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ORIGINAL ARTICLE

A Case-Control Study and Meta-Analysis of the Association of *eNOS* rs1799983 SNP with Stroke Risk

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ABSTRAK

Polimorfisme nitrat sintase endothelial (eNOS) rs1799983 telah dikenalpasti dapat meningkatkan risiko terhadap strok, tetapi tiada data yang dilaporkan dalam negara Malaysia. Oleh itu, kajian ini bertujuan untuk mengkaji perkaitan ini dalam populasi Malaysia dan dalam meta-analisis secara menyeluruh. Pengenotipan eNOS rs1799983 polimorfisme telah dilakukan bagi 241 rakyat Malaysia dengan menggunakan tanda hidrolisis. Nisbah ganjil dengan selang keyakinan 95% telah dikira. Meta-analisis dijalankan dengan perisian Comprehensive Meta-Analysis Ver. 2.2.064. Nilai p kurang daripada 0.05 dianggap signifikan secara statistik. Secara keseluruhannya, kami menunjukkan bahawa alel eNOS rs1799983-T meningkatkan risiko terhadap strok terutamanya dalam kalangan lelaki, mereka yang kerap makan makanan segera dan bangsa Cina Malaysia. Meta-analisis menunjukkan bahawa polimorfisme rs1799983 dikaitkan dengan peningkatan risiko terhadap strok iskemia dalam model resesif dan alelik. Selepas stratifikasi mengikut populasi, perkaitan ini kekal signifikan dalam populasi Asia tetapi tidak signifikan dalam populasi Kaukasia. Kesimpulannya, kajian ini mendapati bahawa terdapat hubungan yang signifikan antara polimorfisme eNOS rs1799983 dengan perbezaan jantina, gaya hidup dan bangsa dalam mempengaruhi risiko terhadap strok di dalam populasi Malaysia. Di samping itu, meta-analisis kami mencadangkan bahawa polimorfisme eNOS rs1799983 dikaitkan dengan peningkatan risiko terhadap strok iskemia.

Kata kunci: eNOS, meta-analisis, polimorfisme nukleotida tunggal, rs1799983, strok

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ABSTRACT

The endothelial nitric oxide synthase (*eNOS*) rs1799983 polymorphism is known to increase the risk towards stroke, but data is under-reported in Malaysian population. Therefore, this study sought to investigate this association in a Malaysian population and in a comprehensive meta-analysis. Genotyping of the *eNOS* rs1799983 polymorphism was performed for 241 Malaysians using a hydrolysis probe. Odd ratio with 95% confidence interval was calculated. Meta-analysis was conducted using the Comprehensive Meta-Analysis software ver. 2.2.064. A *p*-value less than 0.05 was considered statistically significant. Overall, our results showed that the presence of *eNOS* rs1799983-T allele increases the risk towards stroke, particularly in males, fast-food goers and Malaysian Chinese. The meta-analysis showed that the rs1799983 polymorphism is significantly associated with an increase ischemic stroke risk in the recessive and allelic models. After stratified with population, these associations remain significant in the Asian population but not in the Caucasian population. In summary, this study establishes a significant relationship between the *eNOS* rs1799983 polymorphism with gender, lifestyle and ethnicity differences towards stroke risk in the Malaysian population. In addition, our meta-analysis suggests that the *eNOS* rs1799983 polymorphism is associated with an increase risk of ischemic stroke.

Keywords: *eNOS*, meta-analysis, rs1799983, single nucleotide polymorphism, stroke

INTRODUCTION

Stroke is the second leading cause of death and the third leading cause of disability worldwide (World Health Organization 2012), and it is also the leading cause of dementia and depression (Owolabi et al. 2015). It has been proposed that stroke accounts for nearly 5.7 million of deaths globally each year and 87% of these deaths occurred in the low and middle income nations (Strong et al. 2007). In Malaysia, stroke accounts for 7.1% of total death in year 2014, ranking stroke the third cause of deaths in the country after ischemic heart diseases and pneumonia (Department of Statistics Malaysia 2016). Stroke incidence in Malaysia is estimated to increase annually by 29.5% for ischemic stroke and 18.7% for haemorrhagic stroke (Aziz et al. 2015).

Stroke is a multifactorial disorder. Common health and habitual risks of stroke such as dyslipidemia, hypertension, atrial fibrillation, smoking, and diabetes mellitus have been long proposed but only explain a small proportion of the observed clinical events (Sacco et al. 1989). Twin and familial aggregation studies suggest that the risk of stroke is substantially influenced by the genetic component (Dichgans 2007). A recent study had suggested that genetic factors are highly

involved in ischemic stroke incidences through endothelial dysfunction (Meschia et al. 2011). Abnormalities in the endothelial nitric oxide synthase *(eNOS*) activity have been proposed to be one of the factors that contribute to endothelial dysfunction and lead to stroke incidences (Huang 2003).

The *eNOS* gene is located at chromosome 7q36.1 with 26 exons (Marsden et al. 1993). This gene encodes the *eNOS* protein that produces nitric oxides (NO) that dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic guanosine monophosphate in the smooth muscle cells (Forstermann et al. 1994), which helps in maintaining the resting cerebral blood flow and regulates neuronal activity (Faraci & Brian 1994). The NO released also act as a potent inhibitor of platelet aggregation and adhesion, implying that NO provides protection against the onset of atherogenesis and fibrous plaque formation (Fostermann & Munzel 2006) that will eventually lead to stroke incidences.

The *eNOS* rs1799983 single nucleotide polymorphism (SNP) is located in the exon 7 that leads to the substitution of G to T at coding nucleotide position 894, resulting in the conversion of glutamate (Glu) to aspartic (Asp) at codon 298 (Hingorani et al. 1999; Ellul et al. 2011). This SNP was found to be associated with a reduction in the eNOS activity and led to the reduction of basal NO production (Tesauro et al. 2000; Veldman et al. 2002), impairing vasodilation in the endothelium and causing atherosclerosis and stroke incidences.

A large number of studies have been conducted to investigate the association of the *eNOS* rs1799983 SNP with the risk of stroke, however, these studies have yielded inconsistent and conflicting results. Therefore, we conducted a case-control study to investigate this association in Malaysian population and subsequently pooled our results with other similar studies for a meta-analysis that would establish a comprehensive picture of the relationship between the *eNOS* rs1799983 SNP and stroke risk.

MATERIALS AND METHODS

STUDY SUBJECTS AND ETHICS

A total of 103 blood samples were collected from confirmed stroke survivors at Hospital Queen Elizabeth, Sabah, Malaysia with informed consent. The stroke patients are randomly selected and retrospectively collected after being diagnosed with stroke by certified neurologists. In addition, blood samples from 138 healthy individuals who did not have a stroke history, were collected. Information about age, gender, lifestyle (i.e. smoking status, alcohol consumption and fast-food intake) and ethnicity of both stroke patients and healthy controls were recorded. This study was approved by the Medical Research and Ethics Committee of the Ministry of Health, Malaysia with reference no. NMRR-16-38-28777 (IIR).

DNA EXTRACTION AND GENOTYPING

DNA was extracted from the peripheral blood samples using a salting out method with modifications from Sambrook & Russell (2002). Genotyping of the *eNOS* rs1799983 SNP was carried out using 0.5 µL of TaqMan® assay (assay ID: C___3219460_20) (ABI, Foster City, CA), 2 µL of 5 X HOT FIREPol® Probe Universal qPCR Mix (Solis BioDyne, Tartu, Estonia), 1 µL of genomic DNA (10-20 ng/µL) and 6.5 µL of sterile distilled water with the following amplification protocol: preheated at 60°C for 30 seconds, denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 18 seconds, and annealing/extension at 60°C for 1 minutes. The genotyping was carried out using the StepOnePlus™ Real-time PCR System (ABI, USA) and the genotyping results were analyzed using the TaqMan® Genotyper Software Ver. 1.3 (ABI, USA).

META-ANALYSIS

Studies reported on the association between the *eNOS* rs1799983 SNP and stroke risk were retrieved by performing an extensive search in the PubMed database for English or Chinese literatures published before 31 January 2018. Search term combinations are relating to rs1799983 and stroke such as "rs1799983", "G894T" and "Glu298Asp" in combination with "stroke", "cerebrovascular accident" and "cva". We sought eligible studies which met the following criteria: i) original paper published based on case-control design assessing the association between *eNOS* rs1799983 SNP (G894T/Glu298Asp) and stroke risk; ii) providing sufficient data for the calculation of genotypic odd ratio (OR) with 95% confidence interval (95% CI); iii) only ischemic stroke (IS) cases and its subtypes are being considered. The major reasons for exclusion of studies were: i) not case-control study (i.e. review articles, meta-analysis, clinical/case-only studies, etc.); ii) those not reporting the *eNOS* rs1799983 SNP and stroke risk. The most recent and complete studies were chosen for overlapping data.

Titles, abstracts, and full texts of all the retrieved articles were screened carefully by following the inclusion and exclusion criteria stated above. Data such as first author's name, year of publication, country, number of cases and controls, population group, genotyping method and genotype frequency were extracted.

STATISTICAL ANALYSIS

In case-control study, the Hardy-Weinberg equilibrium (HWE) in both cases and controls were assessed using the Chi-square test. OR with 95% CI was calculated using the SPSS V.17.0 (IBM, USA). Association was considered statistical significant when the *p*-value was less than 0.05.

In meta-analysis, OR with 95% CI was used to assess the strength of the association between the *eNOS* rs1799983 SNP with the risk of IS. The Pearson's Chi-square test was used to test the deviation of the *eNOS*

rs1799983 SNP genotype distribution in control groups from the HWE. Association analysis was performed for dominant (GG versus GT+TT), recessive (TT versus GG+GT), allelic (G versus T), heterozygous (GG versus GT) and homozygous (GG versus TT) models. The I^2 method (Higgins & Thompson 2002) was used to assess the heterogeneity among studies. Random effect model (REM) (DerSimonian & Laird 1986) was adopted as the pooling method if substantial heterogeneity was present (I^2>50%) (Higgins et al. 2003), otherwise, the fixed effect model (FEM) (Mantel & Haenszel 1959) was selected as the pooling method. In addition, sources of heterogeneity were investigated by stratifying the meta-analysis based on population subgroup.

A funnel plot and the Egger's linear regression test were used to assess evidence for potential publication bias (Egger et al. 1997). Begg's test was used as an additional measure to detect publication bias (Begg & Majumdar 1994). The Comprehensive Meta-Analysis ver. 2.2.064 (Biostat, Inc., USA) was used to perform the meta-analysis.

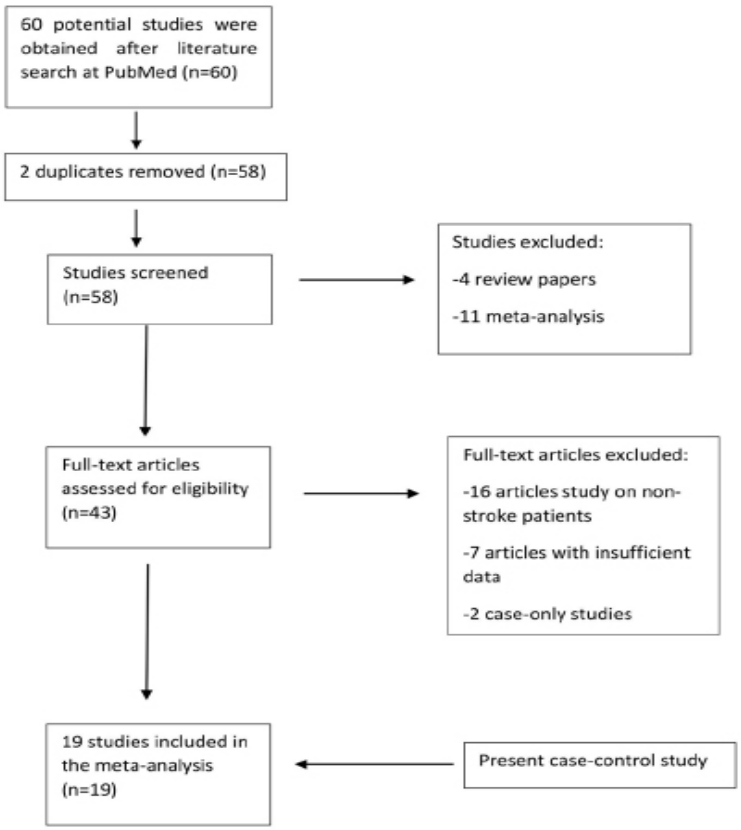

Figure 1: Literature selection process for meta-analysis.

Table 1: Stroke risk association of *eNOS* rs1799983 SNP in the Malaysian population

Reference	Controls	Cases	OR (95% CI)	*p*
	N	N		
Overall				
GG	108	71	1.00 (Reference)	-
GT	26	31	1.81 (0.99-3.31)	0.052
TT	4	1	0.38 (0.04-3.47)	0.392
GT + TT	30	32	1.62 (0.91-2.90	0.103
Gender				
Male	90	66		
GG	71	43	1.00 (Reference)	-
GT	17	22	2.14 (1.02-4.47)	0.044*
TT	2	1	0.83 (0.07-9.38)	0.877
GT + TT	19	23	2.00 (0.98-4.09)	0.058
Female	48	37		
GG	37	28	1.00 (Reference)	-
GT	9	9	1.32 (0.46-3.76)	0.602
TT	2	0	-	-
GT + TT	11	9	1.08 (0.39-2.96)	0.879
Smoking status				
Yes (≥ 2/day)	41	15		
GG	30	10	1.00 (Reference)	-
GT	11	5	1.36 (0.38-4.89)	0.634
TT	0	0	-	-
GT + TT	11	5	1.36 (0.38-4.89)	0.634
No	97	88		
GG	78	61	1.00 (Reference)	-
GT	15	26	2.22 (1.08-4.55)	0.030
TT	4	1	0.32 (0.03-2.93)	0.313
GT + TT	19	27	1.82 (0.92-3.57)	0.083
Alcohol consumption				
Yes (≥ 2/week)	30	12		
GG	24	7	1.00 (Reference)	-
GT	6	5	2.86 (0.67-12.24)	0.157
TT	0	0	-	-
GT + TT	6	5	2.86 (0.67-12.24)	0.157
No	108	91		
GG	84	64	1.00 (Reference)	-
GT	20	26	1.71 (0.88-3.33)	0.117
TT	4	1	0.33 (0.04-3.01)	0.324
GT + TT	24	27	1.48 (0.78-2.80)	0.232

Reference	Controls N	Cases N	OR (95% CI)	*p*
Fast-food intake				
Yes (≥ 2/week)	100	83		
GG	84	58	1.00 (Reference)	-
GT	14	25	2.59 (1.24-5.39)	0.011*
TT	2	0	-	-
GT + TT	16	25	2.26 (1.11-4.61)	0.024*
No	38	20		
GG	24	13	1.00 (Reference)	-
GT	12	6	0.92 (0.28-3.03)	0.895
TT	2	1	0.92 (0.08-11.17)	0.950
GT + TT	14	7	0.92 (0.30-2.86)	0.890
Ethnicity				
Malay	14	9		
GG	10	8	1.00 (Reference)	-
GT	2	1	0.63 (0.05-8.20)	0.721
TT	2	0	-	-
GT + TT	4	1	0.31 (0.03-3.38)	0.338
Malaysian Chinese	68	20		
GG	59	13	1.00 (Reference)	-
GT	8	7	3.97 (1.22-12.91)	0.022*
TT	1	0	-	-
GT + TT	9	7	3.53 (1.11-11.21)	0.033*
Kadazan/Dusun	7	36		
GG	3	24	1.00 (Reference)	-
GT	4	12	0.38 (0.07-1.95)	0.244
TT	0	0	-	-
GT + TT	4	12	0.38 (0.07-1.95)	0.244
Others	49	38		
GG	36	26	1.00 (Reference)	-
GT	12	11	1.27 (0.49-3.32)	0.627
TT	1	1	1.38 (0.08-23.17)	0.821
GT + TT	13	12	1.28 (0.50-3.25)	0.606

* Statistically significant ($p < 0.05$).

RESULTS

CHARACTERISTIC OF SUBJECTS

The case-control study consisted of 103 stroke patients with 58 IS cases and 45 haemorrhagic (HS) cases. The mean age for stroke patients was 53.53 ± 12.72 years while for the controls is 55.51 ± 14.62 years. Both stroke patients and controls are age-matched as no significant mean difference was observed between their ages (p=0.275).

RISK ASSOCIATION IN CASE-CONTROL STUDY

Genotypic distribution for both cases and controls was found to be in HWE (p=0.229 for cases and p=0.133 for controls). No significant difference was found with respect to genotypic (p=0.086) and allelic (p=0.245) frequencies between the cases and controls. Overall, there was no significant association of the *eNOS* rs1799983 SNP with stroke risk in the Malaysian population (Table 1). However, we found that Malaysian males who were inherited the *eNOS* rs1799983-GT genotype had a 2-fold increased risk towards stroke. After stratified to lifestyles, our study revealed that subjects who consumed fast-food regularly and inherited with the T allele of this SNP were significantly associated with more than a 2-fold higher risk of stroke. Besides that, we found that the Malaysian Chinese with the T-allele of the *eNOS* rs1799983 SNP had a nearly 4-fold increased risk of stroke after the stratification based on ethnicity.

RISK ASSOCIATION IN META-ANALYSIS

A total of 19 studies with 4038 IS cases and 4604 controls were included in the meta-analysis (Figure 1). The distribution of genotypes in the control groups were in HWE, with exception of three included studies (Table 2).

In overall, the *eNOS* rs1799983 SNP was significantly associated with an increased risk of IS in the recessive and allelic models. After the exclusion of studies with controls that deviated from the HWE, the association remained significant in these two models. When stratified to different populations, the genotype of the *eNOS* rs1799983 SNP in the control groups were found to be in HWE of all the studies in the Asian subgroup while 3 out of 5 studies in the Caucasian subgroup were found to be deviated from the HWE. Interestingly, an increase risk of IS associated with the *eNOS* rs1799983 SNP was strongly significant in the Asian population in the dominant, allelic and heterozygous models but no significant association was found in the Caucasian population in all the comparison models (Figure 2 and Table 3).

PUBLICATION BIAS ASSESSMENTS IN META-ANALYSIS

Funnel plots revealed no significant publication bias present in all the comparison models (Figure 3), which is further supported by the Egger's and Begg's tests where the p-values were less than 0.05 in all the comparison

Table 2: Characteristics of the studies included in the meta-analysis

Reference	Country	Population group	Genotyping method	No. of cases/ controls	Genotype (Cases/ Controls)			HWE in controls (p)
					GG	GT	TT	
Kumar et al., 2016	India	Asian	PCR-RFLP	250/250	164/186	74/59	12/5	0.02 (0.8875)
Yan et al., 2011	China	Asian	Taqman	545/557	417/446	123/102	5/9	1.25 (0.2636)
Shyu et al., 2017	Taiwan	Asian	Taqman	229/243	151/185	62/51	16/7	2.16 (0.1416)
Esparza-Garcia et al., 2015	Mexico	Mexican	PCR-RFLP	204/204	107/146	86/52	11/6	0.27 (0.6033)
Xiong et al., 2012	China	Asian	SNP microarray	89/102	59/72	19/27	11/3	0.06 (0.8065)
Saidi et al., 2010	Tunisia	African	PCR-RFLP	329/444	98/229	170/174	61/41	0.9 (0.3428)
Guldiken et al., 2009	Turkey	Asian	PCR-RFLP	146/133	82/66	59/61	5/6	3.06 (0.0802)
Szolnoki et al., 2005	Hungary	Caucasian	PCR-RFLP	407/295	205/144	179/137	23/14	6.87 (0.0088*)
Elbaz et al., 2000	French	Caucasian	PCR-RFLP	460/460	212/163	187/232	61/65	1.48 (0.2238)
Diakite et al., 2014	Morocco	African	PCR-RFLP	165/182	83/117	66/58	16/7	0 (1.0000)
Kaur et al., 2015	India	Asian	PCR-RFLP	120/101	84/83	30/17	6/1	0.02 (0.8875)
Türkano lu Ozcelik et al., 2014	Turkey	Caucasian	PCR-RFLP	245/145	82/55	156/80	7/10	7.09 (0.0078*)
Majumdar et al., 2010	India	Asian	PCR-RFLP	172/214	124/159	43/50	5/5	0.2 (0.6547)
Djordjevic et al.,2009	Serbia	Caucasian	PCR-RFLP	26/50	13/18	7/25	6/7	0.13 (0.7184)
Yemisci et al., 2009	Turkey	Asian	PCR-RFLP	67/81	23/22	36/47	8/12	2.58 (0.1082)
Moe et al., 2008	Singapore	Asian	PCR-RFLP	120/207	89/160	26/42	3/5	1.2 (0.2733)
Howard et al., 2005#	USA	Caucasian and America African	SNP microarray	109/198	69/109	33/75	7/14	0.05 (0.8231)
Hassan et al., 2004	UK	Caucasian	PCR-RFLP	297/598	117/242	138/294	42/62	3.95 (0.0469*)
Current Study	Malaysia	Asian	Taqman	58/138	40/108	17/26	1/4	2.26 (0.1328)

*Control genotypes not in Hardy-Weinberg Equilibrium, p-value<0.05.
#Excluded from ethnicity subgroup analysis because consists of multiple population group.

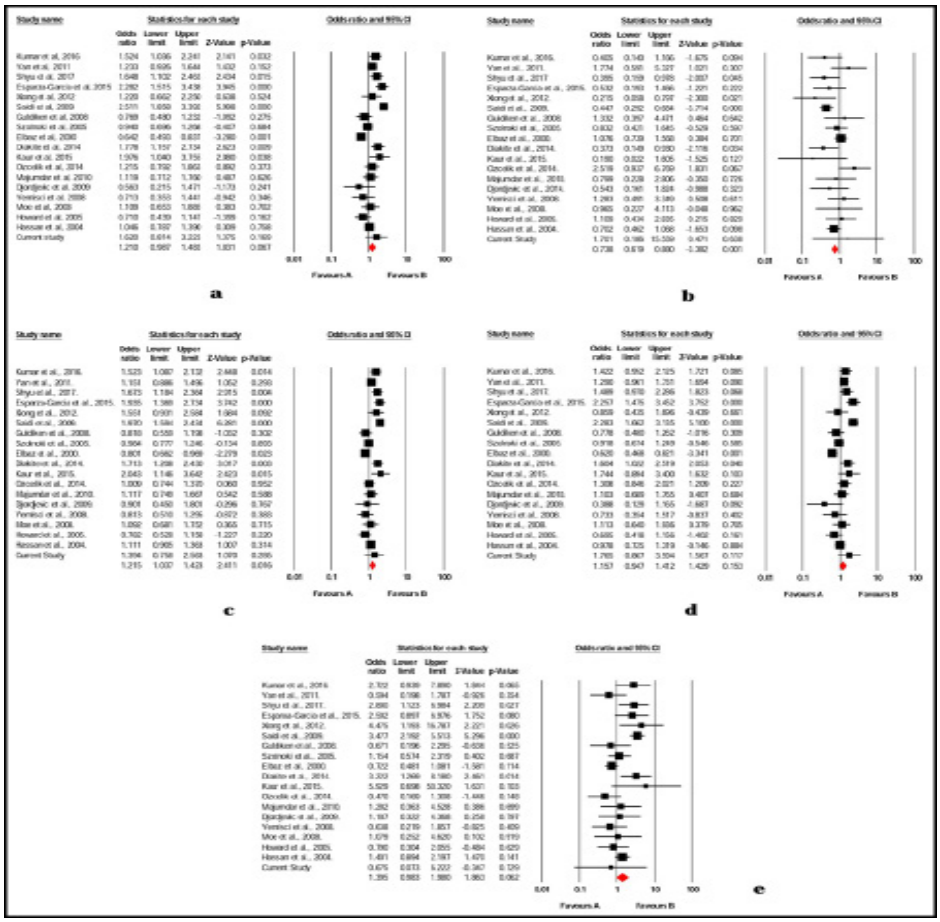

Figure 2: Forest plots for association of *eNOS* rs1799983 SNP with ischemic stroke risk. (a) Dominant model; (b) Recessive model; (c) Allelic model; (d) Heterozygous model; (e) Homozygous model

models.

DISCUSSION

The prevalence of *eNOS* polymorphisms has been established for Caucasian, African-American and Asian populations. However, limited information is available in the Malaysian population. In the present case-control study, no significant association was found between the *eNOS* rs1799983 SNP with stroke risk in the Malaysian population. A previous study based on a Singapore population that share a high similarity with the Malaysian population (in terms of multiple ethnicities) also reported similar findings (Moe et al. 2008). Interestingly, this SNP was found to be associated with a higher risk towards stroke in other Asian populations such as in Indian population (Kumar et al. 2016) and Taiwanese population (Shyu et al. 2017), indicating that the association of this SNP with stroke risk is ethnic-dependent as the populations in Malaysia and Singapore consist of

Table 3: Meta-analysis of eNOS rs1799983 SNP with ischemic stroke risk

Sub-group analysis	No. of study	No. of case/ control	Comparison model	Fixed effect model (FEM)		Random effect model (REM)		I^2 (%)
				OR (95% CI)	p-value	OR (95% CI)	p-value	
Overall	19	4038/ 4602	Dominant	1.198 (1.093-1.314)	0.000	1.210 (0.987-1.483)[S]	0.067	77.591
			Recessive	0.738 (0.619-0.880)[S]	0.001	0.737 (0.562-0.965)	0.027	44.055
			Allelic	1.189 (1.106-1.278)	0.000	1.215 (1.037-1.423)[S]	0.016	76.366
			Heterozygous	1.154 (1.048-1.270)	0.004	1.157 (0.947-1.412)[S]	0.153	74.304
			Homozygous	1.389 (1.154-1.672)	0.001	1.395 (0.983-1.980)[S]	0.062	64.298
Excluded for DHWE#	16	3089/ 3564	Dominant	1.258 (1.132-1.398)	0.000	1.243 (0.968-1.596)[S]	0.089	80.291
			Recessive	0.700 (0.570-0.861)[S]	0.001	0.677 (0.494-0.927)	0.015	41.937
			Allelic	1.248 (1.146-1.359)	0.000	1.261 (1.036-1.534)[S]	0.021	78.847
			Heterozygous	1.206 (1.079-1.347)	0.001	1.178 (0.922-1.505)[S]	0.190	77.211
			Homozygous	1.482 (1.193-1.843)	0.000	1.524 (0.991-2.343)[S]	0.055	67.029
Caucasian	5	1435/ 1548	Dominant	0.873 (0.751-1.015)	0.076	0.887 (0.685-1.149)[S]	0.365	60.204
			Recessive	0.922 (0.721-1.178)[S]	0.514	0.938 (0.649-1.355)	0.733	43.352
			Allelic	0.953 (0.853-1.064)[S]	0.388	0.957 (0.835-1.098)	0.531	28.232
			Heterozygous	0.850 (0.726-0.994)	0.042	0.858 (0.641-1.150)[S]	0.306	65.730
			Homozygous	0.957 (0.737-1.242)[S]	0.740	0.953 (0.650-1.397)	0.805	41.779
Asian	10	1796/ 2026	Dominant	1.255 (1.085-1.452)[S]	0.002	1.249 (1.046-1.491)	0.014	26.772
			Recessive	0.713 (0.486-1.046)[S]	0.083	0.712 (0.441-1.151)	0.166	32.406
			Allelic	1.238 (1.092-1.403)[S]	0.001	1.244 (1.042-1.486)	0.016	45.873
			Heterozygous	1.215 (1.044-1.413)[S]	0.012	1.208 (1.024-1.425)	0.025	11.612
			Homozygous	1.441 (0.975-2.131)[S]	0.067	1.432 (0.854-2.399)	0.173	38.954

[S] Selected effect model based on I^2 value (FEM if I^2<50% and REM if I^2>50%).
#Analysis excluded studies with control genotype deviating from Hardy-Weinberg equilibrium (DHWE)

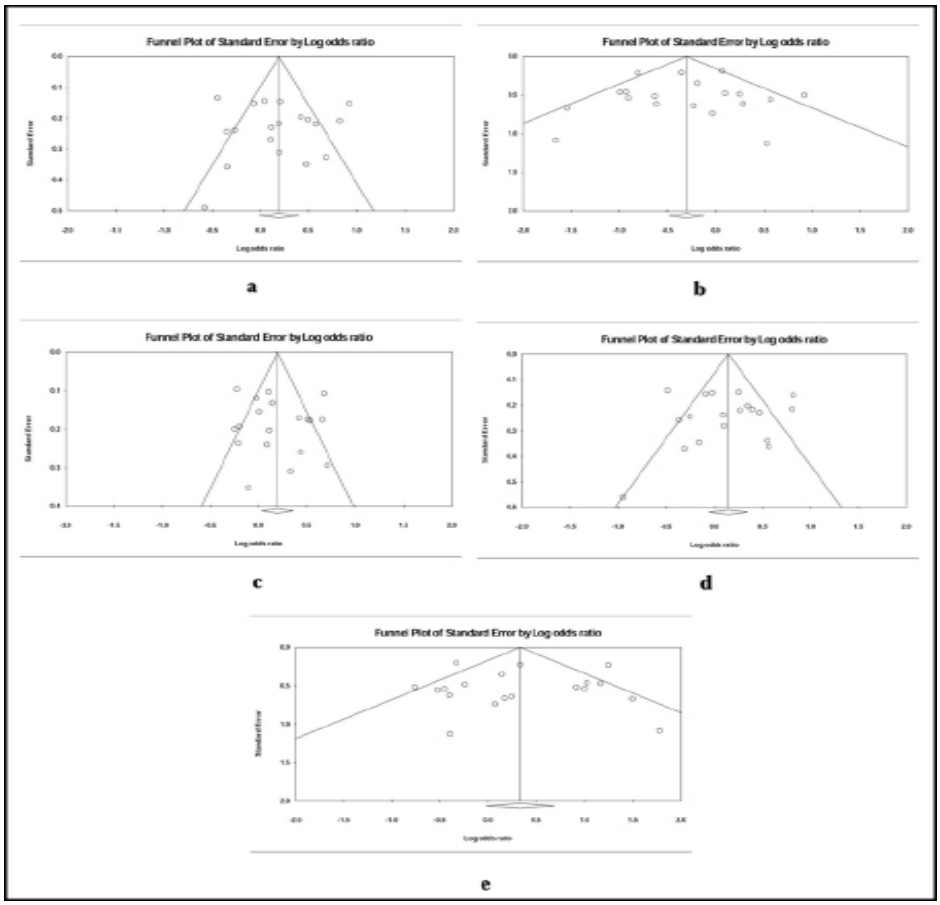

Figure 3: Funnel plots for assessment of publication bias. (a) Dominant model (Egger: p=0.877, Begg: p=0.726). (b) Recessive model (Egger: p=0.936, Begg: p=1.000). (c) Allelic model (Egger: p=0.616, Begg: p=0.780). (d) Heterozygous model (Egger: p=0.960, Begg: p=0.484). (e) Homozygous model (Egger: p=0.937, Begg: p=1.000).

multiple ethnic groups.

After stratification according to gender, we found that Malaysian males with the *eNOS* rs1799983-GT genotype had a significant increase risk towards stroke. However, studies based on other populations such as the Morocco population (Diakite et al. 2014), Tunisian population (Saidi et al. 2010) and German population (Berger et al. 2007) had found that the association of this polymorphism with stroke was independent according to gender. The function of *eNOS* rs1799983 SNP has been well-studied but there are no studies reporting this SNP plays a different role in males and females. It is worth mentioning that the hormone oestrogen that is present in females may increases the production of NO, a potential vasodilator in the body (Kharitonov et al. 1994; Forte et al. 1998) that may aid in reducing stroke risk. Besides that, the study also showed that males have a higher risk of developing vascular diseases such

as stroke than females (Whelton 1994). However, the NO level of the subjects were not measured in this study.

When stratified to lifestyle, we found that subjects who inherited the T allele and consume fast-food regularly had a 2.5-fold increased risk towards stroke. This finding suggests that the risk towards stroke is influenced by the lifestyle factors such as diet. Fast-food are energy-dense meals due to high content of fat, refined starch, and sugar, and correspondingly, lower water content (Benajiba 2016). Furthermore, fast-food is highly processed and prepared using standardised ingredients and production procedures, usually rich in salts as food additives. A habit of consuming fast-food regularly increases the amount of salts intake on a daily basis as fast-food generally contains a relatively higher amount of salts when compared to homemade food. High salt intake from the fast-food and processed food has been associated with a significantly greater risk of both strokes and cardiovascular diseases (Strazzullo et al. 2009).

In addition, it has been proposed that fast-food was associated with a low intake of vegetables, fruits and milk in both adults and children (Paeratakul et al. 2003). Indeed, green leafy vegetables were associated with a reduced likelihood of coronary heart disease and stroke (Joshipura et al. 2001). This may due to the fact that 85% of dietary nitrate (the inorganic nitrate anion, NO_3^-) is derived from vegetables (Gangolli et al. 1994), and these nitrates are important in promoting vasodilatation and preventing vascular dysfunction that will lead to stroke. Studies have shown that nitrate dietary intakes from beetroot juice, beet-enriched bread, and inorganic nitrate supplements have a protective effect against cardiovascular diseases by reducing blood pressure, platelet aggregation inhibition, and prevention of endothelial dysfunction (Kapil et al. 2010; Hobbs et al. 2012). Besides, various natural products present in vegetables and fruits such as genistein improve endothelial nitric oxidase synthase, increase nitric oxide formation, reduce serum cholesterol levels, reduce inflammatory response, and hence, reduce the occurence of atherosclerosis and stroke (Lina et al. 2018).

The T allele of the *eNOS* rs1799983 SNP was associated with a reduced eNOS activity and a lower nitrate concentration in blood (Mahmoodi et al. 2016). Hence, the genetic factors (rs1799983-T allele reduces blood nitrate concentration) together with the dietary habits (high salt intake from fast-food and reduced nitrate intake from vegetables) may be the possible explanation for the significant association between subjects with rs1799983-GT genotype that consume fast-food regularly and a higher risk towards stroke.

In the present case-control study, we also found that Malaysian Chinese carrying the *eNOS* rs1799983-T allele had a nearly 4-fold increased risk of stroke. Our results are in aggreement with previous studies where the T-allele of the *eNOS* rs1799983 SNP was found to be associated with an increased stroke risk in Hans Chinese

(Xiong et al. 2012) and Taiwanese (Shyu et al. 2017). Hence, the *eNOS* rs1799983-T allele might be a potential biomarker for early stroke detection in Chinese ethnicity.

We also performed a meta-analysis for this SNP by pooling our IS cases (n=58) with other similar studies. Only IS cases were included in the meta-analysis as it accounted for 85% of stroke incidences globally and to reduce between study heterogeneity due to different stroke subtypes since other studies included in the meta-analysis only involving IS. A meta-analysis based on the African and Mexican population subgroups was not performed owing to insufficient studies available (number of study: African=2; Mexican=1).

In meta-analysis, we demonstrated that there was a significant association of the *eNOS* rs1799983 SNP and IS risk in the recessive and allelic models. In the population subgroup analysis, the *eNOS* rs1799983 SNP revealed a significant increase risk with IS in the Asian population but not in the Caucasian population. Similar results were reported in the previous meta-analyses (Kumar et al. 2017; Guo 2014; Niu et al. 2013; Yao et al. 2013). Moreover, the TT genotype of the *eNOS* rs1799983 SNP is a higher risk factor for IS in the Asian population but it is protective against IS in the Caucasian population (Niu et al. 2013). These findings indicate that the *eNOS* rs1799983 SNP towards IS susceptibility is population-specific. Further research is warranted to clarify the relevance of this SNP to IS in Caucasian and Asian populations.

Present case-control study and meta-analysis must be carefully interpreted because of certain limitations. First, we do not measure the blood NO level of both stroke patients and controls. Second, this meta-analysis solely focuses the genetic perspective but environmental factors and lifestyles that have been commonly associated with stroke were not included. Third, although we set a precise search strategy for retrieval of eligible studies, we cannot eliminate the possibilities that some studies that published in other databases were not included.

CONCLUSION

In summary, our case-control study suggests that the T allele of the *eNOS* rs1799983 SNP increases the risk of stroke in the Malaysian population, particularly in Malaysian males, that consume fast-food regularly and Chinese ethnicity. In meta-analysis, the *eNOS* rs1799983 SNP is associated with a higher risk of IS in the Asian population but not in the Caucasian population.

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