

Association of *TRPM3* Polymorphism (rs10780946) and Aspirin-Exacerbated Respiratory Disease (AERD)

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Abstract

Introduction Aspirin-exacerbated respiratory disease (AERD) refers to the combination of asthma rhinosinusitis and poliposis; ingestion of aspirin or other non-steroid anti-inflammatory drugs exacerbate asthma-like symptoms. The pathogenesis of AERD is unknown, and genetic and environmental factors contribute to the disease. Our objective is identifying polymorphisms associated with susceptibility in a Mexican mestizo population.

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Methods Primarily we performed custom Illumina gold-engage array-based genotyping of 1512 SNPs, carefully selected from a variety of acute/chronic inflammatory lung conditions previously reported. Four SNPs in *TRPM3* gene showed the lowest *p*-values (rs10780946, rs7025694, rs1889915, and rs7047645). We further selected rs10780946 and rs7025694 for validation using Taqman genotyping (*n* = 743; 288 AERD, 272 ATA, and 183 HC).

Results rs10780946 showed association when compared between AERD and ATA groups under co-dominant (*p* = 0.006), dominant (*p* = 0.002), overdominant (*p* = 0.01), and log-additive (*p* = 0.03) genetic models. AERD showed increased heterozygous TC (rs10780946–rs7025694) haplotype compared to ATA and HC (*p* < 0.05). We could not confirm any association between rs7025694 and AERD.

Conclusion rs10780946 *TRPM3* polymorphism is associated with AERD susceptibility.

Keywords AERD · Aspirin intolerance · Association study · Ca²⁺ channel · *TRPM3*

Introduction

Aspirin-exacerbated respiratory disease (AERD) is characterized by asthma with eosinophilic rhinosinusitis, nasal polyposis, and sensitivity to non-steroid anti-inflammatory drugs such as aspirin (Samter's triad). Patients with AERD develop nasal congestion, rhinorrhea, periorbital edema, and mild to severe asthma attacks along with other range of symptoms within minutes to 3 h of non-steroid anti-inflammatory drugs (NSAIDs) intake [1, 2]. About 20 % of asthma subjects, 30 % of asthma cases with nasal polyposis, and 60–80 % chronic sinusitis cases with nasal polyposis suffer from AERD [3, 4]. Although pathogenesis of the

disease is largely unknown, abnormal arachidonic acid metabolic cascade distortion is well elucidated along with a natural history inconsistent with aspirin-tolerant asthmatics (ATA) distinguishing AERD as a unique entity [1, 5]. Disease exacerbation usually presents itself as reduced prostaglandin E₂ (PGE₂) activity and elevated cysteinyl leukotrienes constitutively and post-aspirin ingestion, thus producing asthma-like symptoms [6, 7]. Reduced PGE₂ activity is thought to be due to under expression of EP2 receptor and signaling defects downstream the receptor [6, 8]. It is also worthwhile to note that the PGE₂ production is slightly higher in AERD compared to ATA subjects post-aspirin challenge [9]. Reduced PGE₂ receptor combined with signaling defects may block the translation of PGE₂ concentration to physiological activity, rendering AERD subjects more susceptible to CysLTs production/activity. Reduced PGE₂ activity can also lead to elevated 5-lipoxygenase (5-LOX) activity by increased 5-lipoxygenase activating protein (FLAP) translocation to the nucleus. Leukotriene C₄ synthase (LTC₄S), the rate-limiting enzyme involved in cysteinyl leukotriene production is overexpressed in AERD subjects [6]. These factors collectively contribute to hyperreactivity of CysLTs in AERD.

Even though eosinophils and mast cells are involved [10] in the disease progression as effector cells, their role is largely unknown. Attenuated PGE₂ activity along with COX 1 inhibition by NSAIDs reduce local inflammatory threshold leading to increased PMN infiltration and activation, predominantly eosinophils and mast cells [11]. Eosinophils and neutrophils are also shown to adhere with platelets, promoting transcellular CysLT production [12]. Most studies point to a TH2 cytokine profile in AERD. In contrast, recently IFN γ was shown to be highly expressed in sinus-derived eosinophils [13]. In this study, we investigate association of AERD with SNPs from genes involved in acute and chronic pulmonary inflammation (pilot study). Following the pilot study, two SNPs from *TRPM3* gene were selected for further validation.

Study Subjects

A total of 743 subjects were enrolled in the study, divided into three groups: 288 AERD patients, 272 ATA, and 183 healthy controls (HC). AERD cases were diagnosed by positive nasal aspirin challenge test using lysine aspirin as previously described [14]. A fall in FEV₁ >20 % from baseline was considered positive. ATA subjects were diagnosed by positive methacholine challenge test (American thoracic society guidelines [15]). HCs were enrolled from general population and were negative for nasal aspirin and methacholine challenge tests. Additionally HCs were checked for sinus/respiratory conditions using radiograms.

Venous blood was collected from the antecubital vein, kept for 30 min to 1 h at 4 °C. Serum was separated by centrifuging at 1500 g for 15 min and stored at –80 °C until further use. Serum IgE levels was determined with uniCap system (Phadia, Valinge, Sweden), following manufacturers instructions. Atopic status was determined using skin prick test using 24 common aeroallergens.

All study subjects were Mexican mestizos, recruited from Immunogenetic and Allergy Department at Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas (INER). The institutional ethical committee approved the study (approval number B14-12) and all subjects provided an informed consent.

Genomic DNA Extraction

Genomic DNA was extracted from peripheral blood using BDtract DNA isolation kit (Maxim Biotech, San Francisco, CA, USA). All DNA samples were stored indefinitely at –80 °C in Tris–EDTA buffer (10 mM Tris–HCl, 2 mM EDTA, pH 8.0) until further use.

SNP Selection and Goldengate Genotyping

An illumina 1536 SNP custom goldengate array was used to genotype 48 AERD patients and 48 HC. The SNPs were selected from a variety of chronic/acute pulmonary inflammatory conditions carefully selected among previous reports from literature.

The selection criteria of SNPs were based on minor allele frequency (MAF) >7 % and Hardy–Weinberg (HWE, $p \geq 1E - 04$) in Mexican Mestizo population. The genomic data regarding mexican mestizos were obtained from the Mexican genome diversity project (MGDP). We included 458 individually associated SNPs with various pulmonary inflammatory diseases based on available literature. Those genes with the most SNP associations were given preference over others. The SNPs included came from 24 candidate genomic regions spanning over 14 chromosomes. Rest of the SNPs included were tag SNPs which were located within a minimum and maximum distance between 60 and 200 bp of each included SNPs, respectively. A total of 825 tagSNPs were included.

The population ancestry was assessed using four reference populations, Caucasian (CEU), East Asian (EA), African (YRI) based on international HapMap project and Amerindian population MGDP (NATIVE). 253 ancestry informative markers (AIMs) were included, which had a minimum difference of $\Delta \geq 0.3$ allele frequencies for each pair of reference population.

253 ancestral SNPs were also used to confirm the genomic homogeneity of studied subjects. 3–6 ng of total DNA was used for genotyping. The genotyping was performed adhering to manufacturer's protocol (Illumina, San Diego, CA, USA) [16]. Only samples with >95 % call rate (genome studio) were included in the study.

Genotyping and Quality Control

Genotyping was conducted using the protocol designed by Illumina for the goldengate platform (Illumina, Inc., San Diego CA, USA) using a Tecan robotic automatic liquid dispenser (Tecan, Trading AG, Switzerland), which operates under the Illumina protocol. The microarrays were read on the BeadArray Reader scanner (Illumina, Inc., San Diego CA, USA). Genotype acquisition and generation of documentation (.ped and .map files) were conducted using the GenomeStudio 2011 v1.0 software (Illumina, Inc., San Diego CA, USA). Subjects who did not comply with the Call Rate criteria (<95 %) were excluded.

Stage 2 Validation

In the validation stage were genotyped SNPs that according to stage 1 proved significantly associated; genotyping was performed by allelic discrimination, were employed Taqman pre-designed probes in a real-time PCR thermocycler (7300 Real-Time PCR Systems, Applied Biosystems, CA, USA).

Conditions of the PCR assay for each probe were: Pre-read 50 °C, 1 min; absolute quantitation: 50 °C, 2 min, 1 cycle; 95 °C, 10 min, 1 cycle; 95 °C, 15 s, 60 °C 1 min, 40 cycles; post-read 50 °C, 1 min. The assignment of genotypes was performed considering the allelic discrimination and confirmed by absolute quantitation; in addition three controls without template (contamination controls) were

included for each genotyping plate and 1 % of the samples included in the study were genotyped in duplicate as control allele assignment. Data interpretation was conducted by Sequence Detection Software (SDS v. 1.4, Applied Biosystems, CA, USA). The fluorophores used were VIC for allele A and FAM for allele B.

SDS software v.2.4 was used for post processing and genotype calls. Samples with call rates <90 % were repeated and reanalyzed. Each Taqman plates were prepared with proportionately equal samples of each group and distributed evenly within the plate. Each Taqman plates were also duplicated and the results were compared. Samples with discordant results were either eliminated or repeated.

Statistical Analysis

HCs were tested for Hardy–Weinberg equilibrium (HWE) employing χ^2 test. t test was used to compare continuous demographic parameters between groups. Categorical variables were compared using χ^2 test (gender, atopy). PLINK software was used to analyze goldengate data [17]. Taqman genotype data were analyzed using SNPstats [18] multiple inheritance models accounting for age and sex covariates (logistic regression). The stringency of the test was further improved by rounding the *p*-values to a single digit after zeros. For e.g., if the *p*-value is 0.045, rounding the *p*-value gives 0.05. All the plots were drawn using R software.

Results

The clinical characteristics of AERD, ATA, and HCs are presented in Table 1. Atopy was less frequent in AERD compared to ATA (*p* < 0.01) but not with HCs (*p* = 0.68). The AERD patients had a significantly higher serum total IgE levels compared to HCs (*p* < 0.01) whereas ATAs had

Table 1 Demographics of study subjects

Characteristics (<i>n</i> = 743)	AERD (<i>n</i> = 288)	ATA (<i>n</i> = 272)	NC (<i>n</i> = 183)	<i>p</i> -value		
				AERD versus ATA	ATA versus NC	AERD versus NC
Age (years)	44.5 ± 22.6	39.1 ± 9.4	30.1 ± 10.7	<0.01	<0.01	<0.01
Gender (male %)*	178 (70 %)	173 (64 %)	119 (65 %)	0.72	0.83	0.54
Atopy (%)*	29.50 %	65.10 %	27.30 %	<0.01	<0.01	0.68
Asthma onset (years)	34.6 ± 23.5	NA	NA	NA	NA	NA
FEV1 (%)	86.5 ± 22.5	89.7 ± 20.3	96.5 ± 11.4	0.75	<0.01	<0.01
Serum total IgE (IU/ml)	178.3 ± 197.8	320 ± 394.9	99.6 ± 52.6	<0.01	<0.01	<0.01

Bold values indicate statistical significance (*p*-value < 0.05)

NA not available, HC Healthy controls, ATA aspirin-tolerant asthmatics, AERD aspirin-exacerbated respiratory disease, FEV1 forced expiratory volume

* *p*-value determined by Pearson's χ^2 test

significantly high serum total IgE levels compared to AERD ($p < 0.01$).

With the purpose of identify potential SNPs in AERD, a pilot study was undertaken in first place employing Illumina goldengate custom microarray with 1536 SNPs (the complete list of goldengate SNP analysis and raw data are available as supplementary material 1): we observed four SNPs with

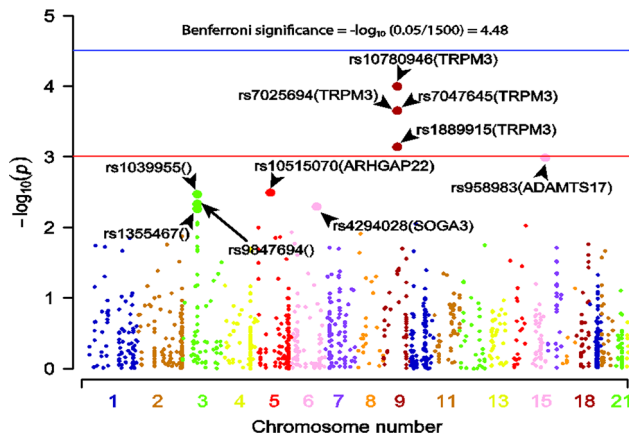


Fig. 1 Goldengate SNP genotyping results from 48 AERD versus 48 HC. [A] Manhattan plot (top 10 SNPs with lowest p -values are highlighted). The blue line shows the Bonferroni significance p -value

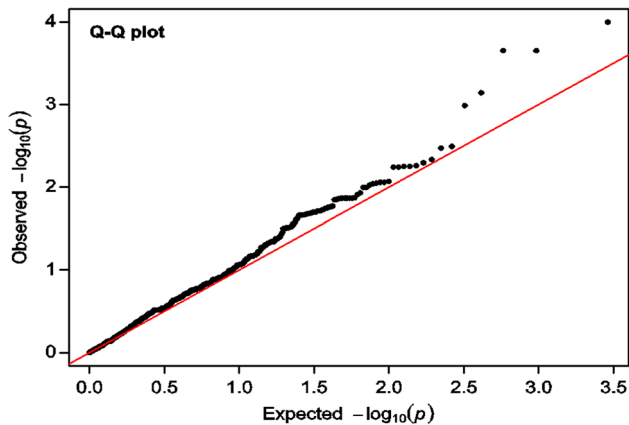


Fig. 2 A Q-Q plot of observed and expected p -values of goldengate SNPs

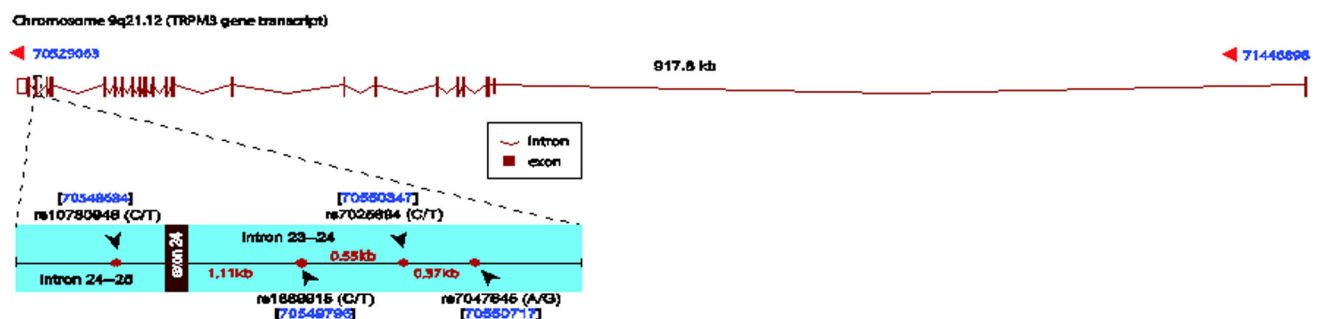


Fig. 3 4 SNPs with the lowest p -value are located in *TRPM3* gene

lowest p uncorr (rs1078094— 1×10^{-4} , rs7025694— 2.2×10^{-4} , rs7047645— 2.2×10^{-4} , rs1889915— 7.2×10^{-4}) values that were located in intron 23–24 and 24–25 of transient receptor potential cation channel subfamily M member 3 (*TRPM3*) gene (Figs. 1, 2, 3). However, Bonferroni correction failed to reach statistical significance may be due to small sample size ($n = 48$ AERD patients). Thus, we next investigated rs1078094 and rs7025694 in a larger population ($n = 743$; 288 AERD, 272 ATA and 183 HC) using Taqman genotyping. Interestingly, rs1078094 showed association when compared between AERD and ATA groups under co-dominant ($p = 0.006$), dominant ($p = 0.002$), overdominant ($p = 0.01$), and log-additive ($p = 0.03$) genetic models (Table 2). Whereas ATA when compared with HC did not show any association. The SNP rs7025694 did not exhibit any association when compared between groups (Table 3). Linkage disequilibrium (LD) among the SNP pairs was measured using Lewontin's value D' (0.9915) and LD coefficient r^2 (0.7481). AERD showed increased heterozygous TC haplotype compared to ATA and HC ($p < 0.05$). Interestingly CT haplotype was only present in ATA but not in AERD or HC (HF = 0.41 %, rare) (Fig. 4).

Discussion

In the present study, we evaluated association of two SNPs from *TRPM3* in Mexican population, employing three study groups AERD, ATA, and HC. The two SNPs (rs10780946 and rs7025694) were selected based on Illumina goldengate association results comparing AERD and HC groups. To our knowledge, this is the first study to explore the association of these intronic polymorphisms in both ATA and AERD.

Inflammation underlying AERD is complex and involve much more than abnormal arachidonic acid metabolism. The immunological and cellular components, even though not well elucidated are prominent in disease sustenance and progression [19]. Although tissue-resident eosinophil activation (both transient and progressive) is known to occur in

Table 2 *TRPM3* rs10780946 (C/T) genotype frequencies (adjusted by sex + age)

<i>p</i> -value	OR (95 % CI)	AERD	ATA	Genotype	Model	Genotype	HC	ATA	OR (95 % CI)	<i>p</i> -value
0.006	1	84 (37.7 %)	63 (25.2 %)	T/T	Co-dominant	T/T	53 (30.6 %)	63 (25.2 %)	1	0.1
	0.50 (0.32–0.77)	89 (39.9 %)	127 (50.8 %)	C/T		C/T	72 (41.6 %)	127 (50.8 %)	1.57 (0.95–2.60)	
	0.60 (0.36–1.00)	50 (22.4 %)	60 (24 %)	C/C		C/C	48 (27.8 %)	60 (24 %)	1.06 (0.60–1.86)	
0.002	1	84 (37.7 %)	63 (25.2 %)	T/T	Dominant	T/T	53 (30.6 %)	63 (25.2 %)	1	0.2
	0.53 (0.36–0.79)	139 (62.3 %)	187 (74.8 %)	C/T–C/C		C/T–C/C	120 (69.4 %)	187 (74.8 %)	1.36 (0.86–2.17)	
0.7	1	173 (77.6 %)	190 (76 %)	T/T–C/T	Recessive	T/T–C/T	125 (72.2 %)	190 (76 %)	1	0.4
	0.91 (0.59–1.41)	50 (22.4 %)	60 (24 %)	C/C		C/C	48 (27.8 %)	60 (24 %)	0.80 (0.50–1.28)	
0.01	1	134 (60.1 %)	123 (49.2 %)	T/T–C/C	Over dominant	T/T–C/C	101 (58.4 %)	123 (49.2 %)	1	0.05
	0.62 (0.43–0.90)	89 (39.9 %)	127 (50.8 %)	C/T		C/T	72 (41.6 %)	127 (50.8 %)	1.53 (1.01–2.33)	
0.03	0.75 (0.58–0.97)	NA	NA	NA	Log-additive	NA	NA	NA	1.04 (0.78–1.38)	0.8

NA not available, HC healthy controls, ATA aspirin-tolerant asthmatics, AERD aspirin-exacerbated respiratory disease

Table 3 *TRPM3* rs7025694 (T/C) genotype frequencies (adjusted by sex + age)

<i>p</i> -value	OR (95 % CI)	AERD	ATA	Genotype	Model	Genotype	HC	ATA	OR (95 % CI)	<i>p</i> -value
0.9	1	58 (24.8 %)	64 (24.7 %)	C/C	Co-dominant	C/C	50 (28.9 %)	64 (24.7 %)	1	0.2
	1.02 (0.66–1.58)	134 (57.3 %)	143 (55.2 %)	C/T		C/T	82 (47.4 %)	143 (55.2 %)	1.41 (0.86–2.31)	
	0.93 (0.54–1.62)	42 (17.9 %)	52 (20.1 %)	T/T		T/T	41 (23.7 %)	52 (20.1 %)	0.93 (0.52–1.69)	
1.0	1	58 (24.8 %)	64 (24.7 %)	C/C	Dominant	C/C	50 (28.9 %)	64 (24.7 %)	1	0.4
	1.00 (0.65–1.52)	176 (75.2 %)	195 (75.3 %)	C/T–T/T		C/T–T/T	123 (71.1 %)	195 (75.3 %)	1.25 (0.78–1.98)	
0.7	1	192 (82 %)	207 (79.9 %)	C/C–C/T	Recessive	C/C–C/T	132 (76.3 %)	207 (79.9 %)	1	0.3
	0.92 (0.58–1.46)	42 (17.9 %)	52 (20.1 %)	T/T		T/T	41 (23.7 %)	52 (20.1 %)	0.75 (0.45–1.23)	
0.8	1	100 (42.7 %)	116 (44.8 %)	T/T–C/C	Over dominant	T/T–C/C	91 (52.6 %)	116 (44.8 %)	1	0.8
	1.05 (0.73–1.51)	134 (57.3 %)	143 (55.2 %)	C/T		C/T	82 (47.4 %)	143 (55.2 %)	1.45 (0.96–2.20)	
0.8	0.97 (0.74–1.28)	NA	NA	NA	Log-additive	NA	NA	NA	0.99 (0.73–1.33)	0.9

NA not available, HC Healthy controls, ATA aspirin-tolerant asthmatics, AERD aspirin-exacerbated respiratory disease

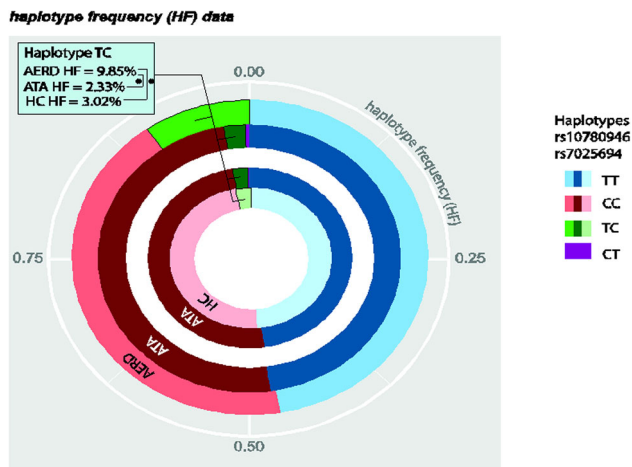


Fig. 4 Haplotype frequency of AERD versus ATA and ATA versus HC plot showing TC haplotype significantly high in AERD compared to both ATA and HCs. * p -value <0.05

AERD, the role of mast cells is less clear [20–22]. However, exacerbations often produce considerable quantities of PGD2 and histamine, indicating mast cell activation [23, 24]. Specifically, over expression of LTC4S is the defining feature of AERD, which is prominent in eosinophils but modest in mast cells [20]. Eosinophils along with neutrophils also have the potential to generate huge concentrations of CysLTs via adherent platelets rich in LTC4S [10]. Such cellular cooperative behaviors need to be explored further in case of mast cells.

In the present study, 1536 SNPs were investigated in AERD patients using a custom goldengate array. We observed four SNPs located in intron 23–24 and 24–25 of *TRPM3* gene (Fig. 3) with lowest p uncorrected although under the stringent Bonferroni correction failed to reach statistical significance because of small sample size. However, validation studies in a larger population demonstrated that rs1078094, has association with AERD, but not with ATA. *TRPM3* is a non-selective cation channel mainly involved in Ca^{2+} flux regulated by osmotic cell swelling, D-erythro-sphingosine, and pregnenolone sulfate [25–27]. It is the largest gene located on chromosome 9 with around 30 exonic regions separated by large intronic sequences [28]. Numerous splice variants are formed from *TRPM3* gene with differential length and functional variability. A splice variant *TRPM3* α 1 is poorly permeable to divalent cations, whereas *TRPM3* α 2 variant readily conducts Ca^{2+} and Mg^{2+} [27]. Recent findings show that aspirin possesses the potential to activate calcium channels in eosinophils and mast cells thereby activating them [29]. However, the study did not find any variation in AERD when compared with HC. It is also interesting that the expression of *TRPM3* is high in lung tissue.

Even though rs1078094 does not fall directly on a splice site, it is very close to one. It may also act as a splicing cis-

regulatory element. However, such a role is unknown at present. The SNPs location also shows strong linkage disequilibrium in the region. In a previous report, an intronic SNP in *ALOX5* was found to be associated with both asthma exacerbations and the patients response to montelukast treatment (rs2115819), suggesting that intronic SNPs may have an impact on disease [30].

In summary, the present study has identified an SNP in the intronic region of the *TRPM3* gene (rs1078094) in AERD patients. A linkage disequilibrium measurement showed increased heterozygous TC haplotype in AERD. Future functional studies will define the role of rs1078094 in AERD.

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Compliance with Ethical Standards

Conflict of Interest None.

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