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RESEARCH ARTICLE

Bader et al: Plasma Sestrin2 in ischemic stroke

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

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ABSTRACT

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 hours 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 considered the second leading cause of death and a major 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 for whom long-term follow-up and intervention are needed [2]. Over the past few decades, there has been a rise in the burden of stroke among those under the age of 65. Globally, the incidence of stroke among adults aged 20 to 64 has increased by 25% [1]. This shift in the incidence of stroke in younger age groups is mainly observed in low- and middle-income countries [1]. Ischemic stroke has much higher incidence rates compared to hemorrhagic stroke. However, the latter has a higher mortality rate [2]. In Southeast Asia (SA) and the Middle East (ME), particularly high rates of Acute Ischemic Stroke (AIS) exist since these regions have high rates of predisposing risk factors such as diabetes and hypertension, as well as rising rates of obesity and metabolic syndrome [3].

Qatar is an ME country on the northeastern coast of the Arabian Peninsula. The population in Qatar comprises less than 15% Qatari nationals, while most of the population consists of migrants from SA (60%). The high percentage of migrant workers in Qatar makes its population relatively young and dominated by males [4]. Even though the United Nations has assigned Qatar as a high-income country, it is still considered a developing country because of its high migrant population [5]. The burden of stroke in Qatar is high, with an estimated overall incidence of stroke of 41 per 100,000 inhabitants per year. The incidence of stroke for people over 45 years of age was 238 per 100,000 inhabitants per year [6]. The average incident age for stroke in Qatar is 55 years, and the estimated age for the first AIS is 52 in 100,000 patient-years, with 50% of the affected population at 50 years of age or younger [7]. Despite the low mortality rate associated with AIS, concerns still exist about the life-changing disabilities and the risk of recurrence [8]. According to the United Nations projections, the life expectancy in Qatar in 2023 is estimated to be around 80.73 years [9]. This means that if the average age for the first incident of stroke in Qatar is 55 years old, stroke survivors would have around 26 more years to live [7]. However, with the high risk of recurrent stroke and a significant mortality rate associated with it, those people have a very low chance of surviving without a disability or compromised quality of

life [7]. Current diagnostic procedures for AIS are mainly non-contrast computed head tomography (CT) and magnetic resonance imaging (MRI) [10].

Reactive oxygen species (ROS) act as signaling molecules and are produced during normal physiological processes such as aerobic respiration and inflammation. Under physiological conditions, a balance in the production and release of ROS exists [11]. In healthy individuals, the production of ROS is counterbalanced by antioxidant activity [12]. However, under pathological states like ischemia, excessive ROS disrupts this balance, leading to oxidative stress and cellular dysfunction [11,12]. ROS plays a role in the pathophysiology of several cardiovascular disorders, including atherosclerosis, cardiomyopathy, hypertension, and stroke [13]. Oxidative stress mainly affects the endothelium of the vasculature, which plays a significant role in maintaining vascular homeostasis [14]. The endothelium secretes several factors and substances, such as nitric oxide (NO) and endothelin-1 (ET-1), which regulate vasodilation and vasoconstriction, cell growth, angiogenesis, and fibrinolysis [15]. Endothelial dysfunction, characterized by reduced NO bioavailability, impaired angiogenic capacity, and a weakened vascular tone [16], is a key driver of vascular damage and atherosclerosis [15].

Sestrin2, a key oxidative stress defense protein, belongs to the sestrin family and maintains cellular homeostasis by regulating the mammalian target of rapamycin (mTOR) signaling and ROS [17,18]. As a stress-inducible protein with an antioxidant role, Sestrin2 is upregulated in response to oxidative and energetic stress. It prevents the unfolded protein response (UPR), inhibits endoplasmic reticulum (ER) stress, and thus promotes cell survival and homeostasis [17]. It has been demonstrated that Sestrin2 is upregulated in ER stress and may play a protective role in ER stress-induced disturbances [19]. Sestrin2 plays a role in the autophagy process by regulating the mTOR/AMP-activated protein kinase (AMPK) signaling pathway, and Sestrin2's deficiency can lead to autophagy impairment and ROS accumulation [17,18]. Sestrin2 has been shown to lower the risk of cardiovascular disease through AMPK activation, mTOR inhibition, and Nuclear factor erythroid 2-related factor 2 (Nrf2) activation [17,18]. Sestrin2 has recently gained attention in ischemic disease. Studies show 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 with induced cerebral ischemia, Sestrin2 overexpression enhances angiogenesis via Nrf2 activation [21]. Sestrin2 overexpression increases the density and overall length of blood vessels and reduces brain injury [21]. Its neuroprotective effects are thought to be mediated through vascular endothelial growth factor (VEGF) upregulation via the Nrf2/heme oxygenase (HO)-1 pathway [20].

Despite the growing interest in Sestrin2 in ischemic diseases, clinical studies investigating the role of circulating Sestrin2 levels in stroke patients and how it correlates to the incidence of stroke are lacking. In this study, our main aim was to evaluate the relationship between Sestrin2 plasma levels and the odds of having AIS in the Qatari population. The secondary objective of this study was to explore the association between plasma total antioxidant capacity (TAC), NO levels, and the odds of stroke. This study further examined the overall risk factors for developing AIS and whether plasma Sestrin2 could have a protective role against AIS.

MATERIALS AND METHODS

Study population and sampling

This is a case-control study involving a group of AIS patients and healthy controls. Patient recruitment was conducted at Hamad General Hospital (HGH), HMC. All patients' samples were processed in the Institute of Neuroscience (HMC) laboratories. Patients were recruited into the study within 48 hours of the onset of the stroke attack. Only adults (>18 years old) who provided written informed consent were enrolled in this study. Stroke was defined according to World Health Organization (WHO) criteria as "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 vascular origin." All AIS patients admitted to the HGH stroke unit were eligible and screened for recruitment. Patients with transient ischemic attack (TIA), mimics of stroke, intracerebral hemorrhage, or cerebral venous sinus thrombosis were excluded. Healthy controls were identified as individuals with no known diagnosed illness and were recruited prospectively during the same period as patient enrollment. Control participants included age-matched healthy individuals who volunteered to participate, but were excluded if they were visiting the outpatient department. All controls were screened to ensure they had no history of stroke, TIA,

or other 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

All baseline demographics and health information of the participants were recorded, including age, gender, employment status, ethnicity, marital status, nationality, smoking status, alcohol consumption, and medical comorbidities. Any history of heart failure, coronary artery disease (CAD), diabetes, peripheral vascular disease, chronic kidney disease, dyslipidemia, and the dosage, frequency, and timing of current prescription and over-the-counter medications were also recorded. Plasma levels of Sestrin2, NO (Nitrite/Nitrate), and TAC were measured at four different time points: within 48 hours of onset and at 5 days, 30 days, and 1 year. However, all other clinical and cardiometabolic parameters were only collected at baseline. The primary study outcome was the odds of having AIS.

Diagnosis of AIS was confirmed through a CT head scan, an echocardiogram, and a conventional (not 3D) carotid ultrasound, which are all part of the standard protocol for the clinical treatment of stroke at HGH. Two vascular neurologists conducted a thorough examination and evaluation of all imaging studies separately. A meeting was convened to resolve a disagreement and achieve a consensus. The level of consensus on diagnosing white matter ischemia and silent infarcts was determined to reach a definitive conclusion. The current study defines SVD as a radiologic representation of vascular pathologies affecting the tiny blood vessels in the brain, including small arteries, arterioles, capillaries, and small veins. These pathologies are collectively known as SVD. The SVD is visualized on MRI as silent lacunar infarctions, white matter alterations, and cerebral microbleeds.

Biochemical analysis of plasma Sestrin2 levels

Plasma levels of Sestrin2 were determined using a human Sestrin2 ELISA Kit (Cat. No. E3437Hu) purchased from Bioassay Technology Laboratory (Shanghai, China), following the manufacturer's protocol [22,23]. Briefly, plasma samples were collected in EDTA tubes and centrifuged for 15 minutes at 2,000-3,000 RPM at 2–8 °C within 30 minutes of collection. Serial dilutions of the standard stock were prepared as per the manufacturer's instructions. Sample and ELISA reagent were added to each well and incubated for 1 hour at 37 ° C. Then, the plate was washed 5 times, followed by

the addition of the substrate solutions A and B and incubation for 10 minutes at 37 °C. A stop solution was then added, and optical density (OD) values were measured within 10 minutes at 450 nm using a BioTek Synergy H1 multimode plate reader (Santa Clara, USA).

Determination of TAC in plasma samples

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

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

Since NO is a free radical with a very short half-life, it is challenging to measure it directly. In this study, the levels of NO oxidation products (Nitrite/Nitrate) were determined using total NO and Nitrite/Nitrate Assay (Cat. No. KGE001) from R&D Systems (Minneapolis, USA) following the manufacturer's instructions. Briefly, plasma samples were collected in tubes containing EDTA and centrifuged for 15 minutes at 1,000 x g within 30 minutes of collection. Collected plasma was then 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 according to the manufacturer's protocol. For the nitrite assay procedure, 50 µL of reaction diluent was added to the blank wells, 50 µL of nitrite standard or sample was added to the remaining wells, 50 µL of reaction diluent was added to all wells, 50 µL of Griess I was added to all wells, 50 µL of Griess II were added to all wells followed by gentle shaking and incubation for 10 minutes at room temperature. OD values were determined at 540 nm (wavelength correction at 690 nm) using a BioTek Synergy H1 multimode plate reader. For the nitrate reduction assay, 50 µL of reaction diluent was added to blank wells, 50 µL of nitrate standard or sample was added to the remaining

wells, 25 µL of NADH was added to all wells, 25 µL of diluted nitrate reductase was added to all wells, followed by incubation for 30 minutes at 37 °C. Then, 50 µL of Griess I and 50 µL of Griess II were added to all wells, followed by gentle mixing and incubation for 10 minutes at room temperature. OD values were determined at 540 nm (wavelength correction at 690 nm) using a 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 Hamad Medical Corporation (HMC) (#15304/15). All participants provided written informed consent before their enrollment in the study.

Statistical analysis

Descriptive statistics and normality tests were used to analyze baseline demographics and cardiometabolic indicators. To test for normality, the Shapiro-Wilk test was used when the sample size was less than 50. At the same time, the Kolmogorov-Smirnov test was used when the sample size was more than 50. Data were presented as mean ± SD or z score ± SD, with "n" indicating the number of individuals. Independent Student's T-test, ANOVA, Mann-Whitney *U,* or Kruskal-Wallis tests were used to estimate the mean difference in plasma sestrin2 levels, TAC, and total NO levels when appropriate (between healthy controls and patients with AIS at onset). Since the parameters studied have different measuring units, z-score ± SD was used when plotting the differences in those parameters at baseline. Z-scores were calculated as an overall standardization and not per study group. To estimate the difference in the studied variables between different time points, and since the data were not normally distributed, we used a Wilcoxon rank test for comparing two time points, and the Friedman test for comparing more than three time points. Data for the parameters studied were not available for all subjects at all time points. Only available data were included in the analysis (Supplementary Table S4 shows all missing data).

Multiple logistic regression was used to determine all the demographic variables and health complication characteristics, including Sestrin2 levels, TAC, and nitrite/nitrates (NO), that are independently associated with AIS. For this part of the analysis, due to the unavailability of demographics and cardiometabolic indicators for the healthy

controls, we used data from patients diagnosed with transient ischemic stroke or stroke mimics (**Supplementary Figure S1** shows a flow diagram of the groups used in each statistical test). First, we analyzed the association between each independent variable and the odds of AIS separately. Then, all variables that had a p-value of 0.25 or less were included in the multiple logistic regression analysis. Followed by a backward, stepwise elimination procedure, with P> 0.1 for the 2-tailed Wald test as the criterion for variable elimination, to build the final multivariate logistic regression model. The final model included only variables that had statistically significant independent associations with the odds of having AIS. To adjust the final model for sociodemographic characteristics, we included age, gender, and nationality, even though they were not statistically significant. The results of the final model were summarized by the AOR of experiencing AIS, with model-based 95% confidence intervals (CIs). All analyses were conducted with SPSS 28.0 software. *P* values of ≤ 0.05 were considered significant.

To estimate if high plasma levels of Sestrin2, NO, and TAC can have a protective effect against AIS, we divided all three parameters into tertiles. Then we ran multiple logistic regressions to assess the odds of developing AIS depending on each tertile. To create the tertiles and perform this analysis, we used data from AIS patients and healthy controls. When applying logistic regression on the sestrin2 and the nitric oxide tertiles, the dependent variable was the odds ratio of stroke. In contrast, the independent variable was either sestrin2 or NO tertiles measured at three levels (T1, T2, and T3, with T1 as the reference). The results were summarized by adjusted odds ratios (AOR) of experiencing AIS when the subject's plasma levels of Sestrin2 or NO belonged to T2 or T3, with model-based 95% CIs. The same method was applied to the tertiles of TAC, but it did not show statistical significance. Therefore, we used logistic regression with stroke as the dependent variable and each tertile of the TAC as a separate independent variable measured at two different levels (0 and 1). The results were summarized by the AOR of experiencing AIS when the subject's plasma levels of TAC belonged to T1, T2, or T3, with model-based 95% CIs.

The tertiles for each one of the parameters 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 between the different tertiles using ANOVA or the Kruskal-Wallis test, when appropriate.

RESULTS

Study population and baseline characteristics

A total of 187 ischemic stroke patients, 30 healthy controls, and 92 TIA or mimics were included in this study. Table 1 shows the baseline demographics for the AIS patients and their metabolic and cardiovascular markers at onset. Most patients were males (93%) with a mean age of 49.4 ± 9.5 years, and most of them were from SA (66.3%). ME patients comprised only 16% of the study population. More than 60% of the patients were either overweight or obese. Eighty-six patients were identified as diabetic, and 137 had hypertension on admission. Only 7.5% of the study population had a known history of stroke, and only two patients had a known history of TIA. Out of the total group of AIS patients, 9.1% had a history of CAD. Dyslipidemia was previously known in 21.4% of the patients and newly diagnosed in 77.5% of them. One hundred and forty patients were confirmed to have small vessel disease. Almost all metabolic markers had a mean value higher than normal: fasting glucose (8.9 ± 4.4), insulin (34 ± 41.1), and HbA1c (7.18 ± 2.31). Moreover, the mean value for cardiovascular markers tended to be in the high-risk ranges: LDL (3.3 ± 1.07), TG (1.75 ± 1), SBP (161.3 ± 30.5), and DBP (96.8 ± 22.2).

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

The mean values and mean 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). Since the data for all three variables were not normally distributed, the Mann-Whitney test was used to conduct the comparison (**Table 2**). The plasma levels of Sestrin2 showed statistically significantly higher values in the healthy controls compared to AIS patients (8.383 ± 7.39 vs 1.434 ± 3.57 ng/ml; $p < 0.001$). TAC was also significantly higher in the healthy controls compared to AIS patients (2.279 ± 0.326 vs 1.88 ± 0.42 nmol/µL; $p < 0.001$). The same pattern was also observed for NO, where levels were higher in healthy controls compared to AIS patients (65.951 ± 44.07 vs 53.5 ± 47.9 µmol/L; p=0.04). **Figure 1** shows the expression levels of the three parameters among all the studied groups presented as z-scores.

To estimate whether the plasma levels of the studied parameters change over time following the introduction of appropriate treatment, their levels were measured at different time points: at the onset of stroke attacks, and at 5 days, 30 days, and 1 year post-acute events. The Wilcoxon Rank sum test or repeated measures ANOVA was used to compare levels between these time points. The levels of Sestrin2 at three different time points were compared: within 48 hours of the onset of attacks, and after 5 days and 30 days following the acute events. A total of 52 stroke patients had their Sestrin2 levels available for the three time points and were included in the analysis. When comparing the Sestrin2 levels at onset and after 5 days, a total of 182 subjects were included based on the availability of their data. As shown in **Figure 2A**, Sestrin2 levels were significantly lower in AIS patients compared to controls at onset and at 5- and 30-days post-attacks. Interestingly, levels of Sestrin2 [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 onset, 5 days, 30 days, and 1 year collectively. For these subjects, we compared Sestrin2 levels across different time points and did not find any significant differences (data not shown). For the TAC and NO, there were only sufficient data points to compare their levels between the onset of attacks and the 5 days' time point. In AIS patients, no statistical significance was found for TAC levels between the onset and 5 days later (data not shown). However, when comparing the TAC levels between the

different time points as separate groups (unpaired), we found a statistically significant difference between the onset of attack and 5 days (**Figure 2B**). However, plasma levels of NO were found to be higher at day 5 compared to onset levels (z = -2.471, *p*=0.01) (**Figure 2C**).

The levels of all three parameters were also compared with those of the healthy controls at each time point. Healthy controls had higher plasma Sestrin2 mean ranks at onset and after 5 days compared to AIS patients (onset: z = -5.107, $p < 0.001$ vs day 5: z = -4.49, $p < 0.001$) (**Figure 2A**). TAC was also significantly higher in controls at onset compared to AIS patients ($p < 0.001$) (**Figure 2B**). Moreover, plasma NO levels were significantly lower in AIS patients at onset compared to healthy controls (*p*=0.04) (**Figure 2C**).

Risk factors associated with AIS

To estimate the risk factors associated with AIS, univariate logistic regression analysis was used to assess the effect of each variable alone on the odds of stroke. Data from AIS, TIA, and stroke mimics were used for the study. All the variables that had a p-value of 0.2 or less are shown in **Table 3**. No statistically significant association between plasma Sestrin2, NO, or TAC and the odds of stroke was observed. Being at a high age, male, South Asian, diabetic, having hypertension, having high NIHSS, having SVD, and being a smoker were all found to increase the odds of AIS, while being from the ME decreased those odds (**Table 3)**.

Multiple logistic regression with backward stepwise elimination, adjusted for age, gender, and nationality, showed that being male, diabetic, having high NIHSS, and the presence of small vessel disease (SVD) all increase the odds of having AIS, while being from ME decreased those odds (**Table 4**).

After estimating the AOR of developing AIS, it was found that being in T3 for Sestrin2, T3 for TAC, and T2 for NO, all decrease the odds of AIS with an AOR of 0.123 ($p < 0.001$), 0.327 (*p*=0.01), and 0.063 (*p*=0.01), respectively (**Table 5**).

Supplementary tables S1, S2, and S3 summarize the comparison of cardiometabolic indicators between the different tertiles for each of the parameters studied. No significant differences were found between the three different 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 S3**).

DISCUSSION

Stroke is the second leading cause of death worldwide [1]. Despite the low mortality rate associated with AIS, great concern arises from the disabilities and impaired quality of life associated with it [7]. Endothelial dysfunction is one of the main underlying mechanisms for cardiovascular diseases like AIS. Endothelial dysfunction occurs under a state of oxidative stress and continuous production of ROS [24]. A reduction in NO bioavailability is the main manifestation of endothelial dysfunction. Sestrin2 is a stress-inducible protein that plays an antioxidant role under oxidative or energetic stress. It has gained a lot of interest in recent years in cardiovascular disease. In this study, we explored the protective role of Sestrin2 in AIS in addition to TAC and NO.

When assessing the plasma levels of Sestrin2, we found it to be significantly higher in healthy controls compared to AIS patients at the time of onset (8.383 ± 7.39 vs 1.434 ± 3.57 ng/ml, $p < 0.001$). Similar results were reported by Tian et al. in diabetic patients with coronary heart disease (CHD) [25]. The study found that plasma levels of Sestrin2 were lower in diabetic patients with CHD compared to those without [11.17 (9.79, 13.14) ng/mL vs 9.46 (8.34, 10.91) ng/mL]. A study by Kishimoto et al., in patients undergoing elective coronary angiography, found that in patients with CAD, plasma Sestrin2 levels were significantly higher than in patients without CAD (median 16.4 vs. 14.2 ng/mL, $p < 0.05$) [26]. Another study, which included 80 controls and 220 congestive heart failure (CHF) patients followed up for 36 months, reported that higher serum Sestrin2 concentrations were found in CHF patients compared to controls [27]. Moreover, in 152 subjects undergoing carotid ultrasonography, Sestrin2 levels were found to be higher in patients with plaque compared to those without (median 14.1 vs. 12.8 ng/ml, $p < 0.02$) [28]. The elevated concentration of Sestrin2 in patients with plaque may indicate a protective role of Sestrin2 in mitigating the progression of carotid atherosclerosis. In this study, we also observed that Sestrin2 levels were significantly lower in acute ischemic stroke (AIS) patients compared to controls. These levels remained low even after the initiation of appropriate therapy, persisting at 5 and 30 days post-acute events. These findings are

consistent with those reported by Gariballa et al. [29]. Moreover, they corroborate our earlier results [3], where we showed that patients with AIS and TIA had elevated levels of extracellular vesicles in their plasma, biomarkers indicative of cellular activation. Specifically, we detected higher levels of extracellular vesicles originating from platelets (CD41+), activated platelets (CD62P+), and pro-coagulant vesicles (Annexin V+) at 5 and 30 days post-acute attacks [3]. In addition, recent transcriptomic analyses have shown that the extracellular vesicles contain specific microRNAs (miRNAs) differentially expressed in stroke patients, that target key genes involved in networks critical for cellular stress responses and recovery [30]. These findings suggest an elevated presence of extracellular vesicles with not only pro-thrombotic activity but also miRNA-mediated regulatory roles in cellular stress responses in these patients. The combination of persistently reduced Sestrin2 levels, diminished TAC, and decreased NO, along with the previously observed elevation in circulating pro-thrombotic extracellular vesicles, may indicate sustained cellular activation in AIS patients even weeks after the initiation of adequate therapeutic management. This prolonged activation could potentially contribute to an increased risk of stroke recurrence in these patients.

TAC is a measure of the cumulative action of all the antioxidants present in plasma and body fluids, offering valuable insights into the delicate balance between oxidants and antioxidants in vivo [31]. Assessing plasma TAC levels can aid in identifying physiological conditions that influence oxidative status in vivo. Reduced TAC has been implicated in ischemic conditions and brain injury, serving as a measure 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 align with those reported by Gariballa et al., who compared TAC levels in ischemic stroke patients, hospitalized non-stroke patients, and healthy controls. Their results demonstrated lower plasma TAC levels in stroke patients relative to the other groups; however, this difference did not reach statistical significance [29]. Conversely, Guldiken et al. assessed TAC levels in diabetic and non-diabetic ischemic stroke patients as well as healthy controls and reported findings that disagree with ours [12]. 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 that investigated the association between dietary TAC and the risk of stroke in women with and without a history of cardiovascular disease at baseline. The study found that higher dietary TAC was associated with a lower risk of stroke [32]. Similarly, a cohort study on 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]. While in a cohort of Iranian stroke patients and controls, after adjusting for dietary intake of fibers and omega-3 fatty acids, it was found that a unit increase in dietary TAC results in 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 an essential endogenous facilitator of cerebral blood flow and cerebrovascular protection [35]. It acts as a neuroprotective in the early stages of ischemic stroke primarily through its vasodilator effect. However, in the later stages of ischemic stroke, neuronal NO synthase induces the overproduction of NO, resulting in increased levels of peroxynitrite, which eventually triggers brain injury [12]. In this study, it was 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. in diabetic stroke patients compared to non-diabetic stroke patients and healthy controls ($p < 0.001$) [12]. It was also reported by Taffi et al. that plasma levels of NO were significantly lower in patients with ischemic stroke compared to healthy controls (51.1 ± 12.5 vs. 115.4 ± 12.4, $p < 0.001$) [36].

Furthermore, this study noted that the plasma levels of NO increased in AIS patients 5 days after the onset of the stroke. However, those levels were still found to be lower compared to healthy controls, although the difference did not reach statistical significance. The increase in NO levels after 5 days of onset in our AIS group could be attributed to the 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 these parameters showed statistical significance. This could be because we only compared AIS patients with mimics and TIA patients, but not with healthy controls. However, after stratifying these parameters into tertiles, we

found that being in the third tertile (T3) for Sestrin2 and the third tertile (T3) for TAC, along with the second tertile (T2) for NO, was associated with a decrease in the odds of stroke by more than 60%. These findings align with a study by Tian et al., which reported that lower serum Sestrin2 levels were related to an increased risk of CHD in type-2 diabetic patients ($p < 0.05$) [25]. Their analysis further demonstrated an inverse relationship between Sestrin2 quartiles and CHD prevalence, with higher Sestrin2 quartiles corresponding to lower CHD occurrence. In contrast, Kishimoto et al. reported that when Sestrin2 levels exceeded 16 ng/ml, the AOR for CAD increased by 1.79 (95% CI: 1.09 – 2.95) [38]. These findings may suggest a possible U-shaped relationship between Sestrin2 levels and the odds of having ischemic disease; however, additional studies are required to assess this.

Multiple logistic regression analysis has shown that being male increases the odds of developing AIS. Our findings agree with reports of the American Heart Association/American Stroke Association, which indicate that the incidence of ischemic stroke is lower in women compared to men [39]. Furthermore, our regression analysis demonstrated that individuals of Middle Eastern (ME) descent exhibited lower odds of AIS. This observation may be influenced by the substantial proportion (60%) of South Asian (SA) labor workers included in this study. Being diabetic was also identified to increase the odds of AIS in our study population. It is well-established that diabetes increases the risk of stroke through various mechanisms, including vascular endothelial dysfunction [40].

Our findings indicate that a higher NIHSS score is associated with increased odds of AIS. The NIHSS is a tool developed by healthcare professionals to quantify the impairment caused by stroke objectively [41]. In addition, we found that the presence of SVD increased the odds of AIS in our study population. SVD is a well-recognized contributor to both stroke and vascular dementia [7].

CONCLUSION

In this study, we report that the plasma levels of Sestrin2, TAC, and total NO were higher in healthy controls compared to AIS patients. Moreover, elevated plasma levels of these markers were found to be associated with reduced odds of stroke. These findings highlight the importance of Sestrin2 as a potential clinical biomarker for predicting the risk of stroke. Additionally, the results reinforce the critical role of

TAC and total NO in the pathophysiology of ischemic stroke. To the best of our knowledge, this is the first study in the region to assess plasma Sestrin2 levels in AIS patients. Given the growing interest in Sestrin2 and the conflicting evidence in the literature, further clinical investigations are warranted to elucidate its precise role in AIS and other cerebrovascular diseases. Future studies should aim to uncover the mechanisms behind Sestrin2's protective role and investigate practical ways to adjust its levels to enhance cerebrovascular health. Strengthening the link between Sestrin2 regulation and better cerebrovascular disease outcomes could greatly influence clinical practices and patient care strategies.

Study limitations

The small sample size limited this study. No power analysis was done to estimate the sample size, and only a sample of convenience was considered. Since the demographic and clinical data of the healthy controls were not attainable, the multivariate logistic regression included only the AIS patients and the TIA and mimics groups as non-AIS subjects. This might have influenced our results, and comparing them with healthy controls could have shown stronger statistical significance. Although this study had a follow-up period to measure the levels of Sestrin2, TAC, and total NO, it did not assess any cardiovascular outcomes or recurrence of stroke, nor did it examine how these may correlate with Sestrin2.

Future directions

While the current study focused on confirming clinical associations between oxidative stress-related biomarkers and having a stroke, we acknowledge the need for further research to improve the biological and translational relevance of these findings. Future research should aim to examine the mechanistic roles of Sestrin2, TAC, and NO in ischemic stroke and vascular dysfunction using in vitro and animal models; investigate longitudinal trends in biomarker levels to better differentiate between pathological and compensatory changes post-stroke; study these biomarkers in larger and more diverse populations to validate their diagnostic or prognostic potential; and evaluate their incorporation with conventional clinical risk factors and imaging data to enhance stroke prediction models. Such efforts will be essential to move from association to causation and to assess 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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Data availability: The data supporting the findings of this study are included in the article. Any additional data may be made available by the corresponding author upon reasonable request.

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TABLES AND FIGURES WITH LEGENDS

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)
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.

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) Score Z	Mean (SD)	Mean (SD) Score Z	
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.

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.

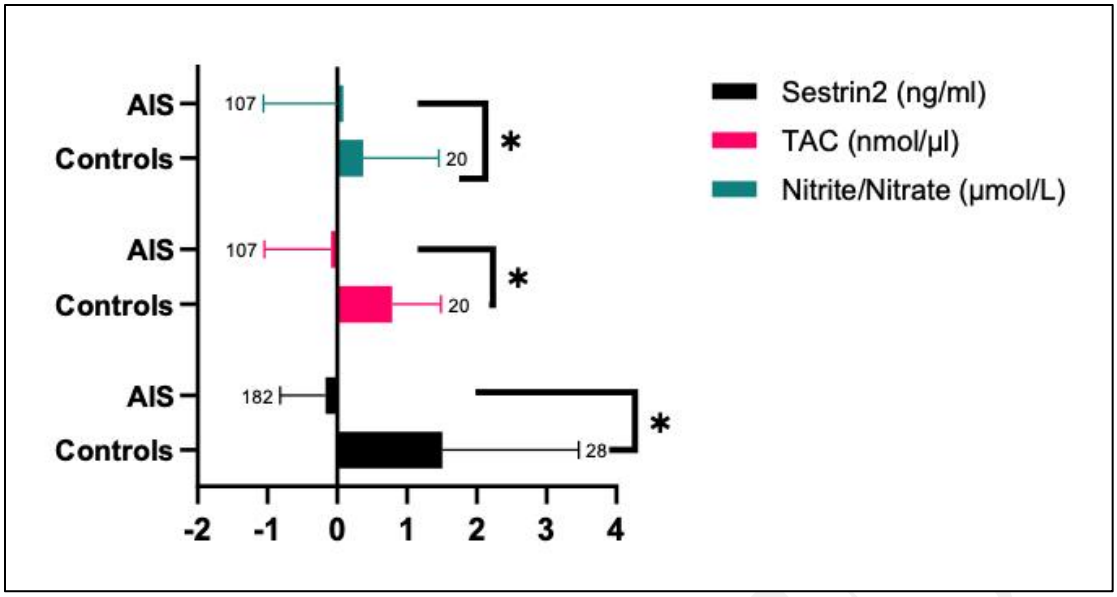

Figure 1. Comparison of plasma levels of Sestrin2, TAC, and nitrite/nitrate (NO) between healthy controls and AIS patients. Plasma levels of Sestrin2, TAC, and nitrite/nitrate (NO) at the onset of AIS are presented as Z-scores. The Mann-Whitney U test was employed to compare the mean Z-scores between 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.

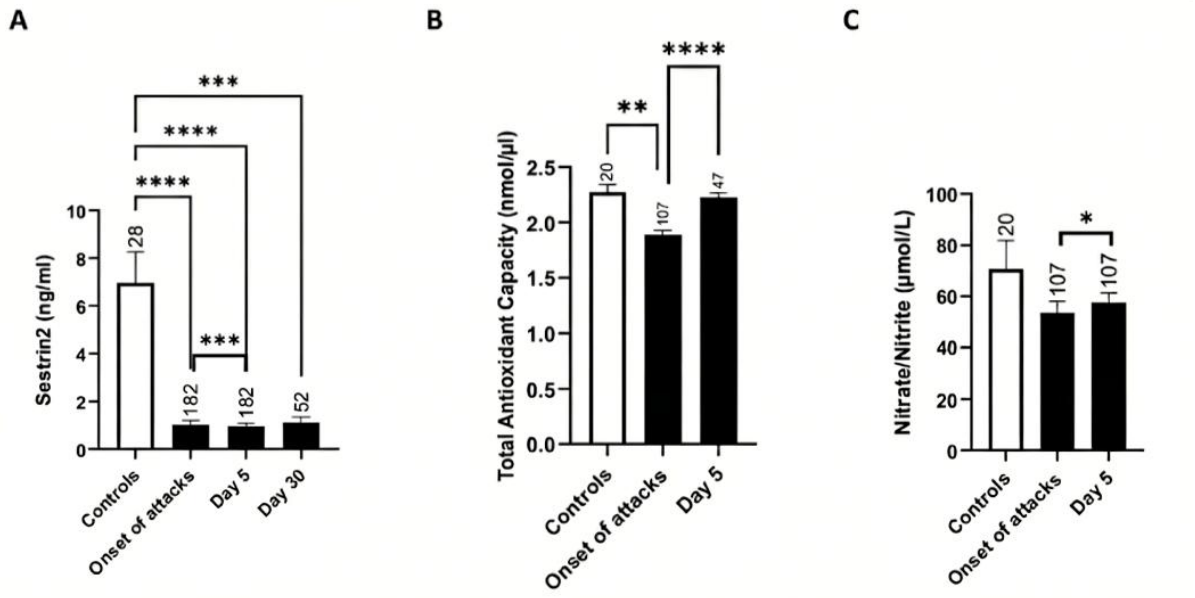

Figure 2. Plasma levels of Sestrin2, TAC, and total nitrite/nitrate (NO) across time points in controls and AIS patients. Plasma concentrations of Sestrin2, TAC, and total nitrite/nitrate (NO) were assessed at various time points and compared between healthy controls and AIS patients. The Wilcoxon rank 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.

SUPPLEMENTAL DATA

Supplemental data are available at the following link:

https://www.bjbms.org/ojs/index.php/bjbms/article/view/12367/4067