



TBX 5 GENE MUTATION ANALYSIS AMONG TANZANIAN CHILDREN WITH CONGENITAL HEART DISEASES USING HIGH-RESOLUTION MELTING ASSAYS.

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ABSTRACT

Early cardiac development is governed by transcription factor genes. TBX5, a T-box transcription factor gene, plays an important role in the development of the second heart field during cardiac septation by promoting cell cycle progression through the enhancement of Cdk6 and hedgehog signaling pathways. TBX5 binds to the promoter region of genes, enhancing the expression of alpha cardiac myosin heavy chain 6 (MYH6), which is a predominant isoform found in human cardiac tissue. TBX5 gene mutations are postulated to cause congenital heart diseases. A case-control TBX5 mutational analysis was performed to provide insight into the etiology of sporadic congenital heart diseases in our setting. We used a magnetic induction cyler (mic-PCR), which is a next-generation tool for polymerase chain reaction-high resolution melting assays, to detect mutations in children with sporadic isolated congenital heart diseases. A retrospective case-control study was conducted at the Jakaya Kikwete Cardiac Institute. The peripheral blood samples were collected, and DNA was extracted using the Quick-DNA Miniprep Kit. The primers were designed using Primer 3 software, validated using the program BLAST, and checked for hairpin and homo-hetero-dimerization using the IDT oligo analyzer. Real-time polymerase chain reaction (PCR)-high-resolution melting assays for screening TBX5 gene mutations were done using a magnetic induction cyler. We found two (2) TBX5 mutations in exon 5, among patients with Atrial-Ventral Septal Defects (ASVD) and Atrial-Septal Defects (ASD) and none among controls. TBX5 exon 5 is a molecular hotspot for isolated congenital heart diseases.

Key words: TBX5 transcription factor, congenital heart diseases, mutation, high-resolution melting assays.

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INTRODUCTION

Congenital heart diseases (CHDs) cause significant morbidity and mortality in low- and middle-income countries (Zimmerman 2020). The prevalence of CHD has increased worldwide to 9.1 per 1000 live births, with significant geographical differences (van der Linde et al., 2011). The prevalence of CHDs varies globally, with an estimation of 11.5 per 1000 births in Australia, 6.5 per 1000 births

in Europe, 6.09 per 1000 births in Iran, 9.78 per 1000 births in Brazil and 8.54 per 1000 births in India (Hansen et al., 2021; Dolk et al., 2010, Nezami et al., 2021; Miyague et al., 2003, Bhat et al., 2013). The CHD prevalence in Africa also varies and is estimated to be 2.3 per 1000 births in Mozambique (Marijon et al. 2006), 14.5 per 1000 births in Sudan (Abdelrahman and Diab 2022), 14.4 per

1000 births in Nigeria (Sadoh et al. 2021), 2.8-4.95 per 1000 births in Botswana (Mazhani et al. 2020), and 1.8 per 1000 births in Kenya (Anabwani and Bonhoeffer 1996). Congenital heart diseases are clinically categorized as cyanotic and acyanotic, the latter being the most common phenotype (Zikarg, Yirdaw, and Aragie 2021). Improvements in prenatal diagnosis and treatment have reduced the burden of congenital heart diseases (Brizard et al. 2017), but their etiology remains poorly understood. Both genetic and environmental factors are implicated in its pathogenesis (Fahed et al. 2013, Leirgul et al. 2016).

CHDs occur as a result of defective early cardiac morphogenesis (Anderson and Wenink, 1988). Early cardiac development is governed by the T-box transcription factor gene, TBX5 (Plageman and Yutzey, 2004). TBX5 is a key regulator for the development of the heart (Steimle and Moskowitz, 2017) and it is involved during cardiac specification (Takeuchi et al., 2003). TBX5 plays an important role in the development of the second heart field during cardiac septation by promoting cell cycle progression through enhancement of the cell cycle progression gene *Cdk6* and the hedgehog signaling pathway (Xie et al., 2012). TBX5 binds to gene promoter regions, enhancing the expression of alpha cardiac myosin heavy chain 6 (MYH6) (Ghosh Tushar et al., 2009), natriuretic peptide A, brain creatine kinase, photoreceptor cadherin, gelsolin, and enhancer of split related 2 (Plageman Jr and Yutzey, 2006).

TBX5 gene mutations are postulated to cause congenital cardiac defects (Behiry et al., 2019b). Studies have demonstrated that TBX5 haploinsufficiency reduces cardiomyocyte differentiation in vitro and also causes severe cardiac malformations in vivo (Kathiriya et al., 2021). Other studies have demonstrated that TBX5 overexpression causes cardiomyocyte proliferation in vivo (Hiroi et al., 2001), and

its knockdown results in severe heart malformations (Mori et al., 2006). Moreover, TBX5 reduced expression affects the alignment of the aorta in the Down syndrome mouse model (Polk et al., 2015).

TBX5 transcription factor gene mutations are postulated to cause CHDs. However, there is little known about TBX5 mutations and the occurrence of CHDs in our settings. Nevertheless, the discovery of contemporary technologies such as high-resolution melting assays (Vossen et al., 2009) and high throughput sequencing techniques (Fox et al., 2009) raises the hope of finding genetic etiological factors that are linked to the pathogenesis of CHDs. Understanding genetic risk factors for CHDs is important for planning CHD risk control measures, providing genetic counseling, and predicting disease prognosis. Therefore, in this study, we aimed to determine the genetic etiology of congenital heart diseases by screening TBX5 gene mutations in patients with CHDs, and hence, bridge the knowledge gap between embryological development of the heart and molecular genetics.

Many previous studies are based on Caucasian subjects. There is scant data regarding the role of cardiac transcription factor mutations and the occurrence of congenital heart diseases in our settings. It is unknown whether TBX5 transcription factor gene mutations responsible for defective cardiac development in Caucasians are also responsible for the development of congenital heart diseases in our population. This is the first study that provides insight into the genetic etiology of congenital heart diseases in our setting. This study is distinct from previous research. Most previous studies used sequencing techniques, while we used high-resolution melting analysis, which is a very sensitive, feasible, and powerful method of detecting mutations. It is more sensitive than Sanger sequencing (Garritano et al. 2009) and denatured high-performance liquid chromatography

(Aguirre-Lamban et al. 2010). The study will also set a benchmark for further genetic studies regarding cardiac transcription

factors and their roles in early cardiac development.

MATERIALS AND METHODS

Research design and setting

A retrospective case-control study was conducted at the Jakaya Kikwete Cardiac Institute (JKCI). We calculated the sample size using the program G*Power 3.1.9.4, which a priori-computes the required sample size. We used one-tailed tests with an error probability (type I error) of 0.05 to achieve 80% power.

Study Subjects

The subjects were Tanzanian children aged under 5 years with isolated CHDs confirmed by ECHO, GE Vivid TM E95 (GE Healthcare, USA). The subjects were obtained at the outpatient clinic, coming for follow-up and screening purposes. Healthy controls were children without congenital heart diseases, obtained at Muhimbili National Hospital when they came for a routine child monitoring clinic. An ECHO and physical examination were performed by an experienced pediatric cardiologist. We excluded children with other congenital malformations unrelated to the heart, children who were very sick before and after surgical repair, and children who refused to sign an informed consent form for any reason. Clinical and social-demographic characteristics were obtained and collected using clinical survey forms and hospital registration cards.

Pilot study

The pretesting clinical survey form for data collection and optimization of qPCR HRM was completed. A DNA sample from five cases and five controls was included in the optimization process. Data collected during the pilot study was used as a guide for the next phase of the study.

Quality assurance

Researchers were instructed on data collection tools as well as how to obtain consent from participants while maintaining confidentiality. We used investigators who were familiar with peripheral venous blood collection using dry blood sample paper (DBS). We stored the samples according to the manufacturer's instructions. DNA extraction and subsequent real-time PCR-HRM assays were done in a molecular laboratory at the Sokoine University of Agriculture. The laboratory is equipped with state-of-the-art equipment such as a magnetic induction cyler, Mic (Biomolecular Systems, Australia), and experienced technicians.

Laboratory work

Peripheral blood samples were collected using Dry Blood Sample (DBS) paper. Each well on DBS paper was filled with a single drop of blood, left to dry in the open air for one hour, and then packed in specialized airtight parcels for transfer and subsequent DNA extraction. Genomic DNA extraction was done as per the Quick-DNA Miniprep Kit protocol following the manufacturer's instructions. The quantity and quality of DNA were measured by a Thermo Scientific Nano Drop™ 2000 Spectrophotometer. We used bioinformatics pipelines to design and validate primers. We used the National Center for Biotechnology Information (NCBI) to identify the reference gene sequence of TBX5 (NG_007373.1). Because of alternative splicing, TBX5 has four transcript variants. Exon numbers 3–9 are the most frequently mutated exons. Therefore, sequences of exon 5 were selected, and both forward and reverse primers were designed using Primer3 software. The primers were validated using

the Program Basic Local Alignment Search tool (BLAST). The primers were also checked for hairpin, homodimer, and heterodimer using Integrated DNA Technologies (IDT) oligo Analyzer software. The primers were as follows: 5'-CTGTACGTGCACCCAGACTC-3' (20bp), Tm: 59.3°C GC content 60% and Reverse Primer 3'-ATGGTCCAGGTGGTTGTT-5' (19bp), Tm: 60.1°C GC content 52.6%. The product size was 100 base pairs. Microgen Europe B.V. in Amsterdam, the Netherlands, manufactured the designed primers (forward and reverse).

We prepared stock primer by mixing it with nuclease-free water (according to the manufacturer's instructions) and storing it at -20°C. A working primer solution was obtained by x10 dilution of the prepared stock primer. Master Mix was obtained by mixing 4µl 5x HOT FIRE Pol Eva GREEN HRM (from Solis BioDyne company, Estonia), working primer solution (consisting of 0.5µl for forward primer and 0.5µl of reverse primer) and 11µl nuclease free water. Then the reaction mixture was obtained by adding 4µl DNA to 16 µl of master mix for the test sample. We also prepared the non-template control by adding 4µl of master mix to 16 µl of master mix and negative control by adding 4µl of nuclease free water to 16 µl master mix. Then the prepared reaction mixture in the Mic PCR tube was loaded into the magnetic induction cycler Mic (Biomolecular Systems, Australia) for Real time PCR-HRMA. PCR conditions were set as follows, activation at 95°C for 12 minutes, followed by three cycles of denaturation at 95°C for 20 seconds, annealing at 58°C for 15 seconds, and elongation at 72°C for 20

seconds. After 35 cycles, holding was at 72°C for 300 seconds, then the temperature was raised to 75°C followed by holding for 90 seconds. There was an increase of 0.1°C per second up to 95°C. The melting assay was analyzed using Mic qPCR Software version 2.8 (www.mic-qpcr.com).

Statistical analysis

SPSS Statistical Software Version 23 and GraphPad Prism 9.1.0 were used to code, enter, clean, and analyze data. The univariate analysis was done for both continuous and categorical variables. We tested the data for normality using the Shapiro-Wilks test, the Kolmogorov-Smirnov test, and Q-Q plots. For normally distributed data, means and standard deviations were calculated, while for non-normally distributed data, the median and percentile were calculated. Frequencies and proportions were calculated for categorical variables. We compared the means between sexes using the Independent T-test for parametric data or the Mann-Whitney U-test for non-parametric data. The differences in categorical variables between the cases and controls and their level of significance were calculated using the Pearson chi-square test. The significance level of less than 0.05 favored the alternative hypothesis. The categorical data were presented using a Chi-square distribution (contingency) table. We used histograms as a diagrammatical representation of different CHD phenotypes. We also compared the mean birth weight and maternal age distributions between cases and controls using box and whisker plots.

RESULTS

The social and demographic characteristics of the study participants are shown in Table 1 below. There was a statistically significant difference in sex between cases and controls. The distribution of CHD phenotypes was as follows: we had eighteen(18) patients with

Ventral Septal defects (VSD), ten(10)patients with Atrial Septal defects(ASD), six (6)patients with Tetralogy of Fallot(TOF), ten patients(10) with Patent Ductus Arteriosus(PDA), two(2) patients with Atrial Ventral Septal Defects (AVSD),

two(2)patients with Double Outlet Right Ventricle (DORV), one(1) patient with Transposition of Greater Vessels (TGV), and one(1) Truncus arteriosus (TA) as shown in figure 1. The most prevalent phenotype was Ventral Septal Defect (VSD). The mean birth weight was 2.99 kg among cases and 2.924 kg among controls. The mean maternal age was 32.66 years among cases and 31.14 years among controls. The gestation age and birth weight distribution for both cases and controls are shown in the plots (Figures 2 and 3). There was no statistically significant difference between the mean birth weights and mean maternal age as the independent T-test yielded an alpha value of 0.725, 95%CI (-0.317-0.221) and 0.187, 95%CI (-0.393-1.402) respectively. The PCR amplification and cycling are shown in figures 4a and 4b. Using normalized curves, the heterozygous mutation was detected by a typical change in the shape of the curve due to hetero-duplex destabilization, while the homozygous mutation was detected by a typical temperature shift. We also plotted the difference between the plots for wild-type and homozygous mutations. The results of high-resolution melting assay curves are shown in figure 5, and difference plots are shown in figures 6 and 7. High-resolution melting analysis of TBX5, exon five (5), reveals two (2) deviated curves in exon five (5), among cases and none among controls. The homozygous mutations were found in two patients with atrial-ventral septal defects (ASVD) and atrial-septal defects (ASD). We, therefore, found two (2) patients with homozygous mutations of the TBX5 transcription factor gene and forty-eight (48) patients who were wildtype. No TBX5 mutations were found among the fifty (50) control subjects.

Table 1 : Social demographic characteristics of children and mothers among cases and control groups

Variables		Cases(n =50)	Control(n=50)	Chi-square	P-value
Sex of child	Male	23(35.4 %)	42(64.6 %)	15.868	0.001
	Female	27(77.1 %)	8(22.9%)		
Maternal age	18-35	34(48.6 %)	36(51.4 %)	0.663	0.38
	36-50	16(53.3 %)	14(46.7 %)		
Maternal Education	Not attended	2(33.3 %)	3(66.7%)	2.356	0.502
	Primary school	23(51.1 %)	22(48.9 %)		
	Secondary school	15(60%)	10(40%)		
	College Education	10(41.7 %)	15(58.3 %)		
Marital status	Single	6(50%)	6(50%)	6.439	0.092
	Married	38(46.3 %)	44(53.7 %)		
	Divorced	1(100%)	0(0%)		
	Widowed	5(100%)	0(0%)		
Occupation	Housewife	13(44.8 %)	16(55.2 %)	0.486	0.784
	Self Employed	23(51.1 %)	22(48.9 %)		
	Employed	14(58.3 %)	12(42.6 %)		

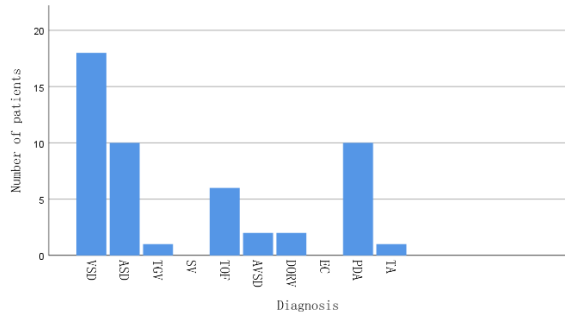


Figure 1. Histogram showing the frequency distribution of different congenital heart disease phenotypes

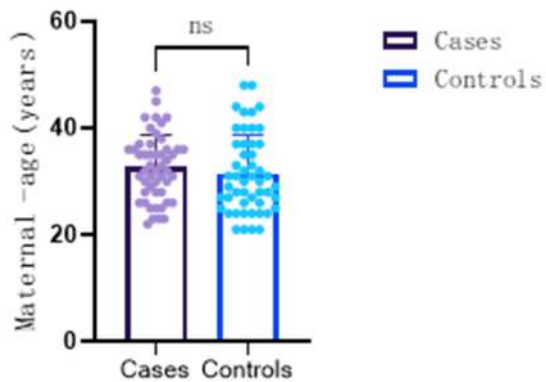
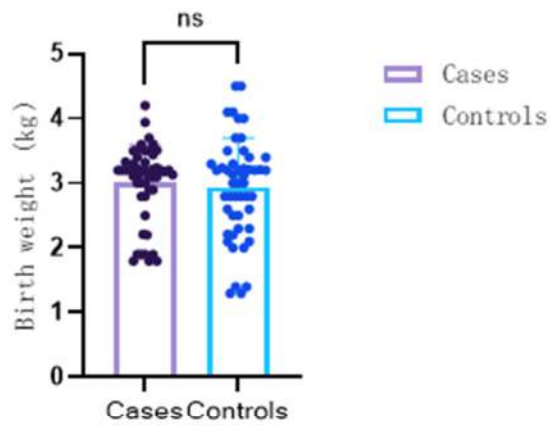


Figure 2 and Figure 3. Plots for birth age and maternal age, mean with standard deviations

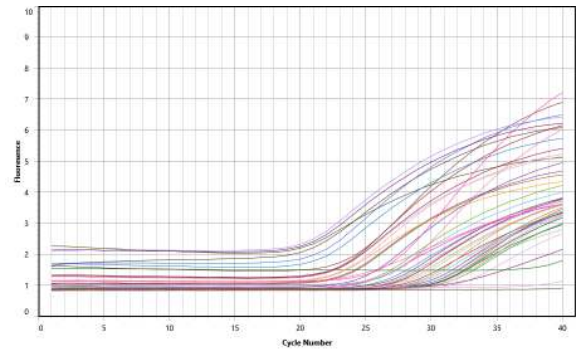


Figure 4a: PCR amplification of the TBX5 gene using Eva green as an intercalated fluorescence dye.

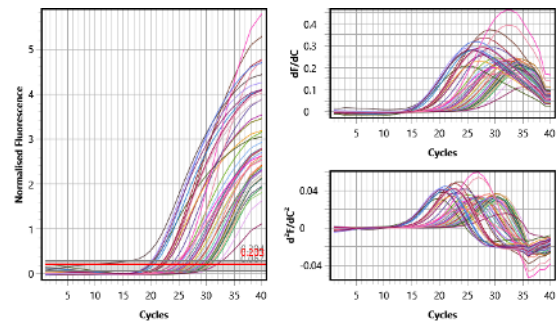


Figure 4b. Cycling analysis of TBX5 gene using Eva green as intercalated fluorescence dye

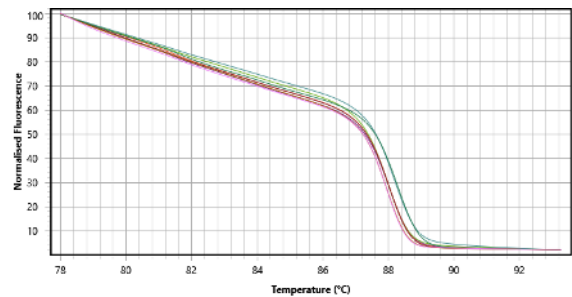


Figure 5: High-resolution melting curve normalized fluorescence for TBX5 exon5

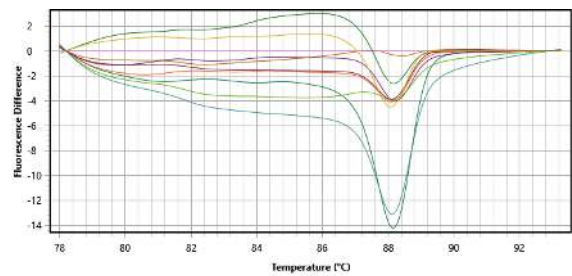


Figure 6: Exon 5 difference plots compared to the wild type.

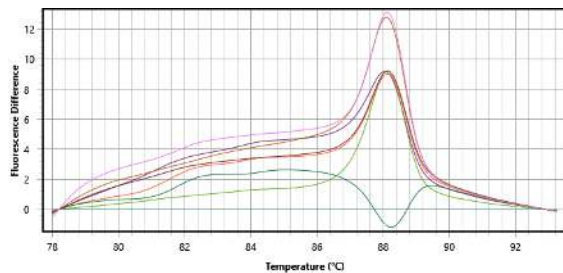


Figure 7: Difference plots for the homozygous mutation exon 5

DISCUSSION

Genetics of isolated congenital heart diseases (CHDs)

The genetic basis of isolated congenital heart disease is still the subject of debate. Nevertheless, the discovery of next-generation state-of-the-art technologies such as next-generation sequencing and real-time PCR-high resolution melting assays gives us an opportunity to study the molecular basis of CHDs. CHDs have multifactorial genetic causes such as chromosomal abnormalities, single gene mutations, and copy number variants (Fahed et al. 2013). CHDs are also postulated to be caused by epigenetic changes such as DNA hypo and hypermethylation of different alleles and histone modifications (Serra-Juhé et al., 2015). Single gene mutations are implicated in both isolated and syndromic CHDs (Nees and Chung, 2020).

TBX5's role in cardiac development has been studied in both human and animal models (Plageman and Yutzey, 2006; Wang et al. 2017). TBX5 mutations are often found in patients with Holt-Oram syndrome (Mori and Bruneau, 2004a; Reamon-Buettner and Borlak, 2004a). However, the role of TBX5 mutations in the etiology of isolated congenital heart disease is still elusive.

TBX5 mutations and congenital heart defects: our findings and comparison to previous research

Congenital heart diseases are linked with mutations of the TBX5 gene in exons 3,4,5,

and 7 (Su et al., 2017). TBX5 novel variations and loss of functions have been associated with different cardiac phenotypes (Yoshida et al., 2016), such as Ventral-septal defects (VSD) (Liu et al., 2009), Atrial septal defects (ASD) and Atrial Ventral Septal defects (AVSD) (Reamon-Buettner and Borlak, 2004a). TBX5 is highly associated with Holt-Oram syndrome (HOS), a syndrome which is characterized by severe heart and limb malformations (Ersoy et al. 2016). TBX5 loss of function is also associated with rare atrial fibrillation (Guo et al. 2016) and familial cardiac defects (Jiang et al. 2020), including familial and sporadic dilated cardiomyopathy (Patterson et al., 2020 ; Zhou et al., 2015). Reamon-Buettner and Borlak (2004) discovered nine (9) TBX5 mutations in non-HOS malformed hearts with ASD and AVSD using direct sequencing. A study among Egyptian children found variants in TBX5 in patients with congenital heart diseases (Behiry et al., 2019a). Another study found the heterozygous missense mutation of TBX5 exon two (2), which results in changes in TBX5 activity (Chen et al., 2017) In a different study, mutations were identified in exon nine (9) and exon eight (8) in patients with tetralogy of Fallot (TOF) (Baban et al., 2014). This is different from our study, which identified the homozygous mutations of the TBX5 gene in exon five (5) in two (2) out of 50 patients with congenital heart diseases. The homozygous mutations were found in patients with atrial septal defects (ASD) and atrial ventricular septal defects (AVSD). Another study among children with CHDs in

the Chinese population found no mutations in exon four (4), five (5), and exon eight (8) (Yang et al., 2017). This is different from our study, which found mutations in exon five (5). The above studies show that there is a wide variation of different mutations involving different exons of TBX5. This warrants the need to do genetically wide-associated studies involving large sample sizes.

CONCLUSION

We found two TBX5 mutations in patients with isolated congenital heart diseases with different phenotypes, namely; Atrial-Ventral Septal Defects (ASVD) and Atrial-Septal Defects (ASD). In our setting, the TBX5 gene exon 5 is a molecular hotspot for isolated congenital heart diseases. It is known that TBX5 gene mutations are associated with Holt-Oram syndrome, a condition that is characterized by both malformations of the limbs and the heart. However, our study demonstrated TBX5 mutations are also associated with patients with isolated congenital heart diseases. Patients with isolated congenital heart diseases should be candidates for genetic screening to rule out the possibility of genetic risk factors and therefore provide family genetic counseling.

RECOMMENDATIONS

Patients with isolated congenital heart diseases should undergo genetic testing and counseling. The TBX5 transcription factor

gene has nine (9) exons and four (4) transcript variants due to alternative splicing. We screened TBX5 exon five (5), which is the most frequently mutated exon, and recommend further studies that will include mutation screening of the other eight (8) TBX5 exons using HRM. We also recommend performing sequence analysis of TBX5 exon five (5) in order to detect specific mutations and subsequently using bioinformatic pipelines such as Clustal W in MEGA 7 software in order to determine if these detected mutations in TBX5 exon five (5) result in changes in amino acids or proteins. In addition, further studies should be done using next-generation sequencing technologies involving a large sample size to screen the entire TBX5 transcription factor gene (whole exon sequencing) in order to detect if there are novel specific mutations associated with CHDs in our settings.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article

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