, *NRP2*, *RERE*, *SH3PXD2A*, *SLC4A8*, *STXBP5*, *ZFHX3*), one was target gene of miR-5196-5p (*SH3PXD2A*), and 3 were target genes of miR-4292 (*CASZ1*, *FURIN*, *STXBP5*). Furthermore, we found that rs79684932 (T>C), located on chromosome 19, ≈15.9 kb upstream of the transcription start site of miR-5196 is nominally associated with stroke in the GWAS data ($P=1.20\times10^{-2}$, $\beta=-0.065$).[3] This SNP was also reported to be associated with the expression of *CD22*, the host gene of miR-5196, in whole blood.[29]

Finally, we sought to explore whether the 3 identified miRNAs are expressed in the brain. We did not find any reports regarding the brain expression levels of the identified miRNAs in literature. Alternatively, we explored the expression in brain of the miRNA host genes as proxy

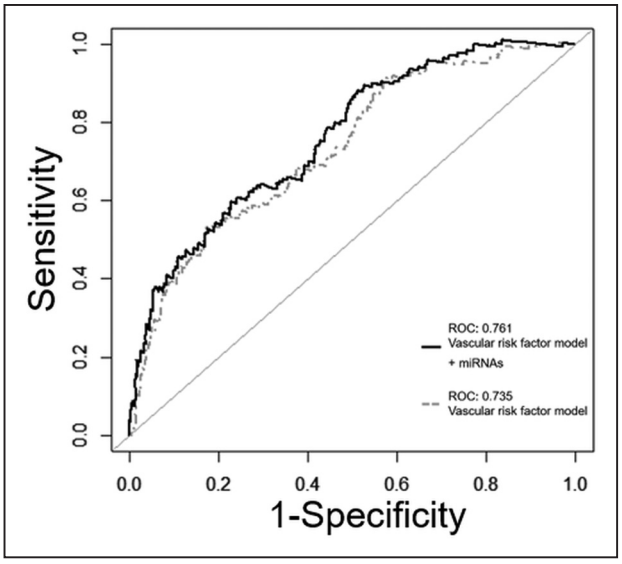

Figure 3. Time-dependent receiver operating characteristics (ROC) curve analysis at 10 y.
The lower line indicates a ROC curve of the vascular risk factor model with an area under the curve (AUC) of 0.735. The upper line indicates a ROC of the vascular risk factor model combined with the plasma values of miR-6214, miR-5196-5p, and miR-4292, with an AUC of 0.761.

for the identified miRNAs. Two of the 3 identified miRNAs (miR-6124 and miR-4292) were reported to be embedded within an intron of the protein-coding genes (*MICAL2* and *RABL6*) in a sense orientation.[28] Moreover, miR-5196-5p is located in the exonic region of *CD22*. All 3 host genes are expressed in brain tissue, of which *MICAL2* has been reported to be expressed at relatively high levels in the brain according to the Human Protein Atlas (https://www.proteinatlas.org/).[26] Out of the 10 stroke-associated target genes, expression levels of 8 genes were detected in the brain (*HDAC9*, *FURIN*, *NRP2*, *RERE*, *SH3PXD2A*, *SLC4A8*, *STXBP5*, *ZFHX3*), of which *SLC4A8* has been reported to be expressed at relatively high levels in the brain.[26]

DISCUSSION

In this longitudinal study, we found that higher plasma levels of 3 miRNAs (miR-6124, miR-5196-5p, and miR-4292) were significantly associated with stroke risk at the population level, suggesting them as potential plasma biomarkers for the disease. In addition, we identified several putative target genes of these miRNAs to be associated with stroke using GWAS data. These observations may suggest that the identified miRNAs are also involved in the pathophysiology of stroke that warrant further investigations.

The findings of this study that circulatory miRNAs are associated with incident stroke is consistent with a previous longitudinal study.[17] In their longitudinal study on ≈2700 participants from the FHS (Framingham Heart Study), Mick et al found plasma levels of miR-656-3p and miR-941 to be associated with incident stroke (n=51 cases).[17] This may confirm that miRNAs are relevant as biomarkers for stroke risk in the general population. But the major differences between Mick et al and the current study include the methodology and data normalization. Therefore, the number of available miRNAs measured by quantitative polymerase chain reaction in the FHS (301 miRNAs) and next-generation sequencing in the current study (591 miRNAs) do not have to overlap completely between studies. Notably, the expression levels of miR-6124, miR-5196-5p, and miR-4292 were not included in the study of Mick et al, and similarly, miR-656-3p and miR-941 were not among the 591 well-expressed miRNAs included in the current study. An independent study from the FHS observed no significant differences of 257 blood-derived miRNA levels between incident stroke cases (n=80) and nonstroke participants.[30] In a similar study, Salinas et al[30] linked the levels of miR-574-3p to prevalent stroke. However, the follow-up time of previous studies was considerably shorter than the current study (3.2 and 2.5 years versus 9.7 years). Given the relatively small effect of a single miRNA, changes in expression levels are likely to be more impactful over a longer period of time. This may explain why Salinas et al[30] found no association between miRNA expression levels and incident stroke. In addition, a different study population is likely to reflect a different miRNA abundance. The FHS participants were 5.2 years younger than the participants of the RS, and age is highly associated with both miRNA expression and stroke risk.[1,31]

A notable observation of previous studies regards the difference in miRNA detection between different types of extracellular biofluids such as, serum, plasma, urine, and saliva but also between extracellular and cellular fluids, like whole blood.[8,32] Most previous studies have measured miRNA expression in whole blood.[11,15,30] Although, blood is an easy accessible tissue, blood biomarkers are more likely to reflect blood-based features than phenomena caused by disease.[33] In particular, cell-derived vesicles, known as exosomes release miRNAs into extracellular fluids, which can be linked to different pathological conditions. We measured cell-free miRNA levels in plasma, which is, therefore, unbiased compared with miRNAs levels in whole blood.

miRNAs can affect the expression of protein-coding genes by complementary binding of their target sequences. Alteration in expression of stroke-associated genes can influence the disease pathophysiology. We found that the 3 identified miRNAs can potentially target 10 genes that are associated with stroke using the GWAS data.[3] Among these, 8 genes are expressed in the brain.[26] Furthermore, the 3 identified miRNAs are located in intergenic regions of coding genes and are, therefore, likely to be expressed together with their host genes in the brain.[27] For instance, miR-6124 is located in the intronic region of *MICAL2*, which has been reported to

Stroke. 2021;52:945–953. DOI: 10.1161/STROKEAHA.120.031543

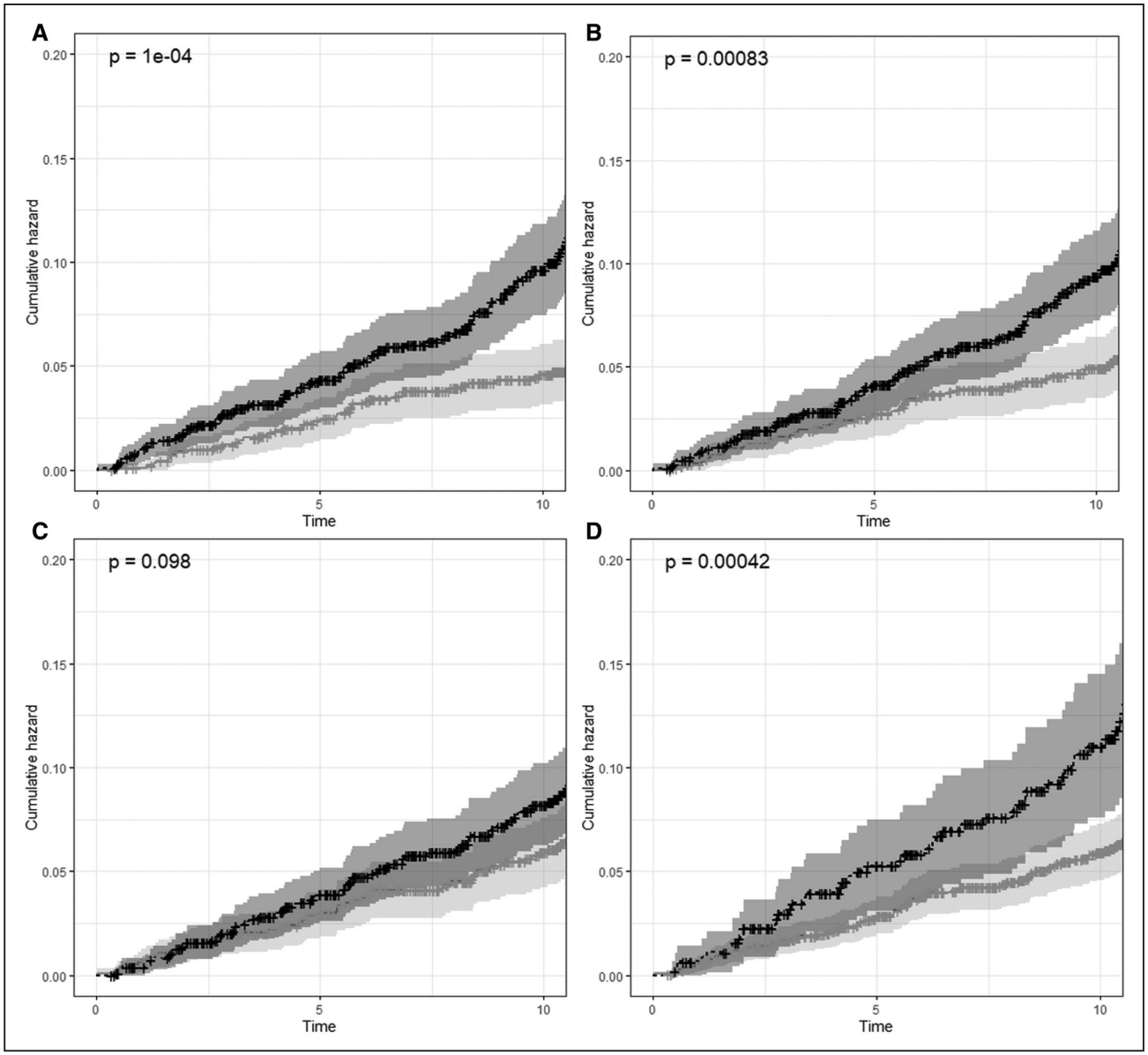

Figure 4. Cumulative hazard curves of log2 counts per million expression of the 3 identified microRNAs (miRNAs) and the risk of incident stroke.

The **upper** curves indicate a high expression level, and the **lower** curves indicate a low expression level based on the log2 median values of individual miRNAs. *P* value indicates difference between the 2 curves. *y* axis indicates cumulative hazard. *x* axis indicates time scale in years. **A**, Cumulative hazard on incident stroke between high and low expression levels of miR-6124. **B**, Cumulative hazard on incident stroke between high and low expression levels of miR-5196-5p. **C**, Cumulative hazard on incident stroke between high and low expression levels of miR-4292. **D**, Cumulative hazard on incident stroke between high and low expression levels of miR-6124, miR-5196-5p, and miR-4292.

be expressed at relatively high levels in brain tissue.[26] To our knowledge, *MICAL2* has not been previously linked to stroke in human. However, Hou et al[34] demonstrated that Mical2 is involved in the endothelial and vascular mechanisms that are disturbed with an ischemic stroke in mice. In addition, according to Malik et al[3] some of the identified putative target genes are related to vascular traits, such as blood pressure (*CASZ1*, *FURIN*), coronary artery disease (*HDAC9*), and atrial fibrillation (*ZFHX3*). The latter gene, *ZFHX3*, is associated with cardioembolic stroke, which accounts for a significant proportion of ischemic strokes.[3] However, it is beyond the scope of this study to elaborate on the pathophysiologic implications of putative target genes. Future studies are needed to experimentally confirm the regulatory interaction between the identified miRNAs and target genes and their relation to stroke subtypes.

Furthermore, we observed differences in the association between the median expression levels of the 3 identified miRNAs and various stroke risk factors (Table II in the Data Supplement). While miR-6124 and miR-5196-5p are associated with nearly all stroke risk

factors, miR-4292 shows only a nominal association with HDL and body mass index. This may indicate that the 2 former miRNAs play a role in the causal pathways of stroke. MiR-4292, unlike miR-6124 and miR-5196-5p, might be involved differently in stroke but is still useful as a predictive biomarker of the disease. Future studies are needed to identify the molecular mechanisms by which these miRNAs promote stroke.

Our study has limitations that need to be considered for interpretation of the results. The pathology of ischemic and hemorrhagic stroke is different, and for risk stratification purposes, it is important to know whether a biomarker is specific for a subtype. With our study design, we were able to elaborate on the associations between some miRNAs and stroke subtypes. However, the number of cases of hemorrhagic stroke was small (n=19) and with a wide 95% CI; future studies with larger samples are needed to provide more certainty. In addition, we used the brain tissue expression levels of the host genes as proxy for the identified miRNAs. Since most tissue-specific expression levels are obtained via quantitative polymerase chain reaction methods, no information was available on the RNA sequencing-measured expression levels of the identified miRNAs in brain tissue. Strengths of our study include the prospective design, almost 10 years of follow-up and the ability to measure virtually all known miRNAs to date with a highly specific and sensitive method. The ideal biomarker is easy assessable, non-invasive, cost-effective, disease-specific and can be detected before the onset of the disease. To this end, plasma-derived miRNAs are favorable for biomarker discovery and unbiased compared with blood-derived miRNAs that might reflect blood-specific features.

CONCLUSIONS

This study indicates that higher plasma levels of miR-6124, miR-5196-5p, and miR-4292 are associated with the risk of stroke in the general population. The elevated levels of these miRNAs may reflect the risk of stroke and, therefore, could serve as plasma biomarkers for early diagnosis of the disease. Future studies with even more samples and experimental validation are warranted to replicate and verify the potential of the identified miRNAs as biomarker of stroke and their roles in the onset of disease.

ARTICLE INFORMATION

Received June 26, 2020; final revision received October 23, 2020; accepted November 26, 2020.

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Acknowledgments

We are grateful to the Rotterdam Study participants, the staff involved with the Rotterdam Study, and the participating general practitioners and pharmacists. We thank the members of lab and bioinformatics teams of the HTG Molecular Diagnostics, Tucson, Arizona for performing the miRNA expression analyses. The author's contributions to this study were as follow: M.M.J. Mens and Dr Ghanbari conceptualization of the study; M.M.J. Mens conducted the analyses; M.M.J. Mens wrote original draft of the article; Drs Heshmatollah, Fani, M.A. Ikram, M.K. Ikram, and Ghanbari provided resources and data; Drs Heshmatollah, Fani, M.A. Ikram, M.K. Ikram, and Ghanbari reviewed article. All authors approved the final article.

Sources of Funding

The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam. miRNA expression analyses by HTG EdgeSeq miRNA whole transcriptome assay (WTA) was funded by Johnson & Johnson.

Disclosures

None.

Supplemental Materials

Tables I–III

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