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Research article

Association between resistin promoter -420C>G polymorphisms and producing ability with type 2 diabetes mellitus

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Abstract: Elevated resistin levels and the polymorphisms located at gene encoding resistin (*RETN*) are associated with diabetic pathogenesis. However, the correlation between *RETN* genotypes and T2DM is controversial due to discrepancies among reports. This study aimed at investigating and clarifying the putative association of *RETN* and T2DM in Taiwanese population. The resistin levels and *RETN* -420C>G genotypes in 244 control and 305 T2DM subjects were examined. Meanwhile, the association between genetic polymorphism of *RETN* -420C>G and resistin levels, as well as between *RETN* -420C>G and subjects' clinical characteristics was statistically analyzed. The *RETN* -420C>G genotypes ($p = 0.01$) and G allele ($p = 0.002$) were significantly associated with T2DM. In addition, concanavalin A-stimulated peripheral blood mononuclear cells from T2DM subjects had higher resistin-secreting ability ($p = 0.044$). Nevertheless, no significant association between the subjects' biochemical data and *RETN* -420 SNPs was found. Our results indicate that *RETN*

-420C>G SNPs and G allele are significantly associated with T2DM. Investigation of *RETN* polymorphisms in T2DM patients from various ethnic populations are crucial and will contribute to the understanding of this gene in the diabetic etiology. The present results may contribute to gain knowledge on the complex genetic heterogeneity of type 2 diabetes.

Keywords: obesity; promoter polymorphism; resistin; type 2 diabetes mellitus

Abbreviations

AC	fasting blood glucose
BMI	body mass index
BUN	blood urea nitrogen
Con A	concanavalin A
CRE	creatinine
HDL-C	high density lipoprotein-cholesterol
MS	metabolic syndrome
PBMCs	peripheral blood mononuclear cells
RELMs	resistin-like molecules
RETN	human resistin gene
SNPs	single nucleotide polymorphisms
SP	systolic pressure
T2DM	type 2 diabetes mellitus
TG	triglyceride

1. Introduction

The etiology of type 2 diabetes mellitus (T2DM), which affects at least 200 million throughout the world, is not entirely disclosed. Though insulin resistance seems to be a central abnormality, the origin of the impaired insulin action and how it explains the many other clinical symptoms and complications of T2DM still await to be investigated. Obesity, primarily characterized by an increased mass of fat, is a major risk factor which leads to the development of hyperlipidaemia, metabolic syndrome (MS), and T2DM. The adipose tissue is traditionally considered to play a passive role in metabolism by acting as a fat storage reservoir. In addition to being the main energy storage organ, adipocytes are involved actively in maintaining metabolic balance [1,2] by secreting several hormones and cytokines [3]. These adipose derived signaling molecules exert potent metabolic effects to distant organs, which are likely to play a key role in the complex inter-organ communication network to modulate metabolism and energy homeostasis [4,5].

Resistin is a 12.5 kDa cysteine-rich peptide that is secreted from adipocytes in rodents and from macrophages in human [6]. Resistin is a member of the resistin-like molecules (RELMs) with a conserved pattern of 11 cysteine residues at its C-terminus [7]. The postulated roles for resistin

include the regulation of glucose homeostasis, adipose tissue mass and inflammation. In murine models, resistin is induced during adipocyte differentiation and its expression is reduced upon treatment with insulin sensitizers. Serum resistin levels are elevated in obese mice, and administration of resistin impairs glucose tolerance in normal mice. Several human studies report serum resistin levels are increased in patients with obesity, insulin resistance, and/or T2DM [8,9,10]. Inflammation is a hyper-resistinemic state in humans, and cytokine induction of resistin may contribute to insulin resistance, obesity, and other inflammatory states [11,12,13]. However, the mechanism and importance of increased resistin levels in human MS and T2DM are still not fully illustrated.

The human resistin gene (*RETN*) is located on chromosome 19p13.3, with several identified single-nucleotide polymorphisms (SNPs) associated with T2DM [14,15]. Two *RETN* 5'-flanking SNPs are associated with obesity among non-diabetic individuals [16,17]. The *RETN* -420C>G variants draw much attention and are most extensively investigated. This SNP is the major determinant of the resistin expression and associated with insulin resistance, and possibly with cellular oxidative stress [18]. Mattevi et al. reported that *RETN* -420C>G is associated with higher mRNA expression in human abdominal subcutaneous fat [19]. Nevertheless, epidemiological studies on the association between the *RETN* -420C>G SNPs and T2DM risk are conflicting and controversial. Most of the resistin-related studies focus on the investigation of the SNPs in Caucasian or Japanese subjects, relatively few corresponding data in Chinese/Taiwanese populations are reported. Therefore, it is tempting for us to investigate whether *RETN* SNPs are linked to T2DM in Taiwanese population for determining the ethnic-dependent variations for T2DM development.

2. Materials and Methods

2.1. Study subjects

Fasting venous blood samples were taken from 244 T2DM patients attending the diabetic clinic in Department of Internal Medicine, Chung Shan Medical University Hospital. Fasting blood samples from 305 non-diabetic control subjects were collected from Physical Check Up Unit. Written informed consent was obtained from all the study subjects after the nature of the procedure was explained. The information of body height, weight, age, fasting blood sugar, renal function index (creatinine [CRE] and blood urea nitrogen [BUN]), etc., was collected and filed for further statistical analysis. The study protocol was approved by the Institutional Review Board of Chung Shan Medical University Hospital.

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was obtained from normal healthy donors, who were receiving no medications nor had any previous history of metabolic disorders, and type 2 diabetic subjects as well. The blood was collected from the antecubital vein in tubes containing EDTA. Samples were immediately processed after blood withdrawal. PBMCs were isolated from buffy coats by layering blood samples onto Ficoll-Paque (Pharmacia Biotech) gradient method in 50 mL conical centrifuge tubes as

described [20,21]. After centrifugation at 400 × g for 30 min at room temperature, the PBMCs were transferred to a new conical centrifuge tube where the cells were washed twice with phosphate buffered saline.

2.3. Analysis of resistin secretion

PBMCs were isolated from whole blood using Ficoll-Paque (Pharmacia Biotech) gradient centrifugation method. After isolation, 2×10^6 PBMCs were cultured in RPMI medium (Hyclone) containing 10% FBS (GIBCO). After 24 h of 10 μg/mL concanavalin A (Con A, Sigma) treatment, secreted resitin levels by the activated PBMCs were determined using ELISA kit (R&D).

2.4. Analysis of RETN promoter genotype

Genomic DNA was extracted from PBMCs using commercially available kit. The 5'-flanking region of the *RETN* gene was amplified by PCR reaction containing 100 ng of genomic DNA, 12.5 pmol of each primer (5'-TGTCATTCTCACCCAGAGACA-3' and 5'-TGGGCTCAGCTAACCAAATC-3'), 1 unit of Taq DNA polymerase and 125 μM dNTP in a total volume of 25 μL, using a PCR thermalcycler. DNA products after PCR amplification were digested using restriction enzyme Bpi *I* to detect the -420C>G polymorphism, followed by separation in 12% polyacrylamide gel.

2.5. Statistical analysis

Data analysis started with descriptive statistics, including mean and standard deviation for continuous variables, and frequency for categorical variables. If necessary, natural logarithm transformation was used to enhance normality for blood biochemistry parameters with skewed distribution. Student's *t* test was applied for comparisons of age, body mass index (BMI), and each of the blood biochemistry parameters between T2DM subjects and controls, and Chi-square test for comparing frequencies of different genotypes and sex between groups. Moreover, one-way analysis of variance was applied to compare means of respective blood biochemistry parameters among subjects with different *RETN* genotypes. Finally, multiple linear regression analysis was used to assess the associations between *RETN* genotypes and each of the biochemistry parameters, with adjustment for diabetes status, age, and sex. An alpha level of 0.05 was used for all statistical tests.

3. Results

Our study aimed at investigating the distribution of the *RETN* -420 SNPs among control and type 2 diabetic subjects to examine the possible correlation between *RETN* genetic polymorphisms and T2DM in Taiwanese population.

3.1. Characteristics of the study subjects (Table 1)

Data regarding biochemical parameters of recruited subjects, including the healthy control

individuals and diabetic patients, were listed in Table 1. Among the biochemical parameters examined, significant differences were observed in fasting blood glucose (AC; 105.2 ± 40.3 v.s 176.4 ± 70.4 mg/dL, $p < 0.001$), BUN (15.0 ± 4.8 v.s 17.2 ± 7.8 mg/dL, $p < 0.001$), systolic pressure (SP, 123.7 ± 17.6 v.s 133.5 ± 18.6 mmHg, $p < 0.001$), high density lipoprotein-cholesterol (HDL-C; 55.5 ± 38.2 v.s 47.8 ± 13.9 mg/dL, $p < 0.001$) and triglyceride (TG; 150.7 ± 129.6 v.s 183.6 ± 129.6 mg/dL, $p = 0.004$) under fasting conditions between patients and controls.

Table 1. Demographic and biochemical data of study subjects in this study.

Item	Normal range	Study subjects		*P*
		Control (n = 244)	T2DM (n = 305)	
Male/female		156/88	159/146	0.003
Age (years)		51.5 ± 13.3	57.6 ± 11.2	<0.001
BMI (kg/m^2)		25.5 ± 5.5	25.4 ± 5.5	0.078
Fasting plasma glucose (AC)	70–110 mg/dl	105.2 ± 40.3	176.4 ± 70.4	<0.001
Blood urea nitrogen (BUN)	7–21 mg/dl	15.0 ± 4.8	17.2 ± 7.8	<0.001
Creatinine	0.6–1.4 mg/dl	1.1 ± 0.3	1.0 ± 0.5	0.066
Systolic pressure (SP)	120–140 mmHg	123.7 ± 17.6	133.5 ± 18.6	<0.001
Diastolic pressure (DP)	70–90 mmHg	78.4 ± 9.6	79.2 ± 11.5	0.521
Cholesterol (CHO)	125–240 mg/dl	193.0 ± 37.5	198.8 ± 46.8	0.055
High density lipoprotein-cholesterol (HDL-C)	>35 mg/dl	55.5 ± 38.2	47.8 ± 13.9	<0.001
Triglyceride (TG)	20–200 mg/dl	150.7 ± 129.6	183.6 ± 129.6	0.004

*Student's t test. †Data are presented as mean ± standard deviation.

3.2. Significant association of RETN -420 genotypes and T2DM (Table 2)

Results regarding the distribution of the *RETN* -420 SNPs in recruited subjects were summarized in Table 2. Among the 244 non-diabetic control individuals, 70 (28.7%), 127 (52.0%) and 47 (19.3%) subjects carried C/C, C/G and G/G genotype, respectively; while the corresponding number in T2DM patients was 124 (40.6%), 139 (45.6%) and 42 (13.8%). Significant difference in distribution of *RETN* -420C>G genotypes between T2DM and control subjects was observed ($p = 0.01$). In addition, the prevalence of *RETN* -420 C and G allele in control individuals was 54.7% and 45.3%, respectively; and that in T2DM counterpart was 63.4% and 36.6%. Significant difference in the distribution of *RETN* alleles between diabetic patients and control subjects was also observed ($p = 0.002$). The above observations demonstrate that the *RETN* -420 SNPs are associated with T2DM subjects in Taiwanese population.

In addition to the significant association between *RETN* -420C>G genotypes with T2DM, we further investigated the correlation between the SNPs and biochemical parameters by stratifying the study subjects according to the criteria of whether their test results were within or beyond the normal range (Table 3). No significant association between the biochemical data and *RETN* -420 SNPs was found.

Table 2. Associations between *RETN* -420 SNPs and clinical parameters of study subjects.

Genotype/allele	Resistin -420C>G SNPs		*P*
	Control (n = 244)	T2DM (n = 305)	
C/C	70 (28.7%)	124 (40.6%)	0.01
C/G	127 (52.0%)	139 (45.6%)	
G/G	47 (19.3%)	42 (13.8%)	
C	267 (54.7%)	387 (63.4%)	0.002
G	221 (45.3%)	223 (36.6%)	

Table 3. Associations between *RETN* -420 SNPs and clinical parameters of study subjects.

Item	Range	Control					T2DM				
		n	C/C n (%)	C/G n (%)	G/G n (%)	*P*	n	C/C n (%)	C/G n (%)	G/G n (%)	*P*
BMI	≥25	97	34 (35.1%)	49 (50.5%)	14 (14.4%)	0.073	74	28 (37.8%)	38 (51.4%)	8 (10.8%)	0.280
	<25	124	29 (23.4%)	65 (52.4%)	30 (24.2%)		54	23 (42.6%)	21 (38.9%)	10 (18.5%)	
SP	≥140	45	9 (20%)	29 (64.4%)	7 (15.6%)	0.162	37	13 (35.1%)	18 (48.7%)	6 (16.2%)	0.794
	<140	175	53 (30.3%)	85 (48.6%)	37 (21.1%)		57	24 (42.1%)	25 (43.9%)	8 (14%)	
DP	≥90	33	8 (24.2%)	18 (54.6%)	7 (21.2%)	0.862	25	10 (40%)	13 (52%)	2 (8%)	0.501
	<90	187	54 (28.9%)	96 (51.3%)	37 (19.8%)		69	27 (39.1%)	30 (43.5%)	12 (17.4%)	
AC	≥110	27	7 (26%)	12 (44.4%)	8 (29.6%)	0.399	186	77 (41.4%)	80 (43%)	29 (15.6%)	0.186
	<110	194	56 (28.8%)	102 (52.6%)	36 (18.6%)		19	4 (21.1%)	10 (52.6%)	5 (26.3%)	
BUN	≥25	2	0	2 (100%)	0	0.388	21	7 (33.3%)	11 (52.4%)	3 (14.3%)	0.634
	<25	219	63 (28.8%)	112 (51.1%)	44 (20.1%)		132	57 (43.2%)	55 (41.7%)	20 (15.1%)	
CRE	≥1.4	17	1 (5.9%)	12 (70.6%)	4 (23.5%)	0.092	27	11 (40.8%)	9 (33.3%)	7 (25.9%)	0.256
	<1.4	203	62 (30.5%)	101 (49.8%)	40 (19.7%)		163	63 (38.7%)	76 (46.6%)	24 (14.7%)	
CHO	≥240	21	4 (19.1%)	14 (66.7%)	3 (14.2%)	0.358	24	13 (54.2%)	6 (25%)	5 (20.8%)	0.103
	<240	199	58 (29.1%)	100 (50.3%)	41 (20.6%)		175	64 (36.6%)	84 (48%)	27 (15.4%)	
HDL-C	>35	196	60 (30.6%)	100 (51%)	36 (18.4%)	0.088	131	52 (39.7%)	58 (44.3%)	21 (16%)	0.390
	≤35	25	3 (12%)	14 (56%)	8 (32%)		37	12 (32.4%)	21 (56.8%)	4 (10.8%)	
TG	≥200	51	14 (27.5%)	24 (47.1%)	13 (25.4%)	0.521	59	26 (44.1%)	24 (40.7%)	9 (15.2%)	0.557
	<200	169	48 (28.4%)	90 (53.3%)	31 (18.3%)		139	50 (36%)	66 (47.5%)	23 (16.5%)	

3.3. RETN -420 genotypes and resistin levels (Table 4)

To further investigate the association of resistin and T2DM, resistin levels produced by ConA-activated PBMCs from 107 control and 58 T2DM study subjects were determined. The results showed that while no significant difference between the resistin levels and *RETN* -420 genotypes was observed, the secretory resisitn levels by Con A-stimulated PBMCs from T2DM were significantly higher than that from control subjects.

Table 4. Associations between *RETN* -420 SNPs and resistin levels among study subjects.

Genotype	Control		T2DM		*P*
	n (107)	Resistin (ng/mL)	n (58)	Resistin (ng/mL)	
C/C	16	2.50 ± 0.44	22	3.95 ± 0.80	
C/G	54	3.94 ± 0.53	24	4.31 ± 0.88	0.044
G/G	37	2.56 ± 0.43	12	4.88 ± 2.31	

Resistin levels secreted from the ConA-activated PBMCs from 107 control and 58 diabetic subjects were analyzed.

4. Discussion

Most of the identified *RETN* SNPs are mapped to the non-coding region, with rs1862513 at position -420 attracts much attention and thus is most widely studied SNPs among different ethnic populations. In addition to the association between *RETN* -420 SNPs with insulin resistance [15], obesity [15,17,19] and T2DM [22,23], the *RETN* -420 G allele is associated with stronger promoter activity [22], higher abdominal fat resistin mRNA levels and plasma resistin concentrations [6,14]. This G allele is also suggested to be an independent predictor for blood glucose deterioration in a Chinese population [24]. Despite the association between *RETN* SNPs with plasma resistin levels and insulin resistance [25], conflicting results are reported. While a meta-analysis indicates that individuals carrying homozygous -420 G allele have a 30% increased odds of developing T2DM [22], another concludes no significant association is observed between *RETN* -420C>G and T2DM risk [26]. Additionally, even varying results are identified in different subjects sampling within the same ethnic origin, possibly due to the difference of patients' clinical profiles or statistical strategies.

Most of the recruited subjects regarding *RETN* genetic studies are populations from Japanese and Caucasian origins. As mentioned above, discrepancies are reported and the associations between *RETN* genotypes and diabetic onset are therefore controversial. To the best of our knowledge, only 1 study regarding the resistin +62G>A genotype in Taiwanese subjects is documented [27]. Therefore, it is tempting for us to investigate the most profoundly studied *RETN* -420C>G SNPs in Taiwanese diabetic patients for examining the putative correlation and involvement of this SNP in our population. Our results indicate that not only the distribution of *RETN* -420C>G genotypes but also G allele is significantly different between diabetic and control subjects. Nevertheless, no significant association between the subjects' biochemical data and *RETN* -420 SNPs is found. Although focusing on different *RETN* SNPs, our data support the report from Tan et al, in which resistin gene polymorphism is an independent factor associated with T2DM [27].

It is intriguing to consider the contribution of *RETN* -420 SNPs from a general prospective by analyzing the distribution of the variants among different ethnic populations. Upon comparing the overall frequency of *RETN* -420C>G variants among populations with different ethnic origins, several interesting phenomena were observed. First of all, the frequencies of *RETN* -420 G allele in Asian population (>30%) are higher than Caucasian subjects (Table 5). The observation supports the finding from Chi et al that the frequency of *RETN* -420 G allele in Chinese population is significantly different from those in European population [28]. Secondly, among the Asian subjects with higher G allele frequency, this SNP is associated with T2DM onset in Japanese and Taiwanese population, but

not in the Caucasian populations (Table 5). Thirdly, the *RETN* genotypes are reported to be associated with circulatory resistin levels in most of the studies from Asian countries (Table 6).

Table 5. Prevalence of RETN -420 SNPs in populations with different ethnic background.

Population/ Subjects	n	SNP			allele		Association	Ref.
		C/C	C/G	G/G	C	G		
Finnish								
Control	409				599 (73.2%)	219 (26.7%)	insulin resistance	[17]
T2DM	781				1141 (73.0%)	423 (27.0%)		
Canadian								
Control	411	212 (51.6%)	169 (41.1%)	30 (7.3%)	593 (72.1%)	229 (27.9%)	BMI	[15]
T2DM	179	90 (50.3%)	78 (43.6%)	11 (6.1%)	258 (72.1%)	100 (27.9%)		
Scandinavian								
Control	433	236 (54.5%)	156 (36.0%)	41 (9.5%)	628 (72.5%)	238 (27.5%)		[15]
T2DM	452	238 (52.7%)	170 (37.6%)	44 (9.7%)	646 (71.5%)	258 (28.5%)		
European-derived Brazilian								
Control	251	125 (49.8%)	101 (40.2%)	25 (10.0%)	351 (69.9%)	151 (30.1%)	BMI waist circumference	[19]
Overweight & obese	334	182 (54.5%)	122 (36.5%)	30 (9.0%)	486 (72.8%)	182 (27.2%)		
Korean								
Control	173	89 (51.5%)	63 (36.4%)	21 (12.1%)	241 (69.7%)	105 (30.3%)	resistin level	[6]
T2DM	411	194 (47.2%)	163 (39.7%)	54 (13.1%)	551 (67.0%)	271 (33.0%)		
Japanese								
Control	564	247 (43.8%)	269 (47.7%)	48 (8.5%)	763 (67.6%)	365 (32.4%)	T2DM	[22]
T2DM	546	216 (39.6%)	254 (46.5%)	76 (13.9%)	686 (62.8%)	406 (37.2%)		
Japanese								
Control	2,502	1,080 (43.2%)	1,123 (44.9%)	299 (11.9%)	3,283 (65.6%)	1,721 (34.4%)	young T2DM	[23]
T2DM	2,610	1,169 (44.9%)	1,144 (43.8%)	297 (11.4%)	3,482 (66.7%)	1,738 (33.3%)		
Taiwanese								
Control	244	70 (28.7%)	127 (52.0%)	47 (19.3%)	267 (54.7%)	221 (45.3%)	T2DM resistin level	this study
T2DM	305	124 (40.6%)	139 (45.6%)	42 (13.8%)	387 (63.4%)	223 (36.6%)		

Numbers in parenthesis of population indicated the number of study subjects in each study.

Several contradictory facts can be raised according to the above observations. First of all, several reports reveal that resistin levels are significantly increased in a genotype-dependent manner based on the *RETN* -420 polymorphisms. Subjects carrying *RETN* -420 G/G genotype had the

highest circulating resistin levels, followed by C/G- and C/C-carrying individuals [29,30,31]. The *RETN* -420 G/G genotype increases T2DM susceptibility by enhancing its promoter activity [32]. On the contrary, no significant difference between the resistin levels and the *RETN* -420 genotypes is found in the present study. Nevertheless, our results show that the resistin levels from T2DM subjects are significantly higher than the control subgroup (Table 4). The reason behinds this discrepancy is perhaps that we examined secretory resistin from ConA-activated PBMCs, since we suggest that the plasma resistin levels would be affected by multiple unidentified factors which are possible to deviate the data and corresponding conclusions. In addition, although it is unlikely that *RETN* is the only gene carrying susceptibility for T2DM development, the other paradox is that the diabetic prevalence of Asian populations with higher frequency of the susceptible G allele is much lower than Caucasian [28]. We previously characterized that certain SNPs identified to be associated with insulin sensitivity in Caucasians are unlikely to be involved in Taiwanese T2DM development [33–36]. Taken together, unique genetic characteristics and distinct factors may play roles in the diabetic pathogenesis among subjects from different racial origins. A given population may have unique protective elements in the genetic reservoir despite the high prevalence of disease-susceptible alleles. Therefore, the possibility of differential protective genetic factors related to a particular ethnicity may lead to the conflicting results among studies.

Table 6. Prevalence of *RETN* genetic variants in Asian populations.

No	Population/Subjects	n	SNP	Association			Ref.
				Serum resistin	T2DM	others	
			Meta-analysis				
1	Control	5,959	-420C>G	–	–		[26]
	T2DM	5,935					
2	Control	529	-638G>A	+	–		[29]
	T2DM	529	-420C>G	–	–		
Chinese							
3	Control	370	-420C>G		–		[28]
	T2DM	318	-394C>G		–		
4	Non-diabetic subjects	624	-420C>G	+		insulin resistance	[24]
			+62G>A			glycemia	
Japanese							
5	Control	286	-420C>G	+		stroke	[30]
	T2DM	349					
6	Community subjects	2,077	-420C>G	+		synergistically with PPAR P12A	[31]
7	Control	2,502	-420C>G		–	young T2DM onset	[23]
	T2DM	2,610					
8	Community subjects	2,078	-420C>G	+		insulin resistance, low HDL high CRP	[37]
9	Obese	60	-638G>A	+			[38]

			-420C>G	+			
10	controls	157	-420C>G	+			[32]
	T2DM	198					
11	Control	406	-420C>G	+	+		[22]
	T2DM	397					
Thais							
12	Control	105	-420C>G		–		[39]
	T2DM	95	+299G>A	+	+		
Korean							
13	Control	173	-537A>C	+			[6]
	T2DM	411	-420C>G	+			

5. Conclusion

This study reports the association between *RETN* -420 polymorphisms and diabetic incidence in Taiwanese population. Our results suggest that *RETN* -420C>G SNPs are associated with diabetic susceptibility, but not subjects' clinical profiles. Investigation of *RETN* SNPs in T2DM patients from various ethnic populations is crucial and will contribute to the understanding of this gene in the diabetic etiology. The present results provide clues to elucidate the contribution of genetic heterogeneity for diabetic development.

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Conflicts of Interest

All authors declare no conflicts of interest in this paper.

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