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Association of CYP2D6 (G1934A) gene and hematological disturbances in chronic pesticide exposures

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ABSTRACT

Cytochrome P450 family genes (CYPs) have common polymorphisms, which are evaluated for pesticide-induced oxidative stress susceptibility. Variant alleles in CYP enzymes may uncover susceptibility biomarkers of environmental toxicity. This study aims to investigate the relationship between CYP2D6 (G1934A) gene polymorphisms and chronic pesticide exposures, and to evaluate the association between CYP2D6 (G1934A) gene polymorphism on biochemical and hematological parameters. A cross-sectional study was carried out at a health promoting hospital, Suphan Buri, Thailand. Rice farmers and a control group ($n = 50$ for each) were recruited and their personal data were collected. Their blood samples were obtained by venepuncture and drawn into plain and Ethylenediaminetetraacetic acid (EDTA) tubes. Serum cholinesterase (SChE), liver function test, kidney function test, and complete blood count were assessed using automatic analyzers; and CYP2D6 (G1934A) genotyping was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). SChE in rice farmers was significantly lower ($p = 0.033$), which may be related to the use of chlorpyrifos. The genotypes were significantly different between rice farmers and the control group ($p = 0.0001$), and the GA variant in rice farmers was more frequent. The CYP2D6 (G1934A) (rs 3892097) gene and SChE level were negatively correlated ($r = -0.258$, $p = 0.009$). However, other biochemical parameters were not different. Blood indices, including mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC), values of rice farmers were significantly lower than the control group; and MCH, MCV, and MCHC values of the GA variant were also significantly lower than the wild type. The GA genotype was associated with hematological toxicity of organophosphate metabolite in chronic exposures.

INTRODUCTION

Pesticides are classified as chemicals which are used to prevent and eliminate weeds or pests. Occupational and environmental exposure can cause adverse health effects and public health risks. Laborers who are exposed to pesticides include farm workers, gardeners, and retailers. People who spray plants with pesticides are frequently exposed at great intensities, which

are potentially harmful to their health (Kim *et al.*, 2017; Suratman *et al.*, 2015). Developing countries use about 20% of the world's pesticides. Thailand is the third ranked user of pesticides in Asia-Pacific (Tawatsin *et al.*, 2015). The reported cases of the toxic effects of pesticides are predominantly from the central region of Thailand. The number of cases usually increases during the growing season of many crops in the rainy season (May–August) each year, and were found mainly in farmers and farm workers (Sapbamrer, 2018). Central Thailand is an intense rice producer when water conditions allow (Grandstaff and Srisupun, 2014). Agricultural workers in Suphan Buri, Central Thailand, have reported that most of the active insecticide substances in commercial products are abamectin, chlorpyrifos, carbofuran, and cypermethrin

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(Prasertung, 2012). Chronic pesticide exposure is associated with various adverse health effects, which include metabolic disorder, neurological toxicity, carcinogenicity, oxidative stress, and endocrine and immune disruption (Androutsopoulos *et al.*, 2015; Corsini *et al.*, 2008; Karami-Mohajeri and Abdollahi, 2011). According to a systematic review, acetylcholinesterase (AChE) inhibitor pesticides can affect biochemical metabolism by reducing AChE activity or by affecting organs directly (Karami-Mohajeri and Abdollahi, 2011). However, serum cholinesterase (SChE) is more commonly tested in the laboratory for the indication of organophosphate (OP) and carbamate intoxication. The decrease in SChE defines the exposure rate, including acute and chronic toxicity on exposure of cholinesterase inhibitors (Giacobini, 2004; Sudjaroen and Suwannahong, 2017). Pesticides also possess hematological toxicity, which is associated with aplastic anemia, agranulocytosis, neutropenia, and thrombocytopenia. Both acute and chronic pesticide exposures are related to hematological abnormalities and thalassemia. OP may interact with the iron binding site of hemoglobin, which leads to anemia (Hundekari *et al.*, 2013; Neghab *et al.*, 2018; Steerenberg *et al.*, 2008; Wafa *et al.*, 2013).

Biological effects of pesticide are involved by oxidative stress, epigenetic controls, and gut microbiological digestion; however, genetic polymorphisms are the main occurrences (Karami-Mohajeri and Abdollahi, 2011; Teodoro *et al.*, 2019). The polymorphism of cytochrome P450 family genes (CYPs) is mainly involved in OP metabolism and is used to evaluate pesticide-induced oxidative stress susceptibility (Kapka-Skrzypczak *et al.*, 2011; Wafa *et al.*, 2013). Variant alleles in CYP enzymes are susceptibility biomarkers of environmental toxicity (Elfaki *et al.*, 2018; Kapka-Skrzypczak *et al.*, 2011). The CYP2D6 (G1934A) (rs 3892097) gene polymorphism leads to a catalytic rate of enzyme activity (Singh *et al.* 2011), and CYP2D6 1934A allele is related to the susceptibility of OP chronic toxicity in Egyptians (Tawfik Khattab *et al.*, 2016). This study aims to investigate the relationship between CYP2D6 (G1934A) gene polymorphisms and chronic pesticide exposures by using PCR-RFLP and SChE, respectively, and to evaluate the association between CYP2D6 (G1934A) gene polymorphism, biochemical parameters, including liver function test (LFT) and kidney function test (KFT), and hematological parameters.

SUBJECTS AND METHODS

Background of research area

The U-Thong District, Suphan Buri Province, is about 150 km west of Bangkok. The main harvested product is rice and its varieties, which include Suphan Buri 1 (SPR1), Suphan Buri 2 (SPR2), and Khao Dawk Mali 105. Rice-growing cycles are 2–3 per year due to a low river plain area. The common pesticides used are chlorpyrifos, carbofuran, paraquat, and glyphosate, respectively. Rice field workers are regularly outsourced pesticide sprayers.

Subject recruitment and data collection

A cross-sectional study was carried out from June 2019 to February 2020, and data were collected annually by the health service program of the health promoting hospital. The study

recruited 100 respondents as follows: 50 rice farmers (risk group) aged 18–65 years, who lived in this area, who handled pesticides regularly or worked in paddy fields for 3 years or more; the control group included 50 respondents who lived near the field area and were listed in house registration and had professions not related to farm work. Respondents with a history of serious conditions, such as liver diseases, severe cardiovascular diseases, and cancer, were excluded. Questionnaire interviewing and blood collection were conducted by well-trained research assistants and medical technicians, respectively. The gathered information concerning long-term pesticide exposures, including personal information, adverse health symptoms, personal protective equipment used, and practical knowledge and use of pesticides were recorded by the questionnaires by personal interviewing. The sample size was estimated using the single proportion formula with 95% confidence interval based on the percentage of abnormal SChE levels in a previous study (Kachaiyaphum *et al.*, 2010). The Ethics Committee of Thammasat University approved this research protocol (COA No. 085/2562). The director of U-Thong district's health promoting hospital, Suphan Buri Province, gave permission to conduct on this study. All participants gave informed consented.

Blood collection, preparation, and storage

From each participant, 4.5 ml of blood sample was obtained by venepuncture of the median cubital vein in the morning (7–9 a.m.) and was drawn into plain and EDTA tubes of 3 and 1.5 ml, respectively. The plain tubes were further centrifuged; and the serum was separated within 2 hours after phlebotomy (Young and Bermes, 1999) for biochemical tests. Whole blood contained in the EDTA tubes were prepared for HbA1c determination and genomic DNA extraction by using the QIAamp blood DNA minikit (QIAGEN Thailand, Bangkok, Thailand). The genomic DNA was stored at -20°C .

Evaluation of biochemical and hematological parameters

The quantitative analysis of SChE, LFT, and KFT was conducted by an automatic analyzer, COBAS c501 (Roche-diagnostics, Rotkreuz, Switzerland), which was carried out in certified clinical laboratories. Hematological parameters (complete blood count) were analyzed by Celltac E MEK-7222 (Nihon Kohden, Tomioka, Japan). The interpretation of biochemical and hematological parameters was carried out by comparing the reference value according to the manufacturer's instructions and the Clinical and Laboratory Standards of the Institute.

CYP2D6 (G1934A) polymorphisms by PCR-RFLP

The DNA template was amplified by polymerase chain reaction (PCR) using forward 5'-GCT TCG CCA ACC ACT CCG-3' and reverse 5'-AAA TCC TGC TCT TCC GAG GC-3' primers, which corresponded to CYP2D6 (G1934A) region (Zayed *et al.*, 2015). PCR was carried out in 25 μl of total volume, which consisted of 1 μl of DNA template, 0.5 μl of each primer (20 pmol/ μl), 5 μl of 10 \times PCR buffer (1.5 mM Mg^{2+}), including dNTP mixture, and 0.2 μl of 1 U AmpliTaq polymerase (Thermo Fisher Scientific, Waltham, MA). PCR was carried out with initial denaturation at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute, and extension at 72°C for 1 minute, followed by

final extension at 72°C for 7 minutes by using a thermal cycler (Applied Biosystems, Waltham, MA). Restriction fragment length polymorphism was carried out and the DNA product was digested by *Bst*NI Restriction Enzyme (New England Bio Labs, Cambridge, UK). The digested DNA fragments were separated in 2% of agarose gel electrophoresis apparatus and then stained with ethidium bromide. DNA electropherogram was read by using an ultraviolet transilluminator (Promega, San Luis Obispo, CA). The CYP2D6 (G1934A) single nucleotide polymorphisms were 104 and 230 bp fragments for the 1934G allele and 334 bp fragment (undigested) for the 1934A allele (Tawfik Khattab *et al.*, 2016). The interpretation of CYP2D6 (G1934A) genotypes was represented by 104 and 230 bp fragments for wild type (GG); 104, 230, and 334 bp fragments for heterozygous (GA); and 334 bp fragment for homozygous (AA). Quality test control was carried out by DNA sequencing, which was randomized from 15% of the samples.

Statistical analysis

Data distribution was analyzed by the Kolmogorov–Smirnov test. Descriptive statistic was represented by frequency and mean. Differentiation of personal information and CYP2D6 (G1934A) genotypes between rice farmer and controls groups were analyzed by the chi-squared test. Correlation of SChE and polymorphisms was analyzed by Pearson’s correlation. The difference between other biochemical and hematological parameters between two groups and between wild type and variant were analyzed by independent *t*-test. Statistical analysis was carried out by SPSS 21.0 program (SPSS, Chicago, IL) and statistical significance was judged at $p < 0.05$.

RESULTS AND DISCUSSION

Pesticide exposure status in rice farmers

The rice farmers were mainly men and were older than 40 years. Gender and risk behaviors, such as alcohol consumption, did not show a statistically significant difference between rice farmers and the control group. All the rice farmers had a long-term pesticide exposure and 60% of them used pesticides for more than 10 years; the rate of pesticide exposure was mainly once or twice per week. The related symptoms of the use of pesticides rarely occurred and almost all of them had knowledge about the awareness of pesticide uses and self-protection. The means of SChE level were significantly different between rice farmers and the control group ($p = 0.033$); however, they were within the reference value. In this study, rice farmers were exposed to pesticides for a long time and used pesticides frequently. However, the occurrence of related symptoms was rare, which may be due to nonspecific symptoms that are difficult to define. Unexpected findings may be due to nonspecific symptoms, imprecise explanations in personal interviews, and tolerance of frequently exposed farmers. The majority of rice farmers had been educated about the health awareness and health protection on pesticide use (Table 1).

SChE enzyme has more variation than AChE. However, it was easier to handle during laboratory tests, especially in field work (Nigg and Knaak, 2000). The differences in SChE levels might be due to the different methods used to measure cholinesterase, such as Elman’s technique and Michel’s change-

Table 1. Personal data and pesticide exposure status of rice farmers.

Personal data/exposing factors	Rice farmer (%)	Control (%)	<i>p</i> -value
Gender : Male	34 (68)	30 (60)	0.405
Female	16 (32)	20 (40)	
Age: < 40 years	22 (44)	40 (80)	0.0001*
≥ 40 years	28 (56)	10 (20)	
Alcohol intake: none	36 (72)	33 (66)	0.517
Drinking	14 (28)	17 (34)	
Duration of pesticide use: 4–9 years	18 (36)	–	–
> 10 years	32 (64)	–	
Frequency of exposure: 1–2 days/weeks	30 (60)	–	–
3–4 days/weeks	4 (8)	–	
5–6 days/weeks	16 (32)	–	
Clinical symptoms: None	36 (72)	–	–
Headache/vertigo	11 (22)	–	
Abdominal cramp	3 (6)	–	
Health education: none	13 (26)	–	–
Educated	37 (74)	–	
Serum cholinesterase (U/l) ^a	7,247.2 ± 1293.3	7,775.9 ± 1152.8	0.033*

^aSChE was screened by paper test before being tested with the automatic analyzer and is represented as mean ± SD (reference value = 5,500–13,000 U/l).

*Statistically significant at $p < 0.05$.

in-pH method (Nigg and Knaak, 2000). Moreover, a variety of laboratory instruments may affect the SChE levels, such as automatic-based or manual-based techniques. Safety behavior and variations in pesticide spraying also affected the SChE level (Shentema *et al.*, 2020). Hence, some studies have reported insignificant or slightly lower SChE levels in chronic exposures (Mathew *et al.*, 2015; Sudjaroen and Suwannahong, 2017). Most of the studies on Thai pesticide exposure had reported exposure of a single type of pesticide; however, a mixture of pesticides is more commonly applied for multicrop cultivation. Thus, the screening of other biomarkers can evaluate chronic pesticide exposure rather than SChE alone, such as pesticide metabolites from blood or urine (Wongta *et al.*, 2018).

CYP2D6 (G1934A) genotype, biochemical, and hematological parameters

The electrophoresis of DNA fragments of the CYP2D6 (G1934A) gene polymorphism in rice farmers included wild type (GG), heterozygote (GA), and homozygote (AA) (Fig. 1). The genotypic polymorphisms of rice farmers and the control group were significantly different ($p = 0.0001$). Rice farmers had a more frequent GA genotype rather than the control group (Table 2) and AA genotype was not detected in either group. The CYP2D6 (G1934A) gene and SChE level were negatively correlated ($r = -0.258$) and were statistically significant ($p = 0.009$) (Table 3). The biochemical parameters were not significantly different between rice farmers and the control group; and they were within the reference values (Table 4). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) values of rice farmers were significantly lower than the control group at $p = 0.04$, 0.045, and 0.03, respectively; and MCHC in rice farmers was lower than the



Figure 1. DNA fragment separation. First lane (from left): DNA ladder; lanes 2, 6, 9, 11, 12, 17, 23, 50, and 59: 104 and 230 bp for GA genotype (variant); and lanes 15, 16, 30, 32, and 42: 334 bp for GG genotype (wild type).

Table 2 Genotypic frequency of CYP2D6 (G1934A) in rice farmers and control group

Genotype	Control		Farmer		p-value
	n	%	n	%	
Wild type (GG genotype)	44	88	22	44	0.0001*
Polymorphism (GA genotype) ^a	6	12	28	56	

^aHomozygous polymorphism (AA genotype) was not detected.

*Statistically significant at $p < 0.05$.

reference value (Table 5). Moreover, MCH, MCV, and MCHC values of GA variant were significantly lower than the wild type at $p = 0.029, 0.017, \text{ and } 0.041$, respectively (Table 6).

CYP2D6 is an important member of cytochrome P450 based on various xenobiotic substrates, which shows a very high degree of variability due to the extensive genetic polymorphism that influences gene expression and enzymatic function (Zanger *et al.*, 2004). CYP2D6 gene is involved in the secondary metabolic pathway of desulphuration of isocarbophos (ICP). ICP enantiomers and its oxidative desulphuration metabolite, isocarbophos oxon have been reported to be inhibitors of AChE (Kaur *et al.*, 2017; Zhuang *et al.*, 2014). The CYP2D6 inhibitors resulted in 50% inhibition of cholinesterase activity for parathion, 38% diazinon and 30% chlorpyrifos as compared to control and SChE is significantly reduced in chronic OP exposures (Sams *et al.*, 2000; Tawfik Khattab *et al.*, 2016). The variants of CYP2D6 alleles at third intron and four exons resulted in missing of transcription and single based deletion of mRNA (Hanioka *et al.*, 1990). Thus, this finding corresponded to previous study in the reduction of SChE activity and the GA genotype was more susceptible to pesticide than wild type in the Thai population,

which was rarely reported. In our study, biochemical parameters in rice farmers were not significantly different; however, lower levels of total protein and albumin in Thai orchid farmers had been report (Aroonvilairat *et al.*, 2015). Other biochemical parameters were also disturbed by chronic pesticide exposure, such as blood glucose and lipid profiles (Patil *et al.*, 2009; Pothu *et al.*, 2019; Ramirez-Vargas *et al.*, 2018). Subclinical change in hematological parameter attracts little attention on pesticide exposure rather than cancer, neurological disorders, epigenetic interaction, oxidative stress, and gene polymorphism (Karami-Mohajeri and Abdollahi, 2011; Neghab *et al.*, 2018; Teodoro *et al.*, 2019). However, hematotoxicity is a biomarker and can indicate occupational exposure along with genotoxicity and oxidative stress occurrence (Ahmadi *et al.*, 2018). In previous studies, abnormal blood indices were found, and the impairment of liver and kidney functions were apparent in pesticide sprayers and agricultural workers (Cortés-Iza *et al.*, 2017; Patil *et al.*, 2009; Sudjaroen and Suwannahong, 2017). Life-long and high-season pesticide use are also associated with lower number of RBC and WBC, particularly lymphocyte and eosinophil (Piccoli *et al.*, 2019). Lower count of B lymphocyte with increments of IgE level in chronic pesticide exposures was also reported and had implication on immunological disturbance (Aroonvilairat *et al.*, 2015).

CYP2D6 has only a small amount of CYPs (< 2%), but it metabolizes approximately 25% of clinically used drugs with significant polymorphisms; it also metabolizes procarcinogens and neurotoxins (Wang *et al.*, 2009). Hence, the variant of CYP2D6 gene was associated with harmful effects of OP metabolite, such as hematological toxicity. Further studies will be conducted on other variants of the CYP2D6 gene, which is associated with the biological effects of other xenobiotics.

Table 3. Liver and KFT between rice farmers and the control group.

Group/parameter	LFT					KFT			
	Total protein (g/dl)	Albumin (g/dl)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	AST (U/l)	ALT (U/l)	ALP (U/l)	BUN (mg/dl)	Creatinine (mg/dl)
Rice farmer	7.29 ± 0.22	4.50 ± 0.04	0.36 ± 0.02	0.13 ± 0.03	23.0 ± 1.2	21.7 ± 2.0	99.9 ± 3.5	10.8 ± 4.6	0.85 ± 0.1
Control	7.45 ± 0.16	4.43 ± 0.04	0.36 ± 0.04	0.11 ± 0.01	23.6 ± 1.3	24.6 ± 1.9	101.2 ± 4.0	9.3 ± 3.5	0.71 ± 0.1
Reference range	6.60–8.70	3.50–5.50	0.30–1.20	0.00–0.50	0–37.0	0–40.0	53–128	5.0–23.0	0.5–1.52
<i>p</i> -value	0.552	0.282	0.964	0.485	0.744	0.308	0.797	0.561	0.152

AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; BUN = blood urea nitrogen.

Table 4. Hematological parameters in rice farmers and the control group^a.

Group/parameter	RBC(10 ⁶ /mm ³)	Hct(%)	Hb(g/dl)	MCV(fl)	MCH (pg)	MCHC(g/dl)	RDW (%)	WBC(10 ³ /mm ³)	Plt (10 ³ /mm ³)
Rice farmer	5.16 ± 0.1	41.8 ± 0.8	13.5 ± 0.3	83.4 ± 1.3	26.0 ± 0.7	30.3 ± 0.2	15.3 ± 0.6	5,895 ± 265	264,421 ± 9,627
Control	5.13 ± 0.1	41.8 ± 1.2	14.5 ± 0.6	88.4 ± 1.6	27.8 ± 0.6	31.7 ± 0.7	15.0 ± 0.5	5,699 ± 241	247,802 ± 10,424
Reference range	4.0–5.5	M = 35–49 F = 32–42	M = 13–18 F = 12–16	80–100	23–33	31–37	12–16	4,000–10,000	140,000–400,000
<i>p</i> -value	0.84	0.984	0.154	0.045	0.04	0.03	0.757	0.587	0.244

WBC = white blood cell; RBC = red blood cell count; Hct = hematocrit; Hb = hemoglobin; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = RBC distribution width; Plt = platelet count.

^aRBC morphology and WBC differentiation with microscopic examination are not shown.

Table 5. Hematological parameters between GG genotype (wild type) and GA genotype (variant)^a.

Genotype/parameter	RBC(10 ⁶ /mm ³)	Hct(%)	Hb(g/dl)	MCV(fl)	MCH (pg)	MCHC(g/dl)	RDW (%)	WBC(10 ³ /mm ³)	Plt (10 ³ /mm ³)
GG genotype	5.2 ± 0.09	42.2 ± 0.7	14.9 ± 0.8	89.4 ± 1.6	28.2 ± 0.5	31.2 ± 0.1	15.4 ± 0.3	5,900 ± 210	246,756 ± 10,622
GA genotype	5.0 ± 0.03	41.0 ± 1.1	13.5 ± 0.3	84.8 ± 1.4	26.2 ± 0.6	29.1 ± 0.5	14.8 ± 0.5	5,598 ± 330	261,133 ± 9,280
Reference range	4.0–5.5	M = 35–49 F = 32–42	M = 13–18 F = 12–16	80–100	23–33	31–37	12–16	4,000–10,000	140,000–400,000
<i>p</i> -value	0.31	0.529	0.131	0.029	0.017	0.041	0.405	0.443	0.244

WBC = white blood cell; RBC = red blood cell count; Hct = hematocrit; Hb = hemoglobin; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = RBC distribution width; Plt = platelet count.

^aRBC morphology and WBC differentiation with microscopic examination are not shown.

Table 6. Hematological parameters between GG genotype (wild type) and GA genotype (variant)^a.

Genotype/parameter	RBC(10 ⁶ /mm ³)	Hct(%)	Hb(g/dl)	MCV(fl)	MCH (pg)	MCHC(g/dl)	RDW (%)	WBC(10 ³ /mm ³)	Plt (10 ³ /mm ³)
GG genotype	5.2 ± 0.09	42.2 ± 0.7	14.9 ± 0.8	89.4 ± 1.6	28.2 ± 0.5	31.2 ± 0.1	15.4 ± 0.3	5,900 ± 210	246,756 ± 10,622
GA genotype	5.0 ± 0.03	41.0 ± 1.1	13.5 ± 0.3	84.8 ± 1.4	26.2 ± 0.6	29.1 ± 0.5	14.8 ± 0.5	5,598 ± 330	261,133 ± 9,280
Reference range	4.0–5.5	M = 35–49 F = 32–42	M = 13–18 F = 12–16	80–100	23–33	31–37	12–16	4,000–10,000	140,000–400,000
<i>p</i> -value	0.31	0.529	0.131	0.029	0.017	0.041	0.405	0.443	0.244

WBC = white blood cell; RBC = red blood cell count; Hct = hematocrit; Hb = hemoglobin; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = RBC distribution width; Plt = platelet count.

^aRBC morphology and WBC differentiation with microscopic examination are not shown.

CONCLUSION

The GA genotype of CYP2D6 (G1934A) gene in chronic pesticide exposures is associated with the alteration of blood indices, including MCV, MCH, and MCHC.

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CONFLICT OF INTEREST

There are no conflicts of interest related to this research study.

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