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Identification of *CYP2D6* allelic mutations in a sub-set of Karachi population

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CYP2D6 gene polymorphism is considered a main obstacle in the process of drug metabolism under clinical diseases that affect pharmacokinetics of ~25% of antidepressants and other drugs. Inter-individual variation occurs in the amount and functional activity of CYP2D6 enzyme produce undesirable side effects. The primary aim of current research is to evaluate gene and genotypic frequencies of CYP2D6 *1 extensive metabolizer, *4 poor metabolizer and *10 intermediate metabolizer allelic variants among depressed patients and compared with normal subjects and other populations. Human genomic DNA was isolated. Genotyping was performed by Polymerase chain reaction followed by restriction endonucleases digestion for variants analysis. The results indicated gene frequency of CYP2D6*1 was 59% (CI 49.6,68.3%) in normal subjects whereas, CYP2D6*4 was 13% (CI 3.7, 22.3%) and CYP2D6*10 was 54% (44.7, 63.3%) predominantly found in depressed patients. The results demonstrate pronounced association of CYP2D6 *4 and *10 allelic variants with patient's drug response activity.

Key words: *CYP2D6* alleles; Polymorphism; Depression; Drug metabolism; Karachi population.

Abbreviations

Extensive metabolizer, EM; poor metabolizer, PM; intermediate metabolizer, Polymerase chain reaction, PCR ; IM; intermediate metabolizer; *Bacillus stearothermophilus*, Bst N1; *Hemophilus parahemolyticus*, Hph1; World health organization, WHO; Hamilton Depression Rating scale, HAM-D Key; Clinical Pharmacogenetics Implementation Consortium, (CPIC).

Introduction

Depressive disorder is a worldwide problem which affects 264 million people and ranked in fourth position among the global burden of diseases. In 2010 study, depressive disorders was reported the second leading cause of mental disability in Canada, the United States and globally [1, 2]. According to world health organization (WHO) about 340 million people above the age of 18 suffer from depressive disorders and 800, 000 people die due to suicide every year. From 2013 to 2016, 10.4% of women were found to have depression as compared to men 5.5% (www.who.int/mental_health). Pakistan is the sixth most populous country in the world comprising 176.7 million people with mixed ethnic groups in that around 20 million people suffer from different forms of mental health condition [3].

The prevalence of depression in Pakistani females were 41.9% as compared to men 29.3% caused by genetic and environmental factors i.e. social distress, poor status, disappointment in relationships [4]. Antidepressant drug therapy decreases the severity of diseases in that fluoxetine and paroxetine are commonly used in Pakistan, China, UAE, UK and USA with recommended dosage 20mg/d whereas 30mg/d paroxetine used in UK, but unfortunately improper drug metabolism process causes serious complications [5].

Inter-individual variability in drug response is a major problem in clinical practice that has been identified by ineffective drug responses. About 80 % of commercialized drugs are metabolized by polymorphic enzymes family i.e. CYP450 enzymes, which are responsible for phase I metabolism and elimination of numerous endogenous substrates and diverse array of clinical used drugs [6]. The CYP2D6 enzyme is a main member of CYP450 enzyme family, encoded by debrisoquine-4-hydroxylase enzyme which is located on chromosome 22 (22q13.1) possess 9 exons with an open reading frame of 1491 base pairs coding for 497 amino acids and 8 introns [7] as shown in figure 1. It is responsible for the biotransformation of psychotropic medications such as antidepressants venlafaxine, fluoxetine, paroxetine and nortriptyline and tricyclic antidepressants [8, 9].

Polymorphism in *CYP2D6* gene comprises 120 allelic variants of which, *CYP2D6**2, *CYP2D6**3, *CYP2D6**4, *CYP2D6**5, *CYP2D6**10, *CYP2D6**17 and *CYP2D6**41 are most apparent [10]. These allelic variants produce drug metabolizing enzyme with varying degree of catalytic activity i.e. normal, reduced, increased and abolished among individuals in different populations leading to inter-individuals variations and such genotypic variability categorized into four phenotypes viz EM carry both functional alleles, IM carry one functional and one mutant allele, PM carry both defective alleles and UM carry multiple functional copies of functional allele produce increased amount of enzyme to be expressed which enhanced biotransformation and elimination rates of drug [11,12].

Around the world, *CYP2D6* polymorphic alleles in different population associated with variable drug metabolizing activity such as *3, *4, *5, *6, *7, *8, *11, *12, *13, *14, *15, *16, *18, *19, *20, *21, *38, *40, *42, *44, *56 and *62 have no enzyme activity [11]. *CYP2D6**4 allele is predominantly appear in Caucasian. However *10, *17, *29, *36 and *41 give rise to substrate-dependent low enzymatic activity due to unstable enzyme product [13]. *CYP2D6**10 and *CYP2D6**36 are frequently found in Asia and East Asia and exhibits reduced substrate affinity. *CYP2D6**17 and *29 alleles associated with African and Afrodescendant populations. *CYP2D6**41 is found in Middle Eastern populations, [14,15]. Besides that, UMs are more

prevalent in the Southern European countries (Spain 7–10 %, Sicily 10 % vs Sweden 1–2 %) [16].

In Pakistan, frequency of *CYP2D6**4 and *CYP2D6**10 was determined by Nazir et al in a group of patients (pre-diagnosed with breast cancer) with severity of Tamoxifen induced hot flushes problem [17]. The study provoked us to examine aforementioned allelic variants in depressed patients who develop undesirable response because lack of the *CYP2D6* metabolizing pathway after taking antidepressants at standard doses [18].

Currently, there is no data published information about *CYP2D6* genetic polymorphisms among depressed patients in Karachi population. The objective of our study was to investigate the frequency of *CYP2D6* allelic mutations including *1, *4 and *10 in Karachi. Of Note, present research approach will generate awareness among clinicians to minimize inadequate antidepressants responses.

Materials and Methods

Biological sample collection:

Firstly, the study was approved by International Center for Chemical and Biological Sciences, Ethic Committee (No. 012-BS-2007), University of Karachi to conduct experiments on human subjects. Blood samples were collected from International Center for Chemical and Biological Sciences, University of Karachi, Pakistan. Patient's samples were collected from different hospitals (Civil Psychiatric Hospital and Asghar Psychiatric Hospital) at Karachi. Written consent was taken from participants.

Materials for PCR-Analysis:

DNA extraction reagents, PCR master kit (Fermentas), primers (*CYP2D6**1F 5'ACAGACCCCTGACCAGTGAC3', *CYP2D6**1R 5'TCCAAGGTTGACTCCTGTCC3') (*CYP2D6**4F5'TGCCGCCTTCGCCAACCCT3', *CYP2D6**4R 5'TCGCCCTGCAGAGACTCCTC3') and (*CYP2D6**10F5'GTGCTGAGAGTGTCCCTGCC3', *CYP2D6**10R5'CACCCACCATCCATGTT TGC 3') (Fermentas). For their restriction analysis the restriction digestion kit (Fermentas) was

used, restriction enzymes BstN1 for *CYP2D6*1*, *CYP2D6*4* and Hph1 for *CYP2D6*1* and *CYP2D6*10* alleles.

Human genomic DNA was extracted from whole blood 5mL by using phenol-chloroform method [19]. PCR amplification was done by using PCR kit. The PCR master mix contained PCR buffer (10x, 5 µl), dNTPs (0.2 mM, 2 µl), Taq polymerase (1U, 2 µl), H₂O (30.5 µl), genomic DNA (250 ng / 5 µl), primers (*CYP2D6*1F* 5'ACAGACCCCTGACCAGTGAC 3', *CYP2D6*1R* 5'TCCAAGGTTGACTCCTGTCC3'), (*CYP2D6*4F* 5'TGCCGCCTTCGCCAACCCT3', *CYP2D6*4R* 5'TCGCCCTGCAGAGACTCCTC3') and (*CYP2D6*10F* 5'GTGCTGAGAGTGTCTGCC3', *CYP2D6*10R* 5'CACCCACCATCCATGTT TGC 3') at fixed concentration (0.35 µM, 1.75 µl). Appropriate concentrations of MgCl₂ for *1 (1.0 mM), *4 (1.5 mM, 3.75 µl) and *10 (3 mM, 7.5 µl) were added. PCR conditions were optimized and reactions was set (30 cycles for 2.5 h duration) undergo the steps of DNA denaturation (94°C for 1 min), primer annealing (62°C for 1 min) DNA extension (72°C for 1 min) and finally, terminal DNA extension (72°C for 10 min). The amplimers of *CYP2D6*1*, *CYP2D6*4* and *CYP2D6*10* were electrophoresed on agarose gel (1%).

Restriction digestion kit (Fermentas) was used to obtain digested DNA bands. The reaction mixture was prepared which contain PCR products (10 µl), nuclease-free water (18 µl), reaction buffer (10x, 2 µl), restriction endonuclease enzymes BstN1 for *CYP2D6*1*, *CYP2D6*4* and Hph1 for *CYP2D6*1*, and *CYP2D6*10* alleles then mixed gently and kept for 5 hours at 37 °C incubation. The digested products were electrophoresed on agarose gel (1%) then visualized under UV gel documentation system (AlphaInnotech-AlphaImager-3400-Transilluminator Gel Imaging Detection System).

Statistical analysis

DNA concentration from normal and depressed patients were analyzed by one way ANOVA with *p value* < 0.05 or less was considered as significant. Hardy-Weinberg equation was applied for determining gene frequencies (*1, *4 and *10) and genotypic frequencies (*1/*1, *1/*4, *1/*10, *4/*10, *4/*4 and *10/*10) where, p (*1) is dominant allele, q (*4) and r (*10) are recessive alleles. The observed and expected *CYP2D6* genotypic frequencies were analyzed by using non-parametric *Chi-square* test.

Results

Demographic study of participants:

We employed survey of psychiatric hospital to enroll depressed patients of both gender (n = 45) mean ages $\pm$ SD (36.6 ± 1.26). Normal subjects of both gender (n = 60) mean ages $\pm$ SD (28.7 ± 1.4) were selected. Patients were diagnosed according to the Hamilton Depression Rating scale (HAM-D Key). They were recommended Fluxit, nauxam, zauxit, paroxetine, taxopar with varying dosages (5-20mg). Females were more affected than males and found to have a history of multiple relapses of depression in medical record file.

Genotyping Results

Depressed patients and normal subjects were genotyped by PCR and the obtained gene and genotypic frequencies of *CYP2D6**1, *4 and *10 alleles were calculated and results showed that mutation is a factor that disrupt the equilibrium of allele frequencies in a population by expressing variants of wild allele. The restriction analysis by BstN1 and Hph1 enzyme produced digested bands of *CYP2D6* polymorphic alleles. Percentages of gene frequency were compared between depressed patients and normal subjects.

The Figure- 2a and b show the digestion pattern of *CYP2D6**1 and *CYP2D6**4 alleles in normal and depressed patients. The BstN1 enzyme digested PCR products yielding two fragments (201bp and 108bp) indicate that individuals are homozygous for wild type allele (*1/*1) whereas, three fragments (309bp, 201bp and 108bp) indicates heterozygous individuals (*1/*4) The individuals homozygous (*4/*4) for mutant allele showed a single undigested band of 309bp due to the absence of BstN1 site on both chromosomes.

Figure- 2 Identification of *CYP2D6*1* and **4* alleles in (a) normal (b) depressed patients.

Lane 1: DNA ladder (100bp-1000bp).

(a) Lanes 2 and 4-18: 201bp and 108bp bands (homozygous wild type *CYP2D6*1/*1* alleles), lane 3: 309bp, 201bp and 108bp (heterozygous mutant *CYP2D6*1/*4* alleles).

(b) Lanes 2, 3, 7-15: 201bp and 108bp (homozygous wild type *CYP2D6*1/*1* alleles), lanes 4-6: 309bp (homozygous mutant *CYP2D6*4/*4* alleles).

The Figure- 3a and b show the digestion pattern of *CYP2D6**1 and *CYP2D6**10 alleles in normal and depressed subjects. The Hph1 enzyme digested PCR products were yielding three fragments 183bp, 80bp and 62bp indicated that individuals are homozygous for wild type (*1/*1) allele whereas, four fragments (300bp, 183bp, 80bp and 62bp) indicates heterozygous individuals. The individuals homozygous for (*10/*10) mutant alleles producing two bands 300bp and 62bp due to the presence of one Hph1 site on both chromosomes.

Figure- 3 Identification of *CYP2D61 and *10 alleles in (a) normal (b) depressed patients**

Lane 1: DNA ladder (100bp-1000bp).

(a) Lanes 2, 4-14, 16-18: 300bp and 62bp (homozygous CYP2D6*10/*10 mutant alleles) whereas, lanes 3, 15 and 19: 300bp, 183bp and 80bp and 62bp (heterozygous mutant CYP2D6*1/*10 alleles).

(b) Lanes 2-6, 12-14: 300bp, 183bp and 80bp and 62bp (heterozygous mutant CYP2D6*1/*10 alleles) whereas, lanes 7-11: 300bp and 62bp (homozygous CYP2D6*10/*10 mutant alleles).

CYP2D6 Allelic frequency in Karachi population

The percent frequencies of *CYP2D6**1, *4 and *10 alleles in normal and depressed patients were determined using Hardy-Weinberg equilibrium equation for three alleles. It has been noticed *1 allele was predominant wild allele in normal subjects 59% (49.6, 68.3%) whereas, *4 allele was highly frequent in depressed 13% (CI 3.7, 22.3%) and nearly zero in normal subjects 0.01% (CI 0, 0.1%). However, *10 allele was predominant mutant allele in depressed 54% (CI 44.7, 63.3%) followed by normal subjects 40% (CI 30.6, 49.3%) in Karachi population. Thus frequency of wild allele is higher and mutant allele is lower in normal subjects than depressed as presented in Table- 1 and graphically shown in figure- 4.

Table- 1 CYP2D6 allelic frequency in Karachi Population

Alleles	Frequency in Normal %	95% CI	Frequency in Depressed %	95% CI
<i>CYP2D6</i>*1	59	49.6, 68.3	32	22.63, 41.3
<i>CYP2D6</i>*4	0.01	0, 0.1	13	3.7, 22.3
<i>CYP2D6</i>*10	40	30.6, 49.3	54	44.7, 63.3

The table represents percent *CYP2D6* allelic frequencies in normal and depressed patients
CI represents confidence interval.

Figure- 4 Comparison of *CYP2D6* alleles (*1, *4 and *10) between normal and depressed patients in Karachi population

CYP2D6 genotypic frequencies in Karachi Population

*1/*1 genotype was predominantly found in normal 53% (CI 46.5, 59.4%) as compared to depressed 16% (CI 11.6, 19.4%) subjects. *1/*4 was appeared 9% (CI 5.1, 12.9%) in depressed. *1/*10 was predominant in depressed 24% (CI 20.1, 27.9%) than normal 10% (CI 3.57, 16.43%). 10/*10 was slightly higher in depressed 42% (CI 38.1, 45.9%) than normal 35% (CI 28.5, 41.4%). None of the normal individual was found with *4/*4 genotype although it was (9% (CI 5.1, 12.9%) in depressed subjects whereas, *4/*10 genotype was absent in all the subjects. Normal individuals with homozygous wild type genotype are comparatively higher

than depressed on the contrary depressed patients with either homozygous or heterozygous mutant genotypes for *4 allele are higher than normal which is shown in Table- 2.

Table- 2 CYP2D6 genotypic frequencies in Karachi Population

Theoretical CYP2D6 Phenotypes	CYP2D6 Genotypes	Normal (n)	Frequency in normal %	95% CI	Depressed (n)	Frequency in Depressed %	95% CI
Extensive metabolizer	*1/*1	32	53	46.5, 59.43	7	15.5	11.6, 19.4
	*1/*4	1	1	8, 4.8	4	9	5.1, 12.9
	*1/*10	6	10	3.57, 16.43	11	24	20.1, 27.9
Intermediate metabolizer	*10/*10	21	35	28.5, 41.4	19	42	38.1, 45.9
	*4/*10	0	0	0	0	0	0
Poor metabolizer	*4/*4	0	0	0	4	9	5.1, 12.9

The table represents genotypic frequencies of CYP2D6 six genotypes in normal and depressed patients as determined by Hardy-Weinberg equilibrium equation. CI represents confidence interval.

Allelic frequency in depressed patients of Karachi, UAE and Japanese Population

The results were also compared with UAE and Japanese population. *CYP2D6**1 allele has been noticed (32%) in Karachi (39.1%) in UAE and (39.4%) Japanese , *CYP2D6**4 allele is (13%) in Karachi (9%) in UAE and (0%) in Japanese indicating similar prevalence in Karachi and UAE

but no appeared in Japanese patients whereas, *10 is (54%) in Karachi, (56.7%) in Japanese but low frequency (3.3%) in UAE as shown in Figure- 5.

Figure- 5 Comparison of *CYP2D6* alleles (*1, *4 and *10) between depressed patients of Karachi with UAE and Japanese population

CYP2D6 alleles: Wild (*1) and variants (*4 and *10)

Populations: Karachi and UAE

Observed and expected gene frequencies of aforementioned alleles were analyzed by *Chi square* test for comparing the differences between normal subjects and depressed patients in Karachi population as shown in Table 3.

Table- 3 Statistical Analysis of CYP2D6 genotypes among normal and depressed patients in Karachi Population

Genotypes	CYP2D6 Genotypes	Normal Subjects			Depressed Patients		
		Observed Individuals	Expected Individuals	Expected Genotypic Frequency	Observed Individuals	Expected Individuals	Expected Genotypic Frequency
p^2	*1/*1,	32	10	0.168	7	0.162	0.0036
$2pq$	*1/*4,	1	0	0	4	1.62	0.036
q^2	*4/*4	0	0	0	4	4	0.09
$2pr$	*1/*10	6	29	0.483	11	3.1	0.07
$2qr$	*4/*10,	0	0	0	0	17	0.384
r^2	*10/*10	21	20.8	0.3481	19	18.4	0.41

p^2 = dominant homozygous frequency

$2pq$ = heterozygous frequency

q^2 = recessive homozygous frequency

$2pr$ = heterozygous frequency

$2qr$ = heterozygous frequency

r^2 = recessive homozygous frequency

Discussion

Tremendous research has been done in the field of pharmacogenomics that emphasized the polymorphic nature of CYP2D6 enzyme contributed high level of inter-individual variation. After administration of drugs patients produce inappropriate response and serious drug induced complications. About 40% to 70% depend on type of drug and 15–30% depends on drug reactions that are developed by *CYP2D6* polymorphism [20]. Hence, there is a need to screen out *CYP2D6* gene variants in a particular population for analyzing their clinical relevance before unraveling the genotype-phenotype associations.

So far few research articles published the work that investigated the frequency of *CYP2D6* polymorphic alleles among Pakistani population but no one report the information of *CYP2D6* polymorphism in depressed patients. Therefore, preliminary study was designed in which three alleles (*CYP2D6*1*, *CYP2D6*4* and *CYP2D6*10*) were assessed.

The results of our study were in agreement with the results of several previously conducted studies. *CYP2D6*1* is a wild allele produces functionally active enzyme which metabolizes 160 therapeutic targets, including antidepressants, antipsychotics, anti-arrhythmic, opioid analgesics, anticancer agents [21]. In our study, in normal subjects (n = 60), the gene frequencies of *CYP2D6*1*, *CYP2D6*4* and *CYP2D6*10* were 59%, 0.01% and 40%, respectively. Another research group reported the gene frequencies of *CYP2D6*1*, *CYP2D6*4* and *CYP2D6*10* as 74%, 1% and 25%, [22] among healthy subjects (n =155) from major ethnic groups in Karachi. The slight differences evident in our studies may be related to small sample size. *CYP2D6*1* was found to be the most frequent in normal subjects of study population as similar as Japanese population and other Asian countries (59% vs 55% vs and 50%) higher than UAE, Koreans and Chinese, (39%, 32% and 30%) but lower than Caucasian and Iranian populations (74% and 83%) [23,24,25,26,27,28].

However, in depressed patients it was 1.84x lower than their normal counterparts in Karachi population but closer to depressed patients of UAE and Japanese population (39.1% and 39.4%) [26, 29]. It has been proved experimentally that *CYP2D6*1* allele in Japanese was closer to Karachiites.

*CYP2D6*4* accounts for ~72% of all null alleles worldwide and reported to be absent in Asian population. Japanese, Chinese, East Asian, UAE, Iranian and Hong Kong populations (0%, 0%, 0.3–0.5%, 2%, 2% and 4.6%) [26, 28, 30]. However, due to the presence of single heterozygous individual (*1/*4) in normal subjects very low or negligible allelic frequency (0.01%) was found in Karachi population. On the contrary, the frequency of *CYP2D6*4* was higher in Caucasians (20%) [23, 29].

The same *CYP2D6*4* allele in depressed patients of Karachi population was found to be (13%) closer to another research group who reported gene frequency in northern Pakistani population including Meo (13.64%), Punjabi (11.96%) and Pathan (10.42%). Low frequency (<4%) of *4 polymorphism was observed in Kalash and Makrani groups, whereas an intermediate frequency

(5-9%) in all the other ethnic groups [31]. In depressed subjects from UAE population *4 is (9%) slightly lower than local population but higher than Japanese population (0%). [24, 25, 28] It may be due to greater number of heterozygous individuals relocate between these regions that may raise the frequency of *CYP2D6**4 allele in our population.

Individuals carrying *CYP2D6**4 mutant allele are more susceptible to develop side-effects with normal doses (20mg/d) of antidepressants fluoxetine and paroxetine as they are poor metabolizer (*4/*4) genotype and hence showed high drug concentrations in their plasma. Therefore, low doses about half the therapeutic doses should be prescribed which will be served for two purposes i.e. minimize the side effects of the drug and cost burden on the patient will also be reduced.

*CYP2D6**10 is the most frequent mutant allele in Asian population (50%). It was found to be (40%) in Karachi closer to Korean, Japanese and East Asian (46.2% and 39%, 37.5–52.5%) but very low in Caucasian populations (1.4%) in accordance with other Asian population. However, in depressed patients of Karachi Population it was found to be (54%) similar in Japanese (56.7%) but significantly higher than UAE (3.3%) [26, 29, 14]. According to the study of Japanese depressed patients *CYP2D6**10 allele showed significant impact on the plasma paroxetine concentrations in Asians. Therefore, it has been proposed for Asian patients with *CYP2D6**10 allele paroxetine drug monitoring is necessary for preventing overdose. The genotyping screening parameters is necessary to optimize dose in each patient [29].

Additionally, in present study difference of *CYP2D6* variants expression was found between patients and normal subjects irrespective of ethnic diversity. Due to study limitation, data was not analyzed for protein translation of *CYP2D6* mutant enzymes in order to confirm genotypic expression into phenotypic drug metabolizing enzyme formation.

The similarities and dissimilarities in the occurrence of various alleles strongly suggests that population based studies should be taken into account before marketing drugs in different countries. Most importantly, same drug dosages cannot be recommended worldwide emphasizing individualized treatment for efficacious and safe therapeutic response.

In Pakistan, multiple research organizations are needed to be established for designing and sharing *CYP2D6* gene testing techniques in different provinces. This perspective might be prolonged to uncover related gene markers that bio-transformed antipsychotic medication to

avoid sufferings of depression [32]. However, PharmVar database was launched in 2018, which is actually gene based catalogue that updated allelic variation of genes impacting on drug metabolism. Clinical Pharmacogenetics Implementation Consortium (CPIC) develops guidelines that captured information of allelic frequencies from different population across the world and doing translation of genetic laboratory test to confirm genetic variants and further specific drugs recommendation [33, 34]. Thereby, we need to initiate extensive *CYP2D6* genetic analysis by utilizing sequence-based approaches to detect maximum number of allelic variants having abolished activity of drug metabolism and update the information. Furthermore, pharmacogenomics (PGx) guidelines need to be implemented for predicting phenotypes before suggest specific drug dosage.

Conclusion

In view of the present findings, *CYP2D6**4 and *10 (13% and 54%) are frequent variants of *CYP2D6* gene, associated with inter-individual variability that induce improper drug metabolism among patients with a family history of depression and using co-medication as a consequence undesirable side effects may develop that impede patient's therapeutic response. To avoid these and for safer therapeutic responses gene screening is demanded. Although, there are some limitations in our study and paucity of available data, extensive research is demanded for characterizing *CYP2D6* gene profile and monitoring patient's drug metabolic activity. In future, study with large sample need to be conducted and expand to national level.

Authorship

Dr. Ahsana Dar participated in study design and supervision, Tamkeen Fatima participated in recruitment of patients, performance of the research, data analysis and writing of the paper, Farah. Zeb participated in the recruitment of patients.

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Declaration of Competing Interest

The authors declare no potential conflict of interest with respect to the authorship and publication of this article.

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