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ORIGINAL ARTICLE

Age-Related Hearing Impairment Associated *NAT2*, *GRM7*, *GRHL2* Susceptibility Gene Polymorphisms and Haplotypes in Roma and Hungarian Populations

Petra Matyas[1,2] · Etelka Postyeni[1,2] · Katalin Komlosi[1,2] · Renata Szalai[1,2] · Judit Bene[1,2] · Lili Magyari[1,2] · Bela Melegh[1,2] · Kinga Hadzsiev[1,2]

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Abstract

Age-related hearing impairment (ARHI) is the most frequent sensory disease in the elderly, which is caused by an interaction between genetic and environmental factors. Here we examined the ethnic differences, allele and genotype frequencies of the *NAT2*, *GRM7*, and *GRHL2* genes pooled samples of healthy Hungarian and healthy and hearing impaired Roma people. Study populations of healthy Hungarian and Roma subjects were characterized for the rs1799930 *NAT2,* rs11928865 *GRM7*, rs10955255, rs13263539, and rs1981361 *GRHL2* polymorphisms and deaf Roma subjects were characterized for the rs1799930 *NAT2,* rs13263539, and rs1981361 *GRHL2* using a PCR-RFLP method. We found significant differences in minor allele frequencies for *GRHL2* rs13263539 and rs1981361 polymorphism between healthy Roma and Hungarian samples (37.9% vs. 51.0% and 43.6% vs. 56.2%, respectively; $p < 0.05$). The differences of homozygous genotype of *GRHL2* rs13263539 and rs1981361 variants, values were also significantly different (13.0% vs. 25.3% and 16.5 vs. 32.3%; $p < 0.05$). The *NAT2* rs1799930 homozygous genotype was 14.0% in healthy Romas and 7.7% in Hungarians, while the minor A allele frequency was 38.0% and 26.7% in Roma and Hungarian population, respectively ($p < 0.05$). Furthermore, the frequency of GGT, GAC and GAT haplotypes was significantly higher in the Hungarian population than in healthy Roma (1.87 vs. 4.47%, 0.91 vs. 2.07% and 1.15 vs. 5.51%, respectively; $p < 0.008$). Present study revealed significant interethnic differences in allele polymorphisms of *NAT2*, *GRM7* and *GRHL2* exhibit quite marked ethnic differences in Roma populations that might have important implications for the preventive and therapeutic treatments in this population.

Keywords *NAT2* · *GRM7* · *GRHL2* · ARHI · Roma · Hungarian

Introduction

Several studies have shown that age-related hearing impairment (ARHI) is one of the most common types of hearing disorders and it is a complex of high-frequency hearing loss (HL) caused by degenerative changes in the inner ear with aging [1, 2]. Age-related hearing loss develops in different age groups with various degree, and it is known that

✉ Bela Melegh
melegh.bela@pte.hu

Petra Matyas
matyas.petra@pte.hu

Etelka Postyeni
etelka14@gmail.com

Katalin Komlosi
komlosi.katalin@pte.hu

Renata Szalai
szalai.renata@pte.hu

Judit Bene
bene.judit@pte.hu

Lili Magyari
magyari.lili@pte.hu

Kinga Hadzsiev
hadzsiev.kinga@pte.hu

[1] Department of Medical Genetics, University of Pecs, Medical School, Szigeti 12, Pecs H-7624, Hungary

[2] Human Genetic and Pharmacogenomic Research Group, Janos Szentagothai Research Centre, Ifjusag 20, Pecs H-7624, Hungary

genetic host factors associate with the degenerative mechanisms [3, 4].

There is raising evidence that aging, noise exposure and medications may impair the inner ear tissues by way of reactive oxygen species (ROS) induced cellular injury [1, 5]. ROS are natural byproducts of aerobic metabolism; and oxidative stress is the result of the accumulation of reactive oxygen species. Bared A. et al. hypothesized that oxidative stress in the inner ear increases ROS production by polymorphisms of antioxidant enzymes making them susceptible to ARHI.

There is a significant correlation between age-related hearing loss and polymorphism in the gene for Nacetyltransferase 2 (*NAT2*) enzyme that has an antioxidant role in the cochlea [3, 6]. *NAT2* is an isoenzyme of N-acetyltransferase (NAT) enzyme, which is a susceptibility gene for age-related hearing impairment. N-acetyltransferases are also known to be responsible for the detoxification of exogenic substrates by N-acetylation or O-acetylation, to be involved in the detoxification of harmful xenobiotics, and NAT are important for the balance of the oxidative status. The enzyme N-acetyltransferase 2 (NAT2) is involved in the formation of reactive oxygen species (ROS). A large amount of ROS contributes to the aging of the inner ear. It is well known from previous studies that certain *NAT2* genotypes may be associated with the risk of age-related hearing impairment [6, 7].

The glutamate metabotropic receptor7 (*GRM7*) gene is located on chromosome 3, and contains 10 exons which translate into a 915 amino acid protein. *GRM7* has a central role in glutamate synaptic transmission and homeostasis in the cochlea at the synapses between the dendrites and hair cells of afferent auditory nerve fibers. The presence of glutamate in large quantity causes neurotoxicity in auditory neurons [8]. The rs11928865 polymorphism of *GRM7* is associated with ARHI and particularly with speech detection in elderly [9].

The grainyhead-like 2 (*GRHL2*) gene is also known as *TFCP2L3* (transcription factor cellular promoter 2-like 3) and *BOM* (brother of mammalian grainyhead). This gene is on chromosome 8q22.3, and is composed of 16 exons which encode a protein of 625 amino acids. *GRHL2* is a transcription factor which is expressed in different epithelial cells, and not only plays a central role in embryonic development, but is also responsible for the maintenance of epithelial cells [10, 11]. In the *GRHL2* gene several SNPs have been associated with age-related hearing loss susceptibility and we selected three of these polymorphisms, rs10955255, rs13263539, and rs1981361 for our study [12].

The size of the Roma population is about 12–15 million in the world and from this 10–12 million Roma people live in Europe. In Hungary there are circa 700.000–1 million Romas [13, 14]. In the Roma population the general morbidity rate is increased, the infant mortality is fourfold elevated, and their lifetime expectance is ten years less compared to Central and Eastern European populations. The relatively highly conserved gene pool of the Roma people is derived from India [15].

The aim of the study was to investigate the *NAT2, GRM7* and *GRHL2* gene polymorphisms as a putative causative factor for ARHI in healthy Roma and Hungarian population samples to assess genetic distribution and interethnic differences between the two groups.

Methods

Study Population

A total of 298 healthy Roma (118 males, 180 females; mean age 42.33 ± 15,51 years) and 298 healthy Hungarian subjects (168 males, 130 females; mean age 37,43 ± 12,53 years) were investigated in case of each polymorphisms. 113 deaf Roma people (57 males and 56 females) were characterized for the rs1799930 *NAT2,* rs13263539, and rs1981361 *GRHL2.* DNA samples with accompanying personal and clinical data were derived from healthy Caucasian and Roma subjects from Hungary. Roma people declared their Roma origin. Written informed consent was obtained from all subjects. The DNA samples of the Roma and the Hungarian population originated from the central Biobank governed by the University of Pecs, as part of the National Biobank Network of Hungary (www.biobanks.hu), which belongs also to the pan-European Biobanking and Biomolecular Resources Research Infrastructure project (http://bbmri.eu/bbmri/). The maintenance and governance principles of the Biobank have been approved by the National Scientific Research Ethics Committee (ETT TUKEB, Budapest, Hungary). The collection and usage of DNA samples and management of data followed the Helsinki Declaration of 1975.

Molecular Methods

Genomic DNA was isolated from peripheral EDTA-anticoagulated blood samples using a standard desalting method. Genotyping was carried out using polymerase chain reaction (PCR) followed by restriction endonuclease digestion (RFLP). For detection of the *NAT2* rs1799930 polymorphism the following primers were used: 5′- CATCTCCTGCCAAA GAAGAAAC -3′ and 5′- TAGAAGGGATCCATCACCAGG -3′. For the amplification of the target sequence of the *GRM7* rs11928865 the 5′- GGTATCTGTCTCCACTCCCAAC -3′ and 5′- CCCAAAATGTTAAGCTTTATCTCC -3′ primers were utilized. For determination of the *GRHL2* rs10955255 the following primers were used: 5′- GGTTAAGGTAGTAG CTGCCAGG -3′, and 5′- GATGGGAACAAAGGCTAAAA AG -3′; to test the rs13263539 SNP the 5′- CGAGCATA GCCATCCTTAAC -3′ and 5′- GCTTTCAGCAATAT CCCTCC -3′ oligonucleotides were used. and for the

detection of rs1981361 SNP: 5′- GATGGTTCCTCAGC TCACTTTG -3′, and 5′- CAGGGTGTTTTCTCATGTAT TCC -3′ were used.

The PCR amplification was carried out in a final volume of 50 μl containing 200 μM of each dNTP, 1 U of Taq polymerase, 5 μl of reaction buffer (10 mM Tris-HCl, pH 9.0, containing 500 mM KCl, 14 mM $MgCl_2$), 0.2 mM of each primers and 1 μg extracted DNA. The PCR amplifications were performed on MJ Research PTC 200 thermal cyclers. PCR conditions were as follows: predenaturation for 2 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C for rs1799930 (*NAT2*) and for rs11928865 (*GRM7*), and 58 °C for rs10955255 and for rs1981361 (*GRHL2*), and 55 °C for rs13263539 (*GRHL2*) primer extension for 30 s at 72 °C, the final extension at 72 °C for 5 min. Digested PCR products were separated by electrophoresis using a 1% agarose gel stained with ethidium-bromide and visualized by UV illumination. The amplicon was digested by allele-specific restriction endonuclease. The amplicon of the *NAT2* rs1799930 SNP was digested with TaqI endonuclease. The MunI was used to cleave of the *GRM7* rs11928865 variant. The PCR product of the *GRHL2* rs10955255, rs13263539 and rs1981361 primers were digested by Hin1II, BseGI and BseNI restriction enzymes, respectively The digested PCR products were separated by electrophoresis using a 3% agarose gel. The amplicon contained an obligatory cleavage site to enable us to control the efficacy of the digestion. Direct sequencing was performed by ABI 3500 Genetic Analyzer (Applied Biosystems [CA, USA]) on random samples to confirm our results.

Statistical Analysis

Statistical analyses were performed using SPSS Statistics 20.0 package for Windows (SPSS Inc., Chicago, IL, USA). We applied the Chi-square test to compare the differences between the studied groups, $p \leq 0.05$ value was considered as statistically significant. For haplotype analysis we used the Phase Program Version 2.1. For linkage disequilibrium analyses we used Haploview software v.3.3.

Results

All allele frequencies and genotype rates were in Hardy-Weinberg equilibrium in both the Hungarian, and Roma groups in the three genes. The genotypes and minor allele frequencies of the examined genes are presented in Table 1.

In the investigated polymorphism of the *NAT2* gene we found significant differences, the AA homozygous genotype ($p = 0.013$) had a higher prevalence in the Roma population (14.1%) compared to the Hungarian cohorts (7.7%). There is an almost two-fold higher incidence of carrying *NAT2* variant allele in Romas than in Hungarians. Furthermore, the minor allele frequency was shown to be significantly higher in the Roma population samples than in Hungarians (38.0% and 26.7%, respectively, $p < 0.05$). We did not find significant differences between healthy Hungarian and Roma subjects neither in the homozygous genotype nor in the minor allele frequency in case of rs11928865 (*GRM7*) and rs10955255 (*GRHL2*). However, we found significant differences for rs13263539 and rs1981361 of *GRHL2* in the minor allele frequency and the homozygous genotype too. The homozygous genotypes in both cases have a lower prevalence in the Roma population than in Hungarians. Furthermore, we did not find significant differences between healthy and deaf Roma populations in case of rs1799930 (*NAT2*), rs13263539 and rs1981361 (*GRHL2*) (Table 2).

Haplotype analysis was performed using the detected SNPs of *GRHL2*. Table 3 summarizes the determined eight haplotypes (ht) and their SNPs and the frequencies of the detected

Table 1 Genotypes and minor allele frequencies of *NAT2* rs1799930; *GRM7* rs11928865; *GRHL2* rs10955255, rs13263539 and rs1981361 polymorphisms in case of healthy Roma and Hungarian populations

NAT2	Roma ($n = 298$)	Hungarian (n = 298)
rs1799930		
GG	114 (38.3%)	162 (54.3%)
GA	142 (47.6%)	113 (38.0%)
AA	42 (14.1%)*	23 (7.7%)
A allele frequency	38%*	26.7%
GRM7	Roma (n = 298)	Hungarian (n = 298)
rs11928865		
AA	165 (55.4%)	165 (55.4%)
AT	113 (37.9%)	112 (37.6%)
TT	20 (6.7%)	21 (7.0%)
T allele frequency	25.6%	25.8%
GRHL2	Roma (n = 298)	Hungarian (n = 298)
rs10955255		
AA	66 (22.3%)	81 (27.2%)
AG	161 (54.0%)	154 (51.7%)
GG	71 (23.7%)	63 (21.1%)
G allele frequency	50.8%	47.0%
rs13263539		
GG	102 (34.2%)	69 (23.2%)
GA	157 (52.7%)	153 (51.3%)
AA	39 (13.1%)*	76 (25.5%)
A allele frequency	37.9%*	51%
rs1981361		
GG	87 (29.1%)	59 (19.8%)
GA	162 (54.4%)	143 (48.0%)
AA	49 (16.5%)*	96 (32.2%)
A allele frequency	43.6%*	56.2%

*$p < 0.05$ vs. Hungarians

Table 2 Genotypes and minor allele frequencies of *NAT2* rs1799930; *GRHL2* rs13263539 and rs1981361 polymorphisms in case of healthy and hearing impaired Roma populations

NAT2	Roma (n = 298)	Hearing impaired Roma (*n* = 113)
rs1799930		
GG	114 (38.3%)	45 (39.8%)
GA	142 (47.6%)	56 (49.5%)
AA	42 (14.1%)	12 (10.6%)
A allele frequency	38.0%	35.4%
GRHL2	Roma (n = 298)	Hearing impaired Roma (n = 113)
rs13263539		
GG	102 (34.2%)	33 (29.2%)
GA	157 (52.7%)	64 (56.6%)
AA	39 (13.1%)	16 (14.2%)
A allele frequency	37.9%	42.5%
GRHL2	Roma (n = 298)	Hearing impaired Roma (n = 113)
rs1981361		
GG	87 (29.2%)	23 (20.4%)
GA	162 (54.4%)	73 (64.6%)
AA	49 (16.4%)	17 (15.0%)
A allele frequency	43.6%	47.4%

*$p < 0.05$ vs. Hungarians

haplotypes in healthy Roma and Hungarian samples. The statistical analysis revealed significant differences in the prevalence rates of GGT, GAC and GAT haplotypes between the Roma and Hungarian populations ($p < 0.008$). The occurrence of GGT and GAC haplotypes were two-fold higher in Hungarian than in Roma samples. In addition, the presence of GAT haplotype was almost five-fold higher in Hungarian than in Roma populations.

Linkage analysis was performed using the detected SNPs. Figure 1 shows the linkage disequilibrium (LD) plot of *GRHL2* rs10955255, rs13263539 and rs1981361 polymorphisms in healthy Roma (A) and Hungarian (B) populations. The LD values were denoted as (|D'|×100). The linkage between the examined SNPs was stronger in Roma populations than in Hungarian samples. The LD values were 68, 70 and 85 in Hungarians and 98, 86 and 92 in Roma samples, respectively.

Discussion

ARHI is the loss of hearing that gradually occurs in most individuals as they grow older. Aging is caused by molecular physiological and biochemical changes, a decrease in mitochondrial function, progressive DNA deterioration, lower concentration of cellular water, reduced cellular membrane elasticity, ionic changes and vascular insufficiency [16, 17]. Many factors redound to aging inter alia genetic mutations with environmental interactions or a significant presence of ROS. It has been shown that in the aging cochlea, blood flow

Table 3 Major haplotypes (ht) and observed frequencies of the detected haplotypes in Roma and Hungarian populations

Haplotypes	SNP			Population	
	rs10955255	rs13263539	rs1981361	Roma (%)	Hungarian (%)
ht1	A	G	C	7.66	5.8
ht2	A	G	T	3.17	3.97
ht3	A	A	C	0.69	0.99
ht4	A	A	T	37.6	44.2
ht5	G	G	C	46.9	34.9
ht6	G	G	T	1.87*	4.47
ht7	G	A	C	0.91*	2.07
ht8	G	A	T	1.15*	5.51

*$p < 0.05$ vs. Hungarians

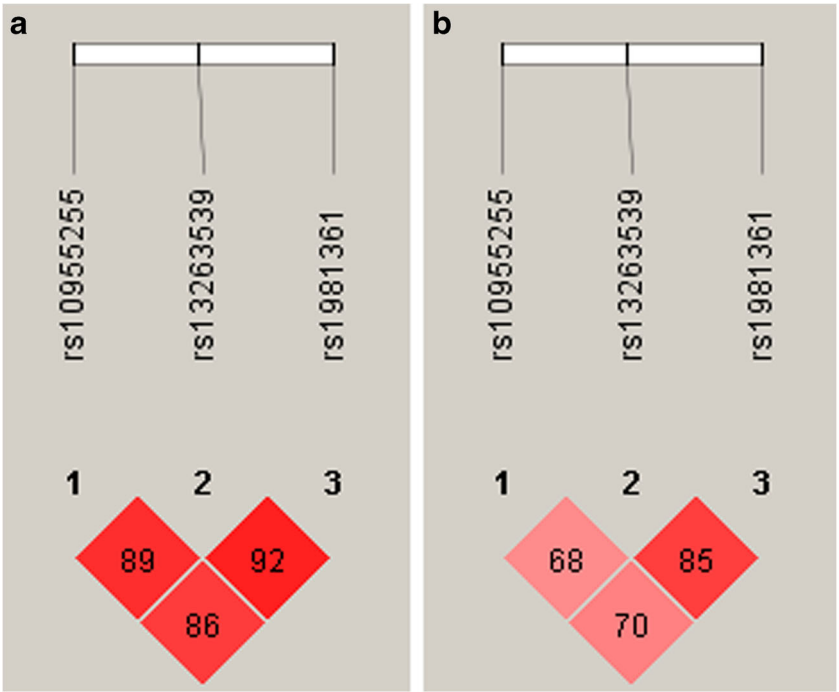

Fig. 1 Linkage disequilibrium analysis for the *GRHL2* rs10955255 (1), rs13263539 (2) and rs1981361 (3) polymorphisms in Roma (**a**) and Hungarian (**b**) populations

is reduced in the inner ear circulatory system [16, 18]. Several tissues in the cochlea react differently to the damaging effect of ROS [3]. Lautermann et al. stated that aging may cause changes in all tissues, but the enzyme activity does not change, and the quantity of glutathione was influenced in the auditory nerve only. Reducing tissue glutathione is a main factor that can damage cellular protection against the toxic effects of ROS and may cause peroxidative cell damage [19].

There are many genes which may contribute to presbycusis [4]. N-acetyltransferase (NAT) enzymes are known to be active in detoxifying xenobiotic toxins. There are 3 types of *NAT2* enzyme metabolism, fast, intermediate, and slow acetylator phenotypes. The mutant allele carriers have decreased acetyltransferase activity and are slow acetylators [3]. The NAT slow-acetylator status appears to be a risk factor of ARHI, several studies propose a significant association [7].

Table 4 Major and minor allele frequencies (af) of *NAT2* rs1799930; *GRM7* rs11928865; *GRHL2* rs10955255, rs13263539 and rs1981361 polymorphisms in case of different populations

Populations	n	NAT2 rs1799930		GRM7 rs11928865		GRHL2 rs10955255		GRHL2 rs13263539		GRHL2 rs1981361	
		G af (%)	A af (%)	A af (%)	T af (%)	A af (%)	G af (%)	A af (%)	G af (%)	C af (%)	T af (%)
EUR	1006	71,8	28,2	72,2	27,8	41,2	58,8	47,1	53,0	40,3	59,7
HUN	298	73,3	26,7	74,2	25,8	53,0	47,0	49,0	51,0	43,8	56,2
Roma	298	62,0	38,0	74,4	25,6	49,2	50,8	62,1	37,9	56,4	43,6
CEU	198	70,2	29,8	70,7	29,3	42,9	57,1	49,0	51,0	43,9	56,1
FIN	198	73,7	26,3	65,7	34,3	31,8	68,2	37,4	62,2	34,8	65,2
GBR	182	72,5	27,5	70,9	29,1	42,3	57,7	47,8	52,2	39,0	61,0
IBS	214	70,6	29,4	73,8	26,2	64,3	53,7	51,9	48,1	43,5	56,5
TSI	214	72,0	28,0	79,0	21,0	73,0	57,9	49,1	50,9	39,7	60,3
SAS	978	64,0	36,0	78,0	22,0	73,0	27,0	74,9	25,1	74,1	25,9
BEB	172	73,3	26,7	76,7	23,3	72,1	27,9	72,1	27,9	69,8	30,2
GIH	176	61,2	38,8	74,8	25,2	69,9	30,1	71,8	28,2	72,3	27,7
ITU	204	65,7	34,3	80,9	19,1	79,4	20,6	82,8	17,2	82,4	17,6
PJL	192	63,5	36,5	80,2	19,8	69,3	30,7	70,3	29,7	69,3	30,7
STU	204	57,8	42,2	77,5	22,5	74,0	26,0	77,0	23,0	76,0	24,0

A high rate of glutamate is neurotoxic, because of its stimulant properties. Glutamate toxicity is involved in different forms of hearing loss, for example in ARHI [20]. *GRM7* variants likely play a role in ARHI through excytotoxicity. Metabotropic glutamate receptors (mGluRs) reduce the release of glutamate. The autoregulation of the glutamate changes in the synaptic gap of the auditory neurons and inner hair cells, which leads to cell death because of the variant of *GRM7* [21].

Results of Bared et al. [7] showed that people with the high-risk genotype (heterozygous and homozygous genotypes) had worse hearing than those individuals with the low-risk (wild type) genotype. In the current study we aimed to reveal the interethnic differences in genotypes and variant allele frequencies of the *NAT2, GRM7* and *GRHL2* genes in the Roma population as compared to the Hungarian population. We observed differences in allele profiles of the examined *NAT2* rs1799930, *GRHL2* rs13263539, and rs1981361 variants between the Hungarian and Roma samples. Our study showed that the prevalence of carrying a mutant allele with an increased risk of ARHI is higher among Romas than among Hungarians in case of *NAT2* polymorphism. However, in case of *GRHL2* rs13263539 and rs1981361 the prevalence of the mutant allele is higher in Hungarian controls compared with Roma samples. Furthermore, the frequency of GGT, GAC and GAT haplotypes was significantly higher in the Hungarian population than in Roma (1.87 vs. 4.47%, 0.91 vs. 2.07% and 1.15 vs. 5.51%, respectively; $p < 0.008$).

The observed Roma and Hungarian major and minor allele frequency of all polymorphisms compared to previously published data from www.ensembl.org in various European and South Asian populations are shown in Table 4. In case of rs10955255 and rs11928865 we can detect, that the allele frequencies of Roma population are almost the same in all European populations. However, in case of rs1799930, rs13263539 and rs1981361 of Roma populations, the allele frequencies are more similar to in South African populations. These observations suggest, that the Roma population which originate from India, is mixed with the Hungarian population during the centuries. The observed populations are the following: European (EUR), Hungarian (HUN), Roma, Utah resident with Northern and Western European ancestry (CEU) , Finnish in Finland (FIN), British in England and Scotland (GBR), Iberian populations ins Spain (IBS), Toscani in Italy (TSI), South Asian (SAS), Bengali in Bangladesh (BEB), Gujarati Indian in Huston, TX (GIH), Indian Telugu in the UK (ITU), Punjabi in Lahore, Pakistan (PJL) and Sri Lankan Tamil in the UK (STU).

It is known from previous examples that a significant difference in minor allele frequency can be observed in Hungarian and Roma population samples of the *GJB2* gene rs104894396, which causes hearing impairment. The significant difference between the Roma and the Hungarian population may initiate personalized medical diagnostics and effective treatments [22].

Conclusion

NAT2, GRM7 and GRHL2 gene polymorphisms are known to be involved in age related hearing loss. Ethnic differences in the allele frequencies of the predisposing variants have important implications for the preventive and therapeutic treatments in different populations in the future. To determine the exact role of this susceptibility SNPs more studies are needed on larger population samples.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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