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RESEARCH ARTICLE

Plasma Sestrin2 levels and risk of acute ischemic stroke: A case-control study

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Sestrin2, a stress-inducible protein with antioxidant properties, is upregulated in response to various stressors, including oxidative and energetic stress. This study examines the relationship between plasma Sestrin2 levels, total antioxidant capacity (TAC), total nitric oxide (NO), and the likelihood of experiencing an acute ischemic stroke (AIS) within the Qatari population. The cohort consisted of 187 AIS patients and 30 healthy controls. Plasma concentrations of Sestrin2, TAC, and nitrite/nitrate (an indirect measure of NO) were evaluated at four intervals: within 48 h of stroke onset, and at 5 days, 30 days, and 1 year post-stroke. At stroke onset, AIS patients exhibited significantly lower plasma levels of Sestrin2 (1.434 ± 3.57 vs 8.383 ± 7.39; $P < 0.001$), TAC (1.88 ± 0.42 vs 2.279 ± 0.326; $P < 0.001$), and nitrite/nitrate (53.5 ± 47.9 vs 65.951 ± 44.07; $P = 0.04$) compared to controls. Sestrin2 levels remained diminished at 5 and 30 days post-stroke, while NO levels increased by day 5 ($P = 0.01$). Multiple logistic regression analysis revealed that male sex, diabetes, high National Institutes of Health Stroke Scale (NIHSS) scores, and small vessel disease (SVD) were associated with increased odds of AIS, whereas Middle Eastern ethnicity correlated with reduced odds. Notably, higher tertiles of Sestrin2, TAC, and NO were linked to decreased odds of AIS, with adjusted odds ratios of 0.123 ($P < 0.001$), 0.327 ($P = 0.01$), and 0.063 ($P = 0.01$), respectively. The observed lower plasma levels of Sestrin2, TAC, and NO at stroke onset and up to 30 days post-event suggest their potential role as biomarkers in stroke occurrence and recovery, with elevated levels associated with a decreased likelihood of AIS.

Keywords: Sestrin2, acute ischemic stroke, ischemia, nitric oxide, total antioxidant capacity.

Introduction

Stroke is recognized as the second leading cause of death and a significant global contributor to disability [1]. According to the World Health Organization (WHO) status report on noncommunicable diseases in 2015, there were 33 million stroke survivors worldwide in need of long-term follow-up and intervention [2]. Over recent decades, the incidence of stroke among individuals under 65 has increased, with a global rise of 25% in stroke cases among adults aged 20–64 [1]. This trend is particularly evident in low- and middle-income countries [1]. Ischemic stroke exhibits much higher incidence rates compared to hemorrhagic stroke; however, the latter presents a higher mortality rate [2]. In Southeast Asia (SA) and the Middle East (ME), the rates of acute ischemic stroke (AIS) are notably elevated due to a high prevalence of risk factors such as diabetes, hypertension, obesity, and metabolic syndrome [3].

Qatar, located on the northeastern coast of the Arabian Peninsula, has a population comprising less than 15% Qatari nationals, with around 60% being migrants from SA. The significant proportion of migrant workers contributes to a relatively young and predominantly male population [4]. Although designated as a high-income country by the United Nations, Qatar is considered a developing nation due to its large migrant population [5]. The burden of stroke in Qatar is substantial, with an estimated incidence of 41 per 100,000 inhabitants annually. For individuals over 45 years of age, the incidence rises to 238 per 100,000 inhabitants per year [6]. The average age for stroke onset in Qatar is 55 years, with the estimated incidence of first AIS at 52 per 100,000 patient-years; notably, 50% of affected individuals are 50 years old or younger [7]. Despite a relatively low mortality rate associated with AIS, there are significant concerns regarding life-altering disabilities and the risk of recurrence [8]. According to United Nations projections, the life expectancy in Qatar for 2023 is estimated at approximately 80.73 years [9]. This implies that stroke survivors, with an average onset age of 55, could have about 26 additional years of life [7]. However, the high risk of recurrent strokes and associated mortality drastically reduces the chances of surviving without disability or a compromised quality of life [7]. Current diagnostic methods for AIS primarily include non-contrast computed tomography (CT) and magnetic resonance imaging (MRI) [10].

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Reactive oxygen species (ROS) serve as signaling molecules produced during normal physiological processes such as aerobic respiration and inflammation. Under physiological conditions, a balance exists between ROS production and antioxidant activity [11]. In healthy individuals, ROS production is counteracted by antioxidant mechanisms [12]. However, in pathological states such as ischemia, excessive ROS disrupts this balance, leading to oxidative stress and cellular dysfunction [11, 12]. ROS is implicated in the pathophysiology of various cardiovascular disorders, including atherosclerosis, cardiomyopathy, hypertension, and stroke [13]. Oxidative stress adversely affects the endothelium, which is crucial for maintaining vascular homeostasis [14]. The endothelium secretes multiple factors, including nitric oxide (NO) and endothelin-1 (ET-1), which regulate vasodilation, vasoconstriction, cell growth, angiogenesis, and fibrinolysis [15]. Endothelial dysfunction, marked by reduced NO bioavailability and impaired angiogenic capacity, is a key factor in vascular damage and atherosclerosis [15, 16].

Sestrin2, a pivotal oxidative stress defense protein belonging to the sestrin family, plays a critical role in maintaining cellular homeostasis by regulating the mammalian target of rapamycin (mTOR) signaling and ROS [17, 18]. As a stress-inducible protein with antioxidant properties, Sestrin2 is upregulated in response to oxidative and energetic stress, preventing the unfolded protein response (UPR), inhibiting endoplasmic reticulum (ER) stress, and promoting cell survival and homeostasis [17]. Research indicates that Sestrin2 is upregulated during ER stress and may provide protective benefits against ER stress-induced disturbances [19]. Furthermore, Sestrin2 is involved in the autophagy process by regulating the mTOR/AMP-activated protein kinase (AMPK) signaling pathway; its deficiency can lead to impaired autophagy and ROS accumulation [17, 18]. Sestrin2 has also been shown to reduce the risk of cardiovascular disease through AMPK activation, mTOR inhibition, and Nuclear factor erythroid 2-related factor 2 (Nrf2) activation [17, 18]. Recently, Sestrin2 has attracted attention for its potential role in ischemic disease. Studies demonstrate that intranasal administration of human recombinant Sestrin2 in neonatal rat pups with hypoxic-ischemic encephalopathy reduces infarct size, brain atrophy, and apoptosis, while improving ventricular area and neurological function [20]. In rats subjected to induced cerebral ischemia, Sestrin2 overexpression enhances angiogenesis through Nrf2 activation [21]. This overexpression increases blood vessel density and length, reducing brain injury [21]. Its neuroprotective effects are believed to be mediated through vascular endothelial growth factor (VEGF) upregulation via the Nrf2/heme oxygenase (HO)-1 pathway [20].

Despite the increasing interest in Sestrin2's role in ischemic diseases, clinical studies investigating the correlation between circulating Sestrin2 levels and stroke incidence in patients are limited. The primary aim of this study was to evaluate the relationship between plasma Sestrin2 levels and the odds of experiencing AIS within the Qatari population. The secondary objective was to explore the association between plasma total antioxidant capacity (TAC), NO levels, and the likelihood of stroke. This study also examined the overall risk factors for developing AIS and assessed whether plasma Sestrin2 could confer a protective role against AIS.

Materials and methods

Study population and sampling

This case-control study involved a cohort of AIS patients and healthy controls. Recruitment occurred at Hamad General Hospital (HGH), Hamad Medical Corporation (HMC), with all patient samples processed in the Institute of Neuroscience laboratories at HMC. Participants were enrolled within 48 h of stroke onset. Only adults over 18 years who provided written informed consent were included. Stroke was defined according to WHO criteria as "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 h or longer or leading to death, with no apparent cause other than vascular origin." All AIS patients admitted to the HGH stroke unit were screened for eligibility. Exclusion criteria included transient ischemic attacks (TIA), stroke mimics, intracerebral hemorrhage, and cerebral venous sinus thrombosis. Healthy controls were defined as individuals without any known diagnosed illnesses and were recruited concurrently with patient enrollment. Control participants were age-matched healthy individuals who volunteered but were excluded if they were visiting the outpatient department. All controls underwent screening to confirm no history of stroke, TIA, or significant neurological or systemic illnesses. All participants received the same research information sheet and provided informed consent prior to enrollment.

Data collection and outcome measures

Baseline demographics and health information were recorded for all participants, including age, gender, employment status, ethnicity, marital status, nationality, smoking habits, alcohol consumption, and medical comorbidities. Relevant medical histories, such as heart failure, coronary artery disease (CAD), diabetes, peripheral vascular disease, chronic kidney disease, and dyslipidemia, were documented, alongside details regarding current prescription and over-the-counter medications. Plasma levels of Sestrin2, nitrite/nitrate (NO), and TAC were measured at four time points: within 48 h of onset, and at 5 days, 30 days, and 1 year post-stroke. Other clinical and cardiometabolic parameters were collected only at baseline. The primary outcome of the study was the odds of having AIS.

Diagnosis of AIS was confirmed through a CT head scan, echocardiogram, and conventional carotid ultrasound, which are standard components of stroke management protocols at HGH. Two vascular neurologists independently evaluated all imaging studies. A consensus meeting was held to resolve discrepancies and establish a unified diagnosis. The study defines small vessel disease (SVD) as a radiological manifestation of vascular pathologies affecting the brain's small blood vessels, including arterioles, capillaries, and small veins. SVD is visualized on MRI as silent lacunar infarctions, white matter changes, and cerebral microbleeds.

Biochemical analysis of plasma Sestrin2 levels

Plasma levels of Sestrin2 were quantified using a human Sestrin2 ELISA Kit (Cat No. E3437Hu) from Bioassay Technology Laboratory (Shanghai, China), adhering to the manufacturer's protocol [22, 23]. Briefly, plasma samples were collected in EDTA tubes and centrifuged for 15 min at 2000–3000 RPM at 2–8 °C within 30 min of collection. Standard stock serial dilutions were prepared according to the manufacturer's instructions. Samples and ELISA reagents were added to each well and incubated for 1 h at 37 °C. The plate was washed five times, followed by the addition of substrate solutions A and B, and then incubated for 10 min at 37 °C. A stop solution was added, and optical density (OD) values were measured within 10 min at 450 nm using a BioTek Synergy H1 multimode plate reader (Santa Clara, CA, USA).

Determination of TAC in plasma samples

TAC was evaluated using an assay kit (Cat No. ab65329) from Abcam (Cambridge, UK), following the manufacturer's protocol. Plasma samples were collected in EDTA-containing tubes and diluted to fit standard curve readings. After solubilizing the Trolox standard, a Trolox standard curve was established (4–20 nmol/well). A Cu^{2+} working solution was prepared (1:50 in assay diluent). A total of 100 μL of the Cu^{2+} working solution was combined with 100 μL of both the standard and the sample, followed by mixing and incubation at room temperature for 90 min, protected from light. Ultimately, OD values were measured at 570 nm using the BioTek Synergy H1 multimode plate reader.

Measurement of nitrite/nitrate levels (indirect measurement of NO) in plasma samples

Due to the short half-life of NO, direct measurement is challenging. In this study, levels of NO oxidation products (nitrite/nitrate) were assessed using a total NO and nitrite/nitrate assay (Cat No. KGE001) from R&D Systems (Minneapolis, MN, USA), following the manufacturer's instructions. Plasma samples were collected in EDTA tubes and centrifuged for 15 min at 1000 × g within 30 min of collection. The collected plasma was filtered (10 kDa MWCO filter) and diluted with reaction diluent at a 1:1 ratio. All reaction reagents and standards were prepared at room temperature in accordance with the manufacturer's protocol. For the nitrite assay, 50 μL of reaction diluent was added to blank wells, while 50 μL of nitrite standard or sample was added to the remaining wells, followed by 50 μL of reaction diluent, 50 μL of Griess I, and 50 μL of Griess II to all wells. After gentle shaking, the mixture was incubated for 10 min at room temperature. OD values were recorded at 540 nm (wavelength correction at 690 nm) using the BioTek Synergy H1 multimode plate reader. For the nitrate reduction assay, 50 μL of reaction diluent was added to blank wells, while 50 μL of nitrate standard or sample was added to the remaining wells. Then, 25 μL of NADH and 25 μL of diluted nitrate reductase were added to all wells, followed by a 30-min incubation at 37 °C. Subsequently, 50 μL of Griess I and 50 μL of Griess II were added to all wells, followed by gentle mixing and a 10-min incubation at room temperature. OD values were determined at 540 nm (wavelength correction at 690 nm) using the BioTek Synergy H1 multimode plate reader.

Ethical statement

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the Institutional Review Board (IRB) of HMC (#15304/15). All participants provided written informed consent prior to their enrollment in the study.

Statistical analysis

Descriptive statistics and normality tests were employed to analyze baseline demographics and cardiometabolic indicators. The Shapiro–Wilk test was utilized for samples with fewer than 50 participants, while the Kolmogorov–Smirnov test was applied for larger samples. Data are presented as mean ± SD or *Z*-score ± SD, with "*n*" denoting the number of individuals. Independent Student's *t*-tests, ANOVA, Mann–Whitney *U*, or Kruskal–Wallis tests were employed to estimate mean differences in plasma sestrin-2 levels, TAC, and total NO levels when appropriate, between healthy controls and patients with AIS at onset. Given that the studied parameters have different measurement units, *Z*-scores ± SD were used for plotting baseline differences. *Z*-scores were calculated for overall standardization and not per study group. To assess differences in the studied variables across various time points, the Wilcoxon rank test was used for comparisons between two time points, while the Friedman test was applied for comparisons involving more than three time points. Data for the studied parameters were not available for all subjects at all time points; only available data were included in the analysis (see Table S1 for missing data).

Multiple logistic regression was utilized to identify demographic variables and health complication characteristics, including sestrin-2 levels, TAC, and nitrite/nitrate (NO), independently associated with AIS. Due to the unavailability of demographic and cardiometabolic indicators for healthy controls, data from patients diagnosed with transient ischemic stroke or stroke mimics were used (Figure S1 illustrates the flow diagram of the groups employed in each statistical test). Initially, we analyzed the association between each independent variable and the odds of AIS separately. Variables with a *P* value of 0.25 or less were subsequently included in the multiple logistic regression analysis. A backward, stepwise elimination procedure was employed, with $P > 0.1$ for the two-tailed Wald test as the criterion for variable removal, to construct the final multivariate logistic regression model. The final model comprised only those variables with statistically significant independent associations with the odds of developing AIS. To adjust for sociodemographic characteristics, age, gender, and nationality were included in the final model, despite not being statistically significant. The results of the final model are summarized by the adjusted odds ratio (AOR) for experiencing AIS, accompanied by model-based 95% confidence intervals (CIs). All analyses were conducted using Statistical Package for the Social Sciences (SPSS) 28.0 software, *with P* values of ≤ 0.05 considered significant.

To evaluate whether elevated plasma levels of sestrin-2, NO, and TAC confer a protective effect against AIS, we divided all three parameters into tertiles. Multiple logistic regressions were then conducted to assess the odds of developing AIS based on each tertile. This analysis utilized data from both AIS patients and healthy controls. In the logistic regression for sestrin-2 and NO tertiles, the dependent variable was the odds ratio for stroke, while the independent variable represented either sestrin-2 or NO tertiles measured at three levels (T1, T2, and T3, with T1 as the reference). Results are summarized by the AOR of experiencing AIS for subjects whose plasma levels of sestrin-2 or NO fell within T2 or T3, along with model-based 95% CIs. The same methodology was applied to the tertiles of TAC, although it did not yield statistical significance. Therefore, logistic regression was performed with stroke as the dependent variable and each tertile of TAC as a separate independent variable measured at two levels (0 and 1). Results are summarized by the AOR of experiencing AIS when subjects' plasma levels of TAC were categorized into T1, T2, or T3, accompanied by model-based 95% CIs.

The tertiles for each parameter were as follows.

Sestrin2:

- Tertile1 (T1): [$n = 69$] < 0.344 (0.261 ± 0.05)
- Tertile2 (T2): [$n = 70$]: 0.344–0.502 (0.416 ± 0.04)
- Tertile3 (T3) [$n = 69$] > 0.502 (4.84 ± 5.65)

TAC:

- Tertile1 (T1) [$n = 42$]: < 1.797 (1.45 ± 0.23)
- Tertile2 (T2) [$n = 43$]: 1.797 - 2.1897 (1.99 ± 0.11)
- Tertile3 (T3) [$n = 42$]: > 2.1897 (2.4 ± 0.19)

Total Nitrite/Nitrate levels (an indirect measure of NO):

- Tertile1 (T1) [$n = 42$]: < 32.575 (19.04 ± 8.81)
- Tertile2 (T2) [$n = 43$]: 32.575 - 57.333 (43.2 ± 7.4)
- Tertile3 (T3) [$n = 42$]: > 57.333 (106.84 ± 53.38)

Cardiometabolic indicators were compared across different tertiles using either ANOVA or the Kruskal–Wallis test, as appropriate.

Results

Study population and baseline characteristics

A total of 187 patients with ischemic stroke, 30 healthy controls, and 92 TIA or mimics were included in this study. Table 1 presents the baseline demographics of the AIS patients, along with their metabolic and cardiovascular markers at the onset of the stroke. The majority of patients were male (93%), with a mean age of 49.4 ± 9.5 years, and 66.3% were of South Asian descent. Patients with metabolic equivalent (ME) conditions constituted only 16% of the study population. Over 60% of patients were classified as overweight or obese. Eighty-six patients were diagnosed with diabetes, and 137 presented with hypertension upon admission. Only 7.5% of the total cohort had a prior history of stroke, and only two patients reported a history of TIA. Among the AIS patients, 9.1% had a history of CAD. Dyslipidemia was previously diagnosed in 21.4% of the patients and newly identified in 77.5%. A total of 140 patients were confirmed to have SVD. Most metabolic markers exhibited mean values above the normal range: fasting glucose

Table 1. Baseline characteristics of patients with AIS

Demographics	Acute ischemic stroke (AIS) $n = 187$, N (%)
Age (years), mean ± SD	49.4 ± 9.5
Gender	
Male	174 (93)
Female	13 (7)
Ethnicity	
ME	31 (16.6)
SA	124 (66.3)
Other	32 (17.1)
Smoking	
Yes	66 (35.3)
No	102 (54.5)
Ex-smoker	19 (10.2)
Diabetes type	
Non-diabetic	78 (41.7)
Unknown diabetes	67 (35.8)
Newly diagnosed	19 (10.2)
Pre-diabetes	23 (12.3)
Diabetes on admission	
Yes	86 (46)
No	101 (54)
Hypertension type	
Unknown	50 (26.7)
Known	105 (56.1)
Newly diagnosed	32 (17.1)
Hypertension on admission	
Yes	137 (73.3)
No	50 (26.7)
Dyslipidemia type	
Known	40 (21.4)
Unknown	2 (1.1)
Newly diagnosed	145 (77.5)
Prior Stroke	
Known	14 (7.5)
Unknown	172 (92.5)
Prior TIA	
Known	2 (1.1)
Unknown	183 (98.9)
CAD	17 (9.1)
SVD	
Absent	39 (21.8)
Present	140 (78.2)

(Continued)

Table 1. Continued

Demographics	Acute ischemic stroke (AIS) $n = 187$, N (%)
Silent infarct	
Absent	123 (68.7)
Present	56 (31.3)
New infarct	170 (95.5)
Metabolic markers	**Mean ± SD/N (%)**
BMI (kg/m^2)	27.3 ± 4.1
Overweight and obese	126 (67.4)
Glucose (mmol/L)	8.9 ± 4.4
Insulin (mIU/L)	34 ± 41.1
HbA1c (%)	7.18 ± 2.31
Homocysteine (μmol/L)	11.9 ± 10.3
Cardiovascular markers	**Mean ± SD/N (%)**
TC (mmol/L	5.04 ± 1.2
HDL (mmol/L)	0.95 ± 0.25
LDL (mmol/L)	3.3 ± 1.07
TG (mmol/L)	1.75 ± 1
DBP (mmHg)	96.8 ± 22.2
SBP (mmHg)	161.3 ± 30.5

Abbreviations: ME: Middle Eastern; SA: South Asian; TIA: Transient ischemic attack; CAD: Coronary artery disease; SVD: Small vessel disease; TC: Total cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglycerides; DBP: Diastolic blood pressure; SBP: Systolic blood pressure.

(8.9 ± 4.4), insulin (34 ± 41.1), and HbA1c (7.18 ± 2.31). Furthermore, cardiovascular markers indicated elevated risk levels: LDL (3.3 ± 1.07), triglycerides (TG) (1.75 ± 1), systolic blood pressure (SBP) (161.3 ± 30.5), and diastolic blood pressure (DBP) (96.8 ± 22.2).

Plasma levels of Sestrin2, TAC, and nitrite/nitrates (NO)

The mean values and Z-scores of plasma levels of Sestrin2, TAC, and nitrite/nitrates (NO) were compared between healthy controls and AIS patients at baseline (onset of attacks). Due to the non-normal distribution of all three variables, the Mann–Whitney test was employed for the comparison (Table 2). Plasma levels of Sestrin2 were significantly higher in healthy controls compared to AIS patients (8.383 ± 7.39 vs 1.434 ± 3.57 ng/mL; $P < 0.001$). Similarly, TAC levels were significantly elevated in healthy controls compared to AIS patients (2.279 ± 0.326 vs 1.88 ± 0.42 nmol/μL; $P < 0.001$). A comparable trend was observed for NO, with levels being higher in healthy controls than in AIS patients (65.951 ± 44.07 vs 53.5 ± 47.9 μmol/L; $P = 0.04$). Figure 1 illustrates the expression levels of these parameters across all studied groups, presented as Z-scores.

To evaluate changes in plasma levels of the measured parameters over time following treatment initiation, their levels were assessed at multiple time points: at stroke onset, and at 5 days,

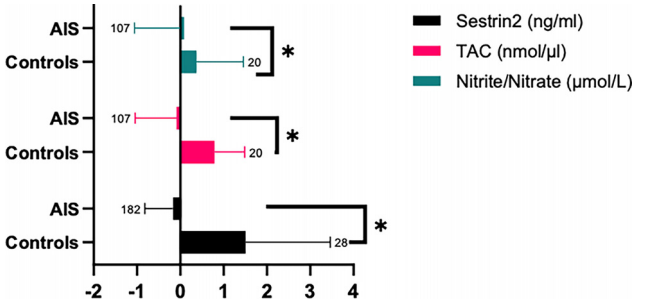

Figure 1. Comparison of plasma levels of Sestrin2, TAC, and nitrite/nitrate (NO) between healthy controls and AIS patients. Plasma levels of Sestrin2, TAC, and nitrite/nitrate (NO) at the onset of AIS are presented as Z-scores. The Mann–Whitney U test was employed to compare the mean Z-scores between healthy controls and AIS patients. Bars represent mean values, while error bars denote SD. The numbers above the error bars indicate the sample size for each group. *$P < 0.05$. Abbreviations: TAC: Total antioxidant capacity; AIS: Acute ischemic stroke; NO: Nitric oxide; SD: Standard deviation.

30 days, and 1 year post-acute events. The Wilcoxon rank-sum test or repeated measures ANOVA was utilized to compare levels across these time points. Sestrin2 levels were specifically analyzed at three intervals: within 48 h of onset, and at 5 days and 30 days post-event. A total of 52 stroke patients had Sestrin2 levels measured at all three time points and were included in the analysis. For comparisons between onset and 5 days post-event, data from 182 subjects were available. As shown in Figure 2A, Sestrin2 levels were significantly lower in AIS patients compared to controls at onset and at both 5- and 30-day follow-ups. Notably, Sestrin2 levels [median (IQR)] were moderately but significantly reduced after 5 days of attacks, [0.4 (0.2) vs 0.39 (0.2), $z = -4.434$; $P < 0.001$] (Figure 2A). Only 10 subjects had their Sestrin2 levels recorded at all time points (onset, 5 days, 30 days, and 1 year). For these subjects, comparisons across different time points yielded no significant differences (data not shown). For TAC and NO, there were sufficient data points to compare levels only between onset and 5 days. In AIS patients, no statistically significant difference in TAC levels was observed between onset and 5 days (data not shown). However, when comparing TAC levels across different time points as separate groups, a statistically significant difference was identified between onset and 5 days (Figure 2B). Plasma levels of NO were significantly higher at day 5 compared to onset levels ($z = -2.471$, $P = 0.01$) (Figure 2C).

The levels of all three parameters were compared to those of healthy controls at each time point. Healthy controls exhibited higher plasma Sestrin2 mean ranks at both onset and after 5 days compared to AIS patients (onset: $z = -5.107$, $P < 0.001$; day 5: $z = -4.49$, $P < 0.001$) (Figure 2A). TAC levels were also significantly higher in controls at onset compared to AIS patients ($P < 0.001$) (Figure 2B). Furthermore, plasma NO levels were significantly lower in AIS patients at onset relative to healthy controls ($P = 0.04$) (Figure 2C).

Risk factors associated with AIS

To identify risk factors associated with AIS, univariate logistic regression analysis was conducted to assess the impact of

Table 2. Comparison of plasma levels of Sestrin2, NO, and TAC between control subjects and AIS patients at onset of symptoms

Variables	Control subjects $n = 30$		Acute ischemic stroke (AIS) $n = 187$		*P* value
	Mean (SD)	Mean (SD) Z Score	Mean (SD)	Mean (SD) Z Score	
Plasma Sestrin2 (ng/mL)	8.383 ± 7.39 $N = 28$	1.5 ± 1.95	1.434 ± 3.57 $N = 186$	−0.16 ± 0.64	**<0.001***
TAC (nmol/μL)	2.279 ± 0.326 $N = 20$	0.78 ± 0.69	1.88 ± 0.42 $N = 107$	−0.08 ± 0.95	**<0.001**
Nitrite/Nitrate (μmol/L)	65.951 ± 44.07 $N = 20$	0.37 ± 1.08	53.5 ± 47.9 $N = 107$	−0.004 ± 1.05	**0.04**

*Bold numbers indicate significant *P* values. Abbreviations: NO: Nitric oxide; TAC: Total antioxidant capacity; AIS: Acute ischemic stroke; SD: Standard deviation.

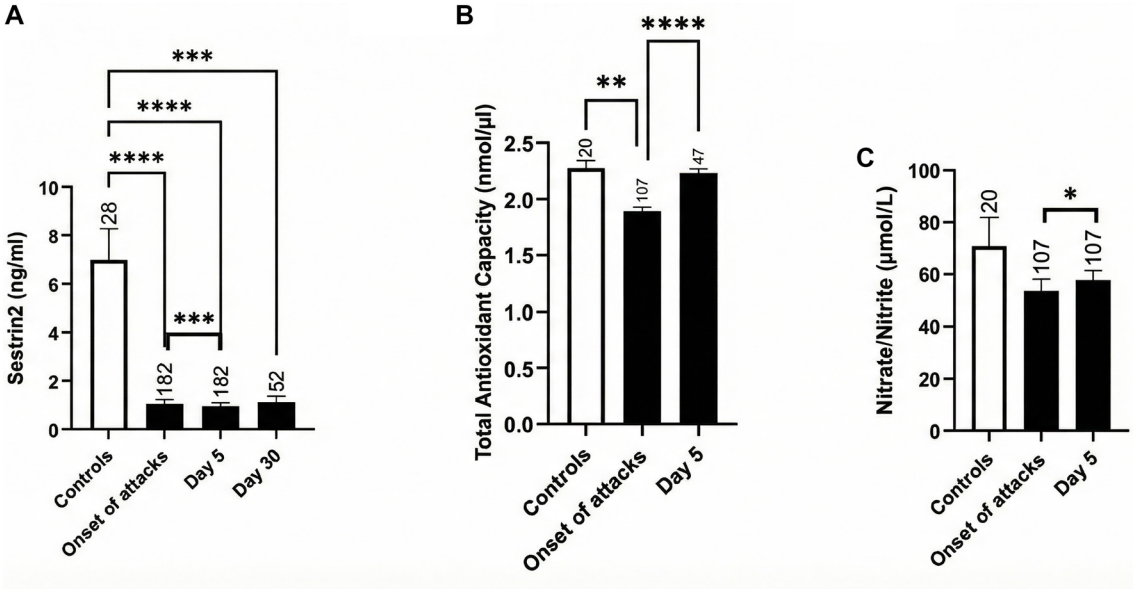

Figure 2. Plasma levels of Sestrin2, TAC, and total nitrite/nitrate (NO) across time points in controls and AIS patients. Plasma concentrations of Sestrin2, TAC, and total nitrite/nitrate (NO) were assessed at various time points and compared between healthy controls and AIS patients. The Wilcoxon rank test was utilized for pairwise comparisons at each time point relative to the onset. For comparisons involving multiple time points, the Friedman test was employed. The Mann–Whitney *U* test was used to compare each time point with the control group. Bars represent mean values, with error bars indicating SD. The numbers above the error bars denote the sample size for each group. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Abbreviations: TAC: Total antioxidant capacity; NO: Nitric oxide; AIS: Acute ischemic stroke; SD: Standard deviation.

each variable on stroke odds. Data from AIS, TIA, and stroke mimics were included in the study. All variables with a *P* value of 0.2 or less are presented in Table 3. No statistically significant association was observed between plasma Sestrin2, NO, or TAC levels and stroke odds. Factors such as advanced age, male gender, South Asian ethnicity, diabetes, hypertension, elevated National Institutes of Health Stroke Scale (NIHSS) score, presence of SVD, and smoking were found to increase the odds of AIS, while being of ME descent appeared to decrease these odds (Table 3).

Multiple logistic regression with backward stepwise elimination, adjusted for age, gender, and nationality, indicated that male gender, diabetes, high NIHSS score, and presence of SVD all increased the likelihood of AIS, whereas being of ME descent decreased these odds (Table 4).

Following the estimation of the AOR for developing AIS, it was determined that being in the third tertile for Sestrin2, third tertile for TAC, and second tertile for NO decreased the odds of AIS, with AORs of 0.123 ($P < 0.001$), 0.327 ($P = 0.01$), and 0.063 ($P = 0.01$), respectively (Table 5).

Tables S2–S4 summarize comparisons of cardiometabolic indicators across the different tertiles for each parameter studied. No significant differences were found between the three tertiles, except for insulin levels between the nitrite/nitrate (NO) tertiles (T1: 21.2 ± 14.5; T2: 25.4 ± 22.1; T3: 50.9 ± 47.3; $P = 0.009$) (Table S4).

Discussion

Stroke is the second leading cause of death worldwide [1]. Although the mortality rate associated with AIS is relatively low, significant concern arises from the resulting disabilities and impaired quality of life [7]. Endothelial dysfunction is a key underlying mechanism of cardiovascular diseases, including AIS. This dysfunction arises from oxidative stress and the continuous production of ROS [24]. A primary manifestation of endothelial dysfunction is the reduction of NO bioavailability. Sestrin2 is a stress-inducible protein that serves an antioxidant function during oxidative or energetic stress and has garnered interest in cardiovascular research. This study investigates the

Table 3. Factors influencing the probability of developing AIS (univariate analysis)

Factor	Coefficient B	AOR	*P* value*	95% CI for Exp B
Age	0.895	2.488	0.05	0.974–6.155
Weight	0.849	2.338	**0.04**	1.036–5.281
Gender (male)	1.11	3.034	**0.01**	1.403–6.56
Ethnicity (ME)	−0.684	0.504	**<0.001**	0.278–0.915
Ethnicity (SA)	0.634	1.884	**0.01**	1.33–3.135
NIHSS	1.378	3.968	**<0.001**	2.107–7.475
Diabetes on admission (yes)	0.615	1.85	**0.02**	1.094–3.129
Hypertension on admission (yes)	0.52	1.682	0.05	0.989–2.861
Smoking (yes)	0.507	1.66	0.08	0.936–2.943
SVD	0.863	2.371	**0.002**	1.361–4.13

*Bold numbers indicate significant *P* values. Abbreviations: AIS: Acute ischemic stroke; AOR: Adjusted odds ratio; CI: Confidence interval; ME: Middle Eastern; SA: South Asian; NIHSS: National Institutes of Health Stroke Scale; SVD: Small vessel disease.

Table 4. Factors influencing the probability of developing AIS (multiple logistic regression)

Factors in the model	Coefficient B	AOR	*P* value*	95% CI for Exp B
Age	0.011	1.011	0.54	0.975–1.049
Gender (male)	0.884	2.420	0.06	0.947–6.184
Ethnicity (ME)	−0.796	0.451	**0.04**	0.213–0.953
NIHSS	0.421	1.523	**<0.001**	1.314–1.765
Diabetes	0.729	2.073	**0.04**	1.032–4.166
SVD	0.786	2.195	**0.03**	1.074–4.483

*Bold numbers indicate significant *P* values. Abbreviations: AIS: Acute ischemic stroke; AOR: Adjusted odds ratio; CI: Confidence interval; ME: Middle Eastern; NIHSS: National Institutes of Health Stroke Scale; SVD: Small vessel disease.

Table 5. Correlation between the odds of AIS and the different tertiles of plasma Sestrin2, TAC, and total NO (AIS vs controls)

Tertile	Coefficient B	AOR	*P* value*	95% CI for Exp B
Sestrin2 (T1 reference)				
T2	1.475	4.369	0.19	0.476–40.108
T3	−2.0.95	0.123	**<0.001**	0.04–0.38
TAC (T1 reference)				
T2	−0.864	0.422	0.10	0.150–1.187
T3	−1.118	0.327	**0.01**	0.143–0.749
Total NO (nitrite/nitrate) (T1 reference)				
T2	−2.764	0.063	**0.01**	0.008–0.511
T3	−2.104	0.122	0.05	0.014–1.040

*Bold numbers indicate significant *P* values. Abbreviations: AIS: Acute ischemic stroke; TAC: Total antioxidant capacity; NO: Nitric oxide; AOR: Adjusted odds ratio; CI: Confidence interval.

protective role of Sestrin2 in AIS, in conjunction with TAC and NO levels.

We assessed plasma levels of Sestrin2 and found them to be significantly higher in healthy controls compared to AIS patients at onset (8.383 ± 7.39 vs 1.434 ± 3.57 ng/mL, $P < 0.001$). Similar findings were reported by Tian et al. [25], who observed lower plasma Sestrin2 levels in diabetic patients with coronary heart disease (CHD) compared to those without (11.17 [9.79,

13.14] ng/mL vs 9.46 [8.34, 10.91] ng/mL). Kishimoto et al. [26] found that in patients undergoing elective coronary angiography, plasma Sestrin2 levels were significantly higher in those with CAD than in those without (median 16.4 vs 14.2 ng/mL, $P < 0.05$). Another study involving 80 controls and 220 patients with congestive heart failure (CHF) reported that higher serum Sestrin2 concentrations were present in CHF patients compared to controls [27]. Additionally, in 152 subjects undergoing carotid ultrasonography, Sestrin2 levels were significantly higher in patients with plaques compared to those without (median 14.1 vs 12.8 ng/mL, $P < 0.02$) [28]. The elevated Sestrin2 concentration in patients with plaques may indicate a protective role in mitigating carotid atherosclerosis progression. Our study also found that Sestrin2 levels were significantly lower in AIS patients compared to controls, remaining low even after the initiation of appropriate therapy, at both 5 and 30 days post-event. These findings align with those reported by Gariballa et al. [29] and corroborate our previous research [3], which demonstrated elevated levels of extracellular vesicles in the plasma of AIS and TIA patients, indicative of cellular activation. Specifically, we detected increased levels of extracellular vesicles derived from platelets (CD41+), activated platelets (CD62P+), and pro-coagulant vesicles (Annexin V+) at 5 and 30 days post-acute events [3]. Recent transcriptomic analyses have revealed that these extracellular vesicles contain specific microRNAs (miRNAs) that are differentially expressed in stroke patients and target key genes involved in cellular stress response networks and recovery [30]. These findings suggest an elevated presence of extracellular vesicles with both pro-thrombotic activity and miRNA-mediated regulatory roles in cellular stress responses among these patients. The combination of persistently reduced Sestrin2 levels, diminished TAC, and decreased NO, alongside previously observed elevations in circulating pro-thrombotic extracellular vesicles, may indicate sustained cellular activation in AIS patients even weeks after initiating appropriate therapeutic management. This prolonged activation could potentially increase the risk of stroke recurrence in these individuals.

TAC quantifies the cumulative action of all antioxidants present in plasma and body fluids, providing valuable insights into the balance between oxidants and antioxidants *in vivo* [31]. Assessing plasma TAC levels can help identify physiological conditions affecting oxidative status. Reduced TAC has been implicated in ischemic conditions and brain injury, serving as an indicator of poor clinical outcomes [29]. In this study, TAC levels were significantly higher in healthy controls compared to AIS patients (2.279 ± 0.326 nmol/μL vs 1.88 ± 0.42 nmol/μL, $P < 0.001$). These findings are consistent with those reported by Gariballa et al., who compared TAC levels among ischemic stroke patients, hospitalized non-stroke patients, and healthy controls. Their results indicated lower plasma TAC levels in stroke patients relative to the other groups; however, this difference did not achieve statistical significance [29]. Conversely, Guldiken et al. [12] assessed TAC levels in diabetic and non-diabetic ischemic stroke patients, as well as healthy controls, and reported findings that contradict our results. Their study showed significantly higher TAC levels in diabetic stroke patients (10.03 ± 1.97 mM) compared to non-diabetic stroke patients (5.97 ± 2.04 mM) and healthy controls (5.44 ± 1.06 mM) ($P < 0.001$ for both comparisons). Further evidence of the role of TAC in stroke risk is provided by a study investigating the association between dietary TAC and stroke risk among women with and without a history of cardiovascular disease. This study found a correlation between higher dietary TAC and a lower risk of stroke [32]. Similarly, a cohort study involving Egyptian AIS patients and healthy controls revealed significantly lower TAC levels in AIS patients (AIS: 1.7 ± 0.30 mmol/L vs Controls: 4.20 ± 0.50 mmol/L, $P < 0.001$) [33]. In a cohort of Iranian stroke patients and controls, after adjusting for dietary intake of fibers and omega-3 fatty acids, a unit increase in dietary TAC was associated with a 29% reduction in the odds of stroke (OR: 0.71; 95% CI: 0.50–1.01, $P = 0.06$) [34].

Endothelium-derived NO is a crucial endogenous facilitator of cerebral blood flow and cerebrovascular protection [35]. It acts neuroprotectively in the early stages of ischemic stroke primarily through its vasodilatory effects. However, during the later stages, neuronal NO synthase can induce NO overproduction, resulting in increased peroxynitrite levels, which may ultimately trigger brain injury [12]. In this study, we found that NO levels were significantly higher in healthy controls compared to AIS patients (65.951 ± 44.07 vs 53.5 ± 47.9 μmol/L, $P = 0.04$). Similar findings were reported by Guldiken et al. [12] in diabetic stroke patients compared to non-diabetic stroke patients and healthy controls ($P < 0.001$). Taffi et al. [36] also reported significantly lower plasma NO levels in ischemic stroke patients compared to healthy controls (51.1 ± 12.5 vs 115.4 ± 12.4, $P < 0.001$).

Furthermore, our study noted an increase in plasma NO levels in AIS patients five days after stroke onset. However, these levels remained lower than those in healthy controls, although the difference was not statistically significant. The increase in NO levels observed in our AIS group may be attributed to overproduction by neuronal and inducible NO synthases, which typically occurs during reperfusion [37].

When estimating the correlation between plasma levels of Sestrin2, TAC, and NO in patients with AIS, none of the parameters demonstrated statistical significance. This may be attributed to our comparison being limited to AIS patients, mimics, and TIA patients, without including healthy controls. However, upon stratifying these parameters into tertiles, we observed that being in the third tertile (T3) for Sestrin2 and TAC, along with the second tertile (T2) for NO, was associated with a reduction in the odds of stroke by more than 60%. These findings are consistent with a study by Tian et al., which indicated that lower serum Sestrin2 levels correlate with an increased risk of CHD in type 2 diabetic patients ($P < 0.05$) [25]. Their analysis further showed an inverse relationship between Sestrin2 quartiles and CHD prevalence, with higher Sestrin2 quartiles associated with lower CHD incidence. Conversely, Kishimoto et al. [26] reported that when Sestrin2 levels exceeded 16 ng/mL, the AOR for CAD increased by 1.79 (95% CI: 1.09–2.95). These findings may suggest a potential *U*-shaped relationship between Sestrin2 levels and the

likelihood of ischemic disease; however, further studies are required to explore this hypothesis.

Multiple logistic regression analysis indicated that male sex is associated with an increased likelihood of developing AIS. Our findings corroborate those of the American Heart Association/American Stroke Association, which report a lower incidence of ischemic stroke in women compared to men [38]. Furthermore, our regression analysis revealed that individuals of Middle Eastern descent exhibited lower odds of AIS. This observation may be influenced by the significant proportion (60%) of South Asian labor workers included in this study. Diabetes was also identified as a factor that increases the odds of AIS in our study population, aligning with established literature demonstrating that diabetes elevates stroke risk through various mechanisms, including vascular endothelial dysfunction [39].

Our findings indicate that a higher NIHSS score correlates with an increased likelihood of AIS. The NIHSS is a standardized tool developed to objectively quantify stroke impairment [40]. Additionally, we found that the presence of SVD increased the odds of AIS in our study population. SVD is a well-known contributor to both stroke and vascular dementia [7].

Study limitations

This study is limited by its small sample size. No power analysis was conducted to estimate the appropriate sample size, and we relied solely on a convenience sample. Due to the unavailability of demographic and clinical data for healthy controls, the multivariate logistic regression was limited to AIS, TIA, and mimic groups as non-AIS subjects. This limitation may have influenced our results, as comparisons with healthy controls could have yielded stronger statistical significance. Although the study included a follow-up period to assess the levels of Sestrin2, TAC, and total NO, it did not evaluate cardiovascular outcomes or stroke recurrence, nor did it investigate how these factors correlate with Sestrin2 levels.

Conclusion

In this study, we found that plasma levels of Sestrin2, TAC, and total NO were higher in healthy controls compared to AIS patients. Furthermore, elevated plasma levels of these markers were associated with reduced odds of stroke. These findings underscore the potential of Sestrin2 as a clinical biomarker for predicting stroke risk and highlight the critical roles of TAC and total NO in the pathophysiology of ischemic stroke. To our knowledge, this is the first study in the region to evaluate plasma Sestrin2 levels in AIS patients. Given the growing interest in Sestrin2 and the conflicting evidence in the existing literature, further clinical investigations are warranted to clarify its precise role in AIS and other cerebrovascular diseases. Future studies should focus on elucidating the mechanisms underlying Sestrin2's protective effects and exploring practical methods to modulate its levels to promote cerebrovascular health. Strengthening the connection between Sestrin2 regulation and improved outcomes in cerebrovascular diseases could significantly influence clinical practices and patient care strategies.

Future directions

While this study primarily aimed to confirm clinical associations between oxidative stress-related biomarkers and the occurrence of stroke, we recognize the necessity for further research to enhance the biological and translational relevance of these findings. Future investigations should focus on examining the mechanistic roles of Sestrin2, TAC, and NO in ischemic stroke and vascular dysfunction using *in vitro* and animal models; exploring longitudinal trends in biomarker levels to better differentiate between pathological and compensatory changes post-stroke; studying these biomarkers in larger, more diverse populations to validate their diagnostic and prognostic potential; and assessing their integration with conventional clinical risk factors and imaging data to refine stroke prediction models. Such efforts will be crucial for transitioning from association to causation and for evaluating the utility of these biomarkers as potential therapeutic targets or clinical tools.

Conflicts of interest: Authors declare no conflicts of interest.

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