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Objective: **Carotid stenosis is a common manifestation of systemic atherosclerosis. Apart from traditional risk factors, genetic determinants, such as polymorphisms of the renin angiotensin system (RAS), may be relevant in modulating the atherosclerotic process leading to carotid stenosis. In this study, we investigated the role of angiotensin-converting enzyme (ACE) I/D and -240A>T, *angiotensinogen* (*AGT*) M235T, and *angiotensin type 1 receptor* (*AGTR1*) 1166A > C polymorphisms in modulating the susceptibility to the disease.**

Methods: **Eight hundred twenty-one consecutive patients with severe carotid stenosis (≥70%) and 847 control subjects were investigated.**

Results: **A significant difference in genotype distribution ($P < .0001$) and allele frequency ($P < .0001$) between patients and controls for the *ACE* I/D polymorphism, but not for the other single-nucleotide polymorphisms investigated, was observed. The *ACE* D allele frequency was significantly higher in patients without traditional risk factors in comparison with that observed in those with at least one risk factor (0.71 vs 0.61; $P = .04$). The *ACE* D allele significantly influenced carotid stenosis under dominant, recessive, and additive model of inheritance at both univariate ($P < .0001$) and multivariate analysis ($P < .0001$). When the combined effect of RAS unfavorable alleles was considered, patients carrying less than three alleles had a lower risk of carotid stenosis (odds ratio [OR], 0.79 [0.63-0.99]; $P = .05$), while carriers of more than four unfavorable alleles had an increased risk (OR, 1.44 [1.12-1.84]; $P = .004$), in comparison with subjects carrying three or four unfavorable alleles. *ACE* D allele frequency was similar in patients with and without additional atherosclerotic localizations (0.61 vs 0.62, respectively).**

Conclusions: **Our findings evidence a role for *ACE* I/D polymorphism in influencing the susceptibility to carotid stenosis, even in the absence of traditional risk factors. Interestingly, our findings provided further information concerning the role of this polymorphism in modulating the atherosclerotic process apart from its different localizations. (J Vasc Surg 2011;54:467-73.)**

Atherosclerosis is a widespread process with a strong heritable component and represents the final common pathway affecting blood vessels in the brain, coronary circulation, and peripheral circulation. Carotid atherosclerosis is a common manifestation of systemic atherosclerosis associated with an increased risk of vascular events.

The renin angiotensin system (RAS) plays a central role in the pathogenesis of atherosclerotic disease as angiotensin II activates intracellular signaling pathways, thus promoting atherothrombosis through inflammation, endothelial dysfunction, growth, altered fibrinolysis, and enhanced LDL oxidation.[1] Functional polymorphisms such as *angiotensinogen* (*AGT*) M235T, *angiotensin type 1 receptor* (*AGTR1*) 1166A > C, angiotensin-converting enzyme (ACE) I/D, and -240A>T in genes encoding for RAS components modulate the atherosclerotic process.[2] In particular, *AGT* M235T polymorphism, by influencing angiotensinogen concentration,[3] has been associated with hypertension[3] and myocardial infarction,[4] but data from literature did not demonstrate its role in cerebrovascular disease.[5-7] The *ACE* I/D polymorphism has been reported to modulate angiotensin II concentrations,[8,9] and an increased mRNA expression in white blood cells from subjects carrying the *ACE* D allele has been demonstrated.[10] This polymorphism modulates atherosclerosis at different localizations such as coronary artery disease,[11] peripheral arterial disease (PAD),[12,13] and abdominal aortic aneurysm (AAA).[14] Conflicting data concerning its role in cerebrovascular disease are reported.[15-17] The ACE - 240A > T polymorphism has been investigated in cardiovascular disease[18] and PAD,[13] but no information in carotid atherosclerosis is available.

Finally, the *AGTR1* 1166A > C polymorphism has been demonstrated to influence the predisposition to CAD,[11] aortic stiffness,[19] and vascular reactivity,[20] but only one study on cerebrovascular disease is reported.[7]

As we previously investigated the possible influence of *ACE* (I/D, -240A>T), *AGT* (M235T) and *AGTR1* (1166A > C) RAS polymorphisms on CAD, AAA, and PAD, we aimed this study to analyze the single and com-

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bined effect of RAS polymorphisms in influencing the predisposition to carotid atherosclerosis.

METHODS

The study population consisted of 835 unrelated consecutive patients admitted to the Unit of Vascular Surgery of the University of Florence to be evaluated for surgical intervention. Fourteen patients refused to consent to the genetic analysis, so the final study group included 821 patients.

Carotid stenosis was assessed by duplex scanning with color-coded echo flow imaging and confirmed by angio computed tomography, according to the North American Symptomatic Carotid Endarterectomy Trial criteria.[21] All patients had severe (defined by a stenosis >70%) carotid stenosis. Preoperative neurological symptoms, defined as ipsilateral cerebrovascular events during 180 days within the intervention, were recorded in 292 patients; the remaining patients were asymptomatic. Patients with nonhemispheric symptoms were considered to be asymptomatic. Thirty-one of 821 patients (3.8%) suffered from AAAs, 72 (8.8%) from CAD, and 179 (21.8%) from PAD.

All patients underwent a clinical cardiological evaluation, electrocardiogram (ECG), and echocardiogram and peripheral arteries echo color Doppler analysis. In patients referring symptoms, possibly related to ischemic heart disease, further investigations have been performed (ECG on exercise stress testing, myocardial scintigraphy, and coronary angiography).

We have enrolled 982 apparently healthy subjects recruited from blood donors and from partners or friends of patients, in order to have a control group. Expert physicians performed, in the frame of a physical examination, a detailed interview addressed to personal and familial history in order to identify disease-free controls and to exclude subjects who were suspected of having any form of vascular disease. We have excluded 135 subjects suspected to have had any form of vascular disease; therefore, 847 apparently healthy subjects represented the control group.

Based on information from physical examinations and detailed interviews, we have investigated the presence/absence of traditional cardiovascular risk factors. The subjects were considered to have hypertension if they had been diagnosed as hypertensives according to the guidelines of European Society of Hypertension/European Society of Cardiology[22] or were taking antihypertensive drugs. Dyslipidemia was defined according to the Third Report of the National Cholesterol Education Program[23] and diabetes in agreement with the American Diabetes Association.[24] Smokers were defined as current or recent (ex-smokers who stopped less than 5 years previously) smokers.

A positive family history was defined as the presence of at least one first-degree relative who had developed cardiovascular disease before the age of 55 years for men and 65 years for women.

Consanguineous subjects were excluded; patients and controls were Caucasian and drawn from the same area (Central Italy); all subjects gave informed consent, and the study complies with the Declaration of Helsinki and was approved by the local ethic committee of the Faculty of Medicine, University of Florence.

Detection of RAS polymorphisms. Genomic DNA extraction was performed from peripheral blood leukocytes by using QIAmp Blood Kit (Qiagen, Hilden, Germany).

RAS polymorphisms [*ACE* I/D (rs4340; **NT_010783.14**: g.20217903_20217904ins5), ACE - 240A > T (rs4291; **NT_010783.14**: g.20206205T > A), *AGTR1* 1166A > C (rs5186; **NT_005612.15**: g.54955134A > C), *AGT* M235T (rs699; **NT_004559.13**: g.7047947A > G)] have been genotyped according to previously reported methods.[11,25]

Statistical analysis. Statistical analysis was performed by using the SPSS (Statistical Package for Social Sciences, Chicago, Ill) software for Windows (Version 11.5). Continuous variables were expressed as median (range), and the nonparametric Mann-Whitney test for unpaired data was used for comparison between single groups, as appropriate. The χ^2 test was used to test for deviation of genotype distribution from Hardy-Weinberg equilibrium, to evaluate the differences in genotype distribution and allele frequency, and to analyze differences in the prevalence of traditional risk factors between patients and controls.

The number of subjects studied was sufficient to detect, with a statistical power of 90% ($\beta = 0.9$) and significance value of 0.05 (α), absolute differences in *ACE* D allele frequencies between patients and controls. The Bonferroni correction was used for multiple testing (the four candidate polymorphisms were treated as four independent statistical tests) by multiplying the nominal *P* value of each test by the number of tests conducted.

The association between *ACE*, *AGTR1*, and *AGT* polymorphisms and carotid stenosis was assessed by using logistic regression analysis under dominant, recessive, and additive genetic model of inheritance.[26] The dominant genetic model compares individuals with one or more polymorphic alleles with a baseline group with no polymorphic alleles (eg, *ACE* DD + ID vs II). The recessive genetic model compares homozygous genotype for rare variant with the combined heterozygous and homozygous genotypes for the wild-type allele (eg, *ACE* DD vs ID+II), forming the baseline group. The additive genetic model assumes that there is a linear gradient in the risk among DD, ID, and II genotypes (II genotype baseline). This is equivalent to a comparison of the D allele versus the I allele (baseline). Cardiovascular traditional risk factors and variables as age and gender were included into the multivariate model. Odds ratio (OR) with 95% confidence interval was determined. In order to investigate the risk of disease for combined effect of variant rare alleles, the study population was divided into three subgroups according to tertiles of their distributions: a low-risk group (0-2 alleles), an intermediate-risk group (3-4 alleles), and a high-risk group (>4 alleles).

Statistical significance was accepted at *P* value <.05 (two-sided *P* value).

Table I. Demographic, clinical, and genetic characteristics of the study population

Variable			*Patients (n = 821)*	*Controls (n = 847)*	P
Age (years)[a]			72 (25-88)	69 (19-83)	.06
Males, n (%)			568 (69)	550 (65)	.07
Hypertension, n (%)			484 (59)	162 (19)	**<.0001**
Dyslipidemia, n (%)			332 (40)	153 (18)	**<.0001**
Diabetes, n (%)			128 (16)	41 (5)	**<.0001**
Smoking habits, n (%)			380 (46)	159 (19)	**<.0001**
Family history of cardiovascular disease, n (%)			101 (12)	76 (9)	**.03**
AAA, n (%)			31 (4)	—	—
CAD, n (%)			72 (9)	—	—
PAD, n (%)			179 (22)	—	—
RAS polymorphisms					
Genotype[b]		*Allele*			
ACE					
	DD		318 (39)	217 (26)	
	ID		395 (48)	431 (51)	
	II		108 (13)	199 (24)	**<.0001**
		D	0.63	0.51	**<.0001**
	-240TT		140 (17)	131 (16)	
	-240AT		405 (49)	390 (46)	
	-240AA		276 (34)	326 (39)	.1
		-240T	0.42	0.39	.06
AGT					
	235TT		166 (20)	153 (18)	
	235MT		407 (50)	425 (50)	
	235MM		248 (30)	269 (32)	.5
		235T	0.45	0.43	.3
AGTR1					
	1166CC		56 (7)	65 (8)	
	1166AC		357 (44)	364 (43)	
	1166AA		408 (50)	418 (49)	.8
		1166C	0.28	0.29	.7

AAA, Abdominal aortic aneurysm; *CAD*, coronary artery disease; *PAD*, peripheral arterial disease; *RAS*, renin-angiotensin system.
P values in bold indicate $P < .05$.
[a]Median (range).
[b]Genotypes are expressed as n (%).

RESULTS

We investigated 568 males, median age 72 years (range, 45 to 86 years), 253 females, median age 73 years (range, 25 to 88 years), and 847 apparently healthy subjects (550 males, median age 70 years [range, 24-83 years], and 297 females, median age 70 years [range, 22-83 years]). Demographic, clinical, and genetic characteristics of the study population are reported in Table I. Traditional risk factors were significantly higher in patients than in controls. No deviation from the expected genotype proportion predicted by Hardy-Weinberg equilibrium was observed for all the polymorphisms analyzed. A significant difference for *ACE* I/D genotype distribution and allele frequency between patients and controls was observed, and a higher, even if not significant, *ACE* -240T allele frequency in patients than in controls was found. As concerning the other single-nucleotide polymorphisms investigated, no significant differences between patients and controls were evidenced (Table I).

At both univariate and multivariate analysis, the *ACE* D allele was significantly associated with the disease under dominant, recessive, and additive model of inheritance (Table II); at univariate analysis, but not after adjustment for traditional risk factors, the *ACE* -240T allele was significantly associated with the disease under dominant model of inheritance; no association between *AGTR1* 1166C and *AGT* 235T alleles and the disease at both univariate and multivariate analysis, was found (Table II).

In smokers and diabetic patients, the *ACE* D allele increased the predisposition to the disease under all genetic models of inheritance (Table III). On the other hand, the other RAS rare variants did not modify the risk in hypertensives, diabetics, smokers, and dyslipidemics (data not shown).

Sixty-three out of 821 (7.7%) patients reported no traditional risk factors; in these patients, the frequency of *ACE* D, but not of the other rare variants, was significantly higher than in patients with at least one risk factor (0.71 vs 0.61; $P = .04$); moreover, *ACE* D allele frequency was significantly different between patients and controls without traditional risk factors (0.71 vs 0.50; $P < .0001$).

Two hundred ninety-two out of 821 patients (35.6%) had symptomatic carotid stenosis (140 transient ischemic

Table II. Univariate and multivariate analysis for renin-angiotensin system polymorphisms

	Univariate analysis					
	Dominant model		Recessive model		Additive model	
	OR (95% CI)	P	*OR (95% CI)*	P	*OR (95% CI)*	P
ACE D	2.03 (1.57-2.62)	**<.0001**[a]	1.83 (1.49-2.26)	**<.0001**[a]	2.7 (2.02-3.61)	**<.0001**[a]
ACE-240 T	1.24 (1.01-1.51)	**.04**[b]	1.12 (0.87-1.46)	.4	1.26 (0.95-1.68)	.1
AGTR1 1166C	0.97 (0.81-1.19)	.9	0.88 (0.61-1.28)	.5	0.88 (0.60-1.29)	.5
AGT 235T	1.07 (0.87-1.32)	.5	1.15 (0.90-1.47)	.3	1.18 (0.89-1.57)	.3
	Multivariate analysis[c]					
	Dominant model		Recessive model		Additive model	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
ACE D	1.98 (1.45-2.70)	**<.0001**[a]	1.98 (1.54-2.55)	**<.0001**[a]	2.81 (1.97-4.00)	**<.0001**[a]
ACE-240 T	1.12 (0.88-1.43)	.4	—		—	
AGTR1 1166C	—		—		—	
AGT 235T	—		—		—	

CI, Confidence interval; *OR,* odds ratio.
P values in bold indicate $P < .05$.
Bonferroni correction:
[a]$P < .0001$; [b]$P = .2$; [c]adjusted for age, sex, hypertension, diabetes, smoking habit, and dyslipidemia.

Table III. Univariate and multivariate analysis for the interaction between ACE I/D polymorphism and traditional risk factors

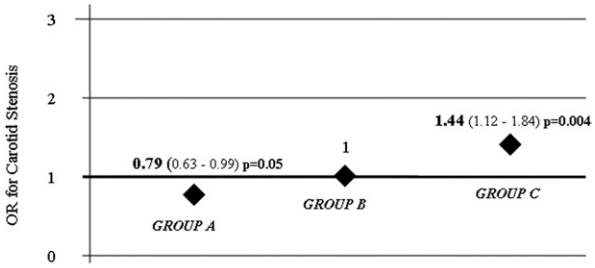
Univariate analysis	*OR (95% CI)*	P	*OR (95% CI)*	P	*OR (95% CI)*	P
Hypertension	6.07 (4.87-7.57)	**<.0001**				
Smoking habit	3.73 (2.99-4.65)	**<.0001**				
Diabetes	3.63 (2.52-5.24)	**<.0001**				
Dyslipidemia	3.08 (2.46-3.85)	**<.0001**				
	Dominant model		*Recessive model*		*Additive model*	
ACE D	2.03 (1.57-2.62)	**<.0001**	1.83 (1.49-2.26)	**<.0001**	2.70 (2.02-3.61)	**<.0001**
ACE D[a] hypertension	5.69 (4.52-7.18)	**<.0001**	5.97 (4.13-8.64)	**<.0001**	7.21 (4.89-10.64)	**<.0001**
ACE D[a] smoking habit	4.45 (3.50-5.67)	**<.0001**	5.24 (3.60-7.64)	**<.0001**	6.04 (4.07-8.96)	**<.0001**
ACE D[a] diabetes	4.11 (2.74-6.16)	**<.0001**	7.45 (3.35-16.57)	**<.0001**	7.42 (3.32-16.60)	**<.0001**
ACE D[a] dyslipidemia	3.27 (2.56-4.16)	**<.0001**	2.97 (2.09-4.22)	**<.0001**	3.15 (2.18-4.56)	**<.0001**
Multivariate analysis[b]						
ACE D[a] hypertension	5.18 (3.99-6.72)	**<.0001**	5.26 (3.5-7.91)	**<.0001**	6.36 (4.13-9.78)	**<.0001**
ACE D[a] smoking habit	4.96 (3.73-6.61)	**<.0001**	6.49 (4.23-9.98)	**<.0001**	7.24 (4.58-11.47)	**<.0001**
ACE D[a] diabetes	3.57 (2.19-5.80)	**<.0001**	5.71 (2.31-14.16)	**<.0001**	5.51 (2.21-13.78)	**<.0001**
ACE D[a] dyslipidemia	2.84 (2.13-3.80)	**<.0001**	2.52 (1.66-3.84)	**<.0001**	2.47 (1.58-3.85)	**<.0001**

CI, Confidence interval; *OR,* odds ratio.
P values in bold indicate $P < .05$.
[a]Contemporary presence of *ACE* D allele and traditional risk factors.
[b]Adjusted for age, gender, and traditional risk factors.

attacks, 97 minor strokes, and 55 major strokes); no difference in *ACE* D allele frequency between symptomatic and asymptomatic patients (0.61 vs 0.62, respectively) and among patients with transient ischemic attack (0.60), minor (0.61), and major stroke (0.61) was observed.

Five hundred forty-nine out of 821 patients (66.9%) showed no additional atherosclerotic localizations (isolated carotid stenosis); in this subgroup, the *ACE* D allele frequency was not different from that observed in patients with other atherosclerotic localizations (0.62 vs 0.61, respectively).

RAS allelic burden. A significant difference between patients and controls according to the number of unfavorable alleles (ranging from 0 to 8) was found ($\chi^2 = 25.65$; $P = .001$). In order to search for the combined effect of these alleles, both patients and controls were divided into three subgroups according to tertiles of their distribution: group A with less than three unfavorable alleles (n = 464), group B with three or four unfavorable alleles (n = 832), and group C with more than four unfavorable alleles (n = 372; Fig).



	< 3 alleles (Group A)	3-4 alleles (Group B)	> 4 alleles (Group C)
Patients (n=821)	200 (24.4%)	406 (49.4%)	215 (26.2%)
Controls (n=847)	264 (31.2%)	426 (50.3%)	157 (18.5%)

χ^2 for distribution 17.95, p=0.0001

Fig. Odds ratio for carotid stenosis in groups A, B, and C, stratified on the basis of number of unfavorable alleles. Group B (3-4 unfavorable alleles), representing the 49.9% of the whole population, is considered as reference group.

By considering group B (3-4 unfavorable alleles), the reference group, subjects with less than three alleles (group A) showed a lower risk of carotid atherosclerosis, while subjects with more than four alleles (group C) showed an increased risk (Fig). By comparing the two extreme groups (C vs A group), subjects with more than four alleles showed a significantly higher risk of carotid stenosis at both univariate and multivariate analysis (OR, 1.81; 95% CI, 1.37-2.38; $P < .0001$; OR, 2.04; 95% CI, 1.46-2.86; $P < .0001$, respectively).

DISCUSSION

This study provides evidence that polymorphisms in genes encoding for components of the renin angiotensin system are associated with the predisposition to carotid atherosclerosis. Most importantly, in the absence of traditional risk factors, and in subjects carrying more than four genetic rare variants. These results suggest that, beyond single polymorphisms, allele-allele interactions may have a synergistic impact on atherosclerotic process leading to carotid atherosclerosis.

These novel findings strengthen the well-known effect of RAS in modulating the atherosclerotic process and support the impact of the combined effect of several common susceptibility polymorphisms in identifying patients at different risk of developing atherosclerosis.

Interestingly, in this study we observed that *ACE* D allele frequency was similar in patients with isolated carotid stenosis and with other atherosclerotic localizations in addition to carotid stenosis. Present findings and other previous studies evidence that *ACE* D allele frequency is similar among subgroups of patients suffering from atherosclerosis at different anatomical localizations, thus strengthening the role of the renin angiotensin system and in particular, the *ACE* D allele, in modulating atherosclerotic process (Table IV).

To date, there is good evidence from experimental and clinical studies that RAS, via its active peptide angiotensin II, may contribute to atherosclerosis development and progression: data from literature documented an association between RAS candidate genes and coronary artery disease,[27] abdominal aortic aneurysms,[28] PAD,[12] and cerebrovascular disease.[29] In particular, the *ACE* I/D polymorphism, the most widely studied polymorphism among RAS polymorphisms, has been reported to be a prognostic factor for cerebrovascular events in patients with carotid atherosclerosis,[16] but few data concerning the role of the other RAS polymorphisms in carotid stenosis are available.[6,7]

In the present study, we observed that the *ACE* gene is able to influence the risk of carotid atherosclerosis in the absence of traditional risk factors. The relevance of this datum might be explained by increased angiotensin II levels, which are primarily modulated by *ACE* I/D polymorphism, by affecting the atherosclerotic process, independently of the well-known cardiovascular risk factors. Really, among polymorphisms in RAS candidate genes, the *ACE* I/D polymorphism is relevant due to its functional role, and the mechanisms underlying the apparent association between the *ACE* gene and atherosclerosis are intriguing. The *ACE* I/D polymorphism is known to be responsible for 47% of the total phenotypic variance of serum ACE,[8] even if data derived from an in vitro assay suggested that the *ACE* intron 16 sequence by itself had no effect in regulating transcription.[30] This datum might suggest that ACE level variability is referred to another locus in linkage disequilibrium with the I/D polymorphism. Actually, experimental data from *ACE* gene expression analysis evidenced that the *ACE* D allele was associated with higher *ACE* mRNA levels.[10]

Our findings did not demonstrate a role for *AGT* and *AGTR1* polymorphisms in modulating carotid stenosis; these results are in keeping with other studies that evaluated their role as markers in carotid stenosis[7] and ischemic cerebrovascular disease.[6]

Of interest, our study evidenced that the contemporary presence of several unfavorable alleles represents a marker of atherosclerotic risk, thus possibly suggesting an allelic burden able to influence the susceptibility to the disease.

This study suffers from these limitations: first, the lack of information concerning intima-media thickness (IMT) measurement; data from literature reported a positive relationship between *ACE* I/D polymorphism and common carotid IMT[15]; nevertheless, recently it has been demonstrated that despite IMT being predictive for cardiovascular end points, it did not consistently improve the risk classification of subjects.[31] Second, the study did not provide data concerning intermediate phenotypes, which may result from RAS polymorphisms, thus not revealing the mechanisms linking the genetic variants and the disease. Finally, the control group consisted of subjects with no clinical evidence of atherosclerotic disease, whereas the optimal comparable group would be represented by atherosclerotic subjects with no carotid stenosis. Moreover, some controls may still have carotid artery disease without any imaging screening of controls.

In conclusion, in this study, we demonstrated that the *ACE* I/D polymorphism is associated with carotid athero-

Table IV. Role of ACE I/D polymorphism in the atherosclerotic process

	Case/controls (n)	*ACE D allele frequency*	*Reference*
Coronary artery disease	205/209	0.62/0.47	Fatini et al[11]
Abdominal aortic aneurysm	250/250	0.63/0.49	Fatini et al[14]
Peripheral arterial disease	281/485	0.59/0.51	Fatini et al[13]
Carotid stenosis	821/847	0.63/0.51	The present study

sclerosis also in the absence of traditional cardiovascular risk factors, and provided evidence of a relationship between multiple unfavorable RAS genetic variants and the risk of disease. We are aware of the evidence from our results that carotid stenosis patients should not be screened for RAS polymorphisms; on the other hand, as carotid stenosis is a polygenic disease, data from the present study might contribute to highlight the role of candidate genes in modulating the susceptibility to the disease. This study may have a pathophysiological relevance by evidencing the contribution of RAS genes in the atherosclerotic process, as we previously demonstrated, and our results might suggest that in the future, it will be possible to apply the knowledge gained by genetic analyses to better tailor and optimize the therapy in atherosclerotic patients.

Further studies investigating a larger number of genetic markers on larger samples are needed to confirm our results, and replication of our findings in other populations would further strengthen the claim of causality for RAS unfavorable alleles and increased susceptibility to carotid stenosis.

AUTHOR CONTRIBUTIONS

Conception and design: ES, RA, CF
Analysis and interpretation: ES, FS, GP, CF
Data collection: IR, GP, RP, CP
Writing the article: ES, FS, CF
Critical revision of the article: ES, CP, RA, CF
Final approval of the article: ES, IR, FS, GP, RP, CP, RA, CF
Statistical analysis: FS, IR
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