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ARTICLE

Fetal polymorphisms at the *ABCB*1-transporter gene locus are associated with susceptibility to non-syndromic oral cleft malformations

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ATP-binding cassette (ABC) proteins in the placenta regulate fetal exposure to xenobiotics. We hypothesized that functional polymorphisms in *ABC* genes influence risk for non-syndromic oral clefts (NSOC). Both family-based and case–control studies were undertaken to evaluate the association of nine potentially functional single-nucleotide polymorphisms within four *ABC* genes with risk of NSOC. Peripheral blood DNA from a total of 150 NSOC case-parent trios from Singapore and Taiwan were genotyped, as was cord blood DNA from 189 normal Chinese neonates used as controls. In trios, significant association was observed between the *ABCB1* single-nucleotide polymorphisms and NSOC ($P < 0.05$). Only *ABCB1* rs1128503 retained significant association after Bonferroni correction (odds ratio (OR) = 2.04; 95% confidence interval (CI) = 1.42–2.98), while rs2032582 and rs1045642 showed nominal significance. Association with rs1128503 was replicated in a case–control analysis comparing NSOC probands with controls (OR = 1.58; 95% CI = 1.12–2.23). A comparison between the mothers of probands and controls showed no evidence of association, suggesting NSOC risk is determined by fetal and not maternal *ABCB1* genotype. The two studies produced a combined OR of 1.79 (95% CI = 1.38–2.30). The T-allele at rs1128503 was associated with higher risk. This study thus provides evidence that potentially functional polymorphisms in fetal *ABCB1* modulate risk for NSOC, presumably through suboptimal exclusion of xenobiotics at the fetal–maternal interface.
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INTRODUCTION

The placenta represents the first fetal organ exposed to exogenous substances including drugs and potential fetotoxins present in maternal blood circulation.[1] The placenta is formed by both fetal (chorionic plate and chorionic villi) and maternal (decidua basalis) tissues. The fetal placenta consists of syncytiotrophoblast and cytotrophoblast (or Langerhans) layers. Transporter proteins, such as ATP-binding cassette (ABC-transporter) are located at apical and basolateral surfaces of the syncytiotrophoblast and endothelial cells of fetal capillaries.[2] These proteins are able to efflux environmental toxicants or drugs ingested by the mother into the maternal circulation. Therefore, the expression of transporter proteins within these structures is important in protecting the fetus from toxic xenobiotics. Expression and activity of these proteins in the placenta depend on gestational age and genetic polymorphisms. Functional single-nucleotide polymorphisms (SNPs) in *ABC*-transporter genes may influence expression and activity of these proteins in the placenta, leading to altered fetal exposure to xenobiotics, and subsequent increase in the risk of complex genetic disorders or birth defects.

Orofacial clefts (ie, cleft lip, cleft lip and palate, or cleft palate alone) are common craniofacial anomalies with an incidence of 1.0/1000–2.21/1000.[3] In Asians, the prevalence rate of non-syndromic cleft lip with or without cleft palate has been reported as 1.19 per 1000 live births.[4]

Fetal lip development is completed at ∼5 to 6 weeks of gestational age, and the palate will close ∼10 weeks after gestation. Being a complex genetic disorder, orofacial malformations can be influenced by both genetic and environmental factors. It has been hypothesized that a cleft lip/palate forms when potential environmental risks, such as maternal cigarette smoking and, possibly, disruptions in the folate biosynthetic pathway[5] interact with a genetically susceptible genotype, during the early stage of fetal development. This is a critical period when fusion of embryonic processes forming the upper lip and palate takes place. Studies have shown a positive association between formation of cleft lip/palate and maternal medication,[6] maternal smoking,[7] and alcohol use.[8] Moreover, maternal infection,[9] and folic acid deficiency[10] may also contribute to orofacial malformations. Hence, we hypothesized that functional polymorphisms within *ABC*

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genes expressed in placenta can potentially increase the risk for orofacial clefts through impairing the protective role of the placenta. Here, we examine the association between important functional polymorphisms in four major members of ABC-transporter family, *ABCB1*; *ABCC1*; *ABCC2*; *ABCG2*, with non-syndromic orofacial clefts.

MATERIALS AND METHODS
Study Design and Subjects
This study used two formats of family-based and case–control studies. As the prevalence of non-syndromic oral clefts (NSOC) in Asians has been reported to be as low as 1.19 per 1000 live births,[4] and trio designs are more powerful than case–control designs for rare diseases with prevalence of 0.1%,[11] we first performed the family-based analysis. The Quanto program[12] was used to estimate the number of case-parent trios needed to detect genes exerting modest to strong levels of relative risks. Thus, a total of 150 case-parent trios were recruited through treatment centers in Singapore (KK Women's and Children's Hospital) and Taiwan (Chang Gung Memorial Hospital) with prior approval from the respective Institutional Review Boards (IRBs). They consisted of 128 Chinese, 10 Malay and 4 Indian trios, with an additional 8 trios of mixed or other ethnicity.

SNPs showing significant association with NSOC in the family-based study were validated in a case–control study. For the case–control analysis, 128 affected cases and 129 mothers of Chinese ethnicity from the trios were separately compared against unrelated controls consisting of 189 healthy Chinese neonates. Non-Chinese cases and mothers were excluded to minimize confounding due to population stratification and differences in genetic background. Ethnicity of the samples was confirmed via patient's medical files and hospital records.

SNP Selection
Nine potentially functional SNPs were selected from the four *ABC*-transporter genes to be evaluated for association with NSOC in our family-based study. SNPs showing significant association with NSOC were selected for case–control analysis. The selected SNPs have either been previously reported to be associated with drug response/function, or showed evidence of recent positive selection, or were reported to affect transcriptional regulation/splicing or the protein structure/function. Table 1 provides detailed information on the SNPs used in this study. Of note, we selected the three most commonly studied *ABCB1* SNPs (rs1128503, rs2032582, rs1045642), which are in strong linkage disequilibrium with high minor allele frequencies (>10%) in Chinese, Malays and Indians.[13,14]

Genotyping
Using extracted DNA, SNP genotyping was performed using multiplex minisequencing and TaqMan SNP genotyping assays. In addition, DNA sequencing was employed to validate any borderline or ambiguous genotype data obtained from the multiplex minisequencing or TaqMan assays.

A single triplex PCR was designed to amplify fragments of *ABCB1*, *ABCC1*, and *ABCG2* containing the three SNPs, rs2032582, rs504348, and rs2231137, respectively. Triplex PCR amplifications were carried out in a reaction volume of 10 μl containing PCR buffer (Qiagen, Valencia, CA, USA), 1.5 mM MgCl$_2$, 0.5 ng of genomic DNA, 200 μM of each dNTP, 0.2 μM of *ABCB1*, 0.4 μM of *ABCG2*, 0.5 μM of *ABCC1* primers, and one unit of HotStarTaq Polymerase (Qiagen) (PCR primer sequences are available in Supplementary Table S1). PCR conditions were as follows: initial cycle of 95 °C for 15 min, followed by 35 cycles of 94 °C for 1 min, 65 °C for 30 s, and 72 °C for 1 min, and a final cycle of 72 °C for 5 min. After verifying the PCR products on 1.5% agarose gel, PCR products were purified and subjected to multiplex minisequencing (minisequencing primer sequences are available in Supplementary Table S2). Genotyping of the remaining six SNPs was accomplished using the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA).

To validate borderline/ambiguous results obtained from TaqMan or minisequencing assays, genotyping was repeated by sequencing the DNA fragments containing the SNPs in the forward or reverse direction using the ABI PRISM BigDye V3.1 sequencing kit (Applied Biosystems). For *ABCB1* rs2032582 and *ABCG2* rs2231137, 0.16 μM of the reverse PCR primer and 0.16 μM of the forward PCR primer were used, respectively. For *ABCC2* rs2273697, the DNA fragment of interest was PCR-amplified in a reaction

Table 1 Selected potentially functional ABC SNPs.

Gene	SNP	Reference SNP no.	Description	Association with function or disease phenotype	Reference numbers
	e12/C1236T	rs1128503	G412G	Increased exposure to irinotecan in cancer patients	36
				Altered ABCB1 expression in 3t3 isogenic fibroblasts	33
				Lower ABCB1 expression	34
ABCB1	e21/G2677T e21/G2677A	rs2032582	A893S A893T	Less placental trophoblast ABCB1 expression in T and A carriers	29
	e26/C3435T	rs1045642	I1145I	Alteration in the rate of translation	37
				Lower ABCB1 expression	34
				Affects mRNA stability	38
ABCC1	5'FR/G-260C	rs504348	5'UTR	Evidence of recent positive selection in European-Americans	39
ABCC2	e1/C-24T	rs717620	−24C>T	Influenced ABCC2 mRNA expression in normal kidney cortex and liver samples but not placenta	22,30,39
				Evidence of recent positive selection	39
	e10/G1249A	rs2273697	V471I	Decreased ABCC2 mRNA levels in preterm placentas	22
				Evidence of recent positive selection	39
ABCG2	e5/C421A	rs2231142	Q141K	Reduced ABCG2 activity	40
				Decreased ABCG2 protein level in placenta	41
				Evidence of recent positive selection	39
				Substrate recognition and/or transport of drugs	42
	e2/G34A	rs2231137	V12M	Reduced ABCG2 activity	40
				Substrate recognition and/or transport of drugs	42
	I9/T-357C	rs2054576	Intron	Alteration of pre-mRNA splicing	43

volume of 10 μl containing 1 ng of genomic DNA, 5 μl 2× PCR master mix buffer (Qiagen), and 0.5 μM of each primer (information of PCR primers are available in Supplementary Table S3). PCR conditions were as follows: initial cycle of 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, and a final cycle of 72 °C for 5 min. The PCR product was then sequenced using 0.16 μM of the PCR reverse primer.

Statistical Analysis

Genotype data from the oral cleft patients and their biological parents were assessed for Mendelian consistency within each family, as well as for deviation from Hardy–Weinberg equilibrium (HWE) among the parents. Family-based analyses were performed using the transmission disequilibrium test (TDT) for biallelic SNPs, while the extended TDT (ETDT) developed by[15] was used for tri-allelic SNP rs2032582. Alpha level was set to 0.05.

Case–control comparisons were performed using the χ^2-test, and odds ratios (ORs), as well as 95% confidence intervals (CIs) were calculated. An online software http://ihg.gsf.de/cgi-bin/hw/hwa1.pldeveloped by T.M. Strom and T.F. Wienker, was employed to check for deviations from HWE and also to test for allelic and genotypic associations with NSOC under different genetic models, and alpha level was set to 0.05.

To provide an overall estimate of the effect size of genetic risk found for NSOC in our family-based and case–control studies, we performed a combined analysis on the results of the two studies using the method described previously for integrated analysis on family-based and case–control studies.[16]

RESULTS

Family-Based Study

No deviation from HWE was observed for any of the SNPs examined in this study. TDT analysis of the nine selected SNPs in 150 trios revealed only *ABCB1* SNPs rs1128503, rs2032582, and rs1045642 were significantly associated with NSOC ($P < 0.05$), although among these only rs1128503 retained its significance after Bonferroni correction for multiple testing (Table 2). For rs1128503 (C>T) and rs1045642 (C>T), further allelic analysis revealed a preferential transmission of

their minor alleles (T) over the wild-type alleles (C) (Table 2). For the tri-allelic SNP rs2032582 (G>T, A), a χ^2-statistic test (df = 1) showed preferential transmission of the T and A alleles over the G-allele from heterozygous parents to the NSOC probands. After correction for multiple testing, however, only the T-allele (not the G or the A alleles) reached statistical significance ($P < 0.05$) (Table 3).

Case–Control Study

We performed two sets of case–control comparisons to validate the family-based associations observed with SNPs rs1128503, rs2032582, and rs1045642. Firstly, the allele and genotype frequencies of the SNPs were compared between the Chinese NSOC probands ($n = 128$) and the healthy neonate controls ($n = 189$). The genotype distribution of the three SNPs were in HWE in the control group ($P > 0.05$). χ^2-Analysis revealed significant allelic association between NSOC and *ABCB1* rs1128503, but not with rs2032582 or rs1045642 (Table 4A). Armitage's trend test produced a similar result (see Supplementary Table S4). Moreover, genotype-based analysis of rs1128503 revealed a higher risk for NSOC in carriers of at least one T-allele compared with the homozygous CC genotype (OR = 2.32; 95% CI = 1.09–4.91; $P = 0.025$). Conversely, a lower risk was observed for carriers of at least one C-allele compared with the homozygous TT genotype (OR = 0.62; 95% CI = 0.40–0.97; $P = 0.038$).

Secondly, the allele and genotype frequencies of the *ABCB1* SNPs were compared between Chinese mothers of NSOC probands ($n = 129$) and the neonate controls ($n = 189$), which disclosed no significant association between NSOC and any of the SNPs (Table 4B).

Joint Analysis

The joint analysis revealed evidence of homogeneity in ORs obtained from family-based and case–control studies for association of *ABCB1* rs1128503 with NSOC. Integrating the ORs from both studies, we

Table 2 Family-based TDT analysis.

Gene	SNP	Reference SNP no.	n1	n2	Transmitted/ Not-transmitted	P-value Observed	P-value Corrected[a]	OR	95% CI
ABCB1	e12/C1236T	rs1128503	150	108	T: 92/45 C: 45/92	**5.1 × 10⁻⁵**	**4.6 × 10⁻⁴**	2.04	1.45–2.99
	e21/G2677T e21/G2677A	rs2032582	150	111	G: 78/91 T: 96/64 A: 23/42	**0.008**	0.072	1.50[b]	1.08–2.09
	e26/C3435T	rs1045642	150	110	C: 56/87 T: 87/56	**0.009**	0.081	1.55	1.10–2.21
ABCC1	5'FR/G-260C	rs504348	150	7	G: 03/04 C: 04/03	0.705	–	1.33	0.23–9.10
ABCC2	e1/C-24T	rs717620	150	76	C: 41/50 T: 50/41	0.345	–	1.24	0.811.93
	e10/G1249A	rs2273697	150	53	G: 34/22 A: 22/34	0.107	–	1.55	0.88–2.77
	e5/C421A	rs2231142	150	110	C: 70/62 A: 62/70	0.486	–	1.13	0.79–1.62
ABCG2	e2/G34A	rs2231137	150	105	G: 54/71 A: 71/54	0.127	–	1.32	0.91–1.91
	I9/T-357C	rs2054576	150	91	T: 59/51 C: 51/59	0.445	–	1.16	0.78–1.72

Abbreviations: CI, confidence Interval; OR, odds ratio; n1, number of family samples which were successfully genotyped; n2, number families with at least one heterozygote parent.
P-value of <0.01 are highlighted in bold.
[a]After adjustment for multiple comparisons (N = 9).
[b]OR for T-allele.

obtained a combined OR = 1.79 (95% CI = 1.38–2.30; *P*-value <0.0001) for association between rs1128503 and NSOC.

DISCUSSION

During fetal development, modulation of maternal exposure to environmental factors, as well as modulation of fetal exposure to potentially toxic xenobiotics digested by the mother is vital. The ABC-transporter proteins ABCB1, ABCC1, ABCC2, and ABCG2 are known to be expressed in maternal barrier organs (intestine, kidney, liver, lung) and the fetal barrier organ (placenta). ABCB1 is highly expressed in human placenta during early gestation,[17–19] while ABCG2 is expressed more in mid gestation.[20] During late gestation, ABCC1[21] and ABCC2[22] show the highest placental expression. Therefore, we can speculate that ABCB1 is vital in protecting the early fetus from xenobiotics insult,[23,24] such as maternal pharmacotherapy and even fetal waste products, while ABCC1, ABCC2, and ABCG2 can provide better protection to the mature fetus. Given lip and palate formation is completed by the first trimester of pregnancy, the role of placental ABCB1 appears to be most crucial. This may explain why we observed association of oral clefting with only *ABCB1*, and not *ABCC1*, *ABCC2*, or *ABCG2*, SNPs (*P* > 0.05).

The *ABCB1* gene has been associated with cleft palate in the mouse model. Lankas *et al*[25] examined homozygote (− / −) and heterozygote (+ / −) *ABCB1* knockout mice exposed to a teratogenic agent (avermectin), and found homozygote − / − fetuses were 100% susceptible to cleft palate when exposed. In contrast, heterozygotes (+ / −) were less sensitive (30% of fetuses had cleft palate) and homozygotes + / + fetuses were insensitive at the tested doses. Hence, ABCB1, which is present at the maternal–fetal interface, may have an important role in regulating the traffic of teratogenic agents across the placenta, and the degree of fetal exposure to chemicals could be dependent on the expression of placental ABCB1. In this regard, homozygous *ABCB1* knockout mice showed many fold higher transplacental transport of ABCB1 substrates such as digoxin, saquinavir, and paclitaxel compared with wild-type mice.[26]

It is possible fetal ABCB1 has a crucial role during the formation of lips and palate by minimizing fetal exposure to potentially fetotoxic substances. Fetal *ABCB1* polymorphisms could increase the risk of NSOC by altering ABCB1 expression or activity. Interestingly, the mean expression of ABCB1 has been found to be two times higher in the early (13–14 week) compared with the late (full-term) gestation human placenta, which is mostly fetal in origin.[17–19]

We observed significant association with *ABCB1* SNPs rs1128503, rs2032582, and rs1045642, although only rs1128503 remained significant after Bonferroni correction. It should be noted, however, that SNPs, rs1128503, rs2032582, and rs1045642, are not independent but are in strong linkage disequilibrium,[14] therefore application of

Table 3 Transmission of *ABCB1* rs2032582 alleles.

Allele	A	G	T
Number of times transmitted (T)	23	78	96
Expected value for T[a]	32.5	84.5	80
Number of times not-transmitted (NT)	42	91	64
Expected value for NT[a]	32.5	84.5	80
Observed *P*-value	0.019	0.317	0.011
Bonferroni corrected *P*-value	0.057	0.951	**0.033**

[a]Expected values were calculated as (T + NT)/2. *P*-value of <0.05 is indicated in bold.

Table 4 Case–control χ^2-analysis.

Gene	SNP	Subjects (n)	Genotype frequencies						HWE P-value	Allele frequencies(n (%))			Allelic association P-value	OR (95% CI)
(A) Oral cleft probands vs controls														
ABCB1	e12/C1236T		TT	TC	CC					T	C		**0.007 (0.021)[a]**	1.60 (1.13–2.26)
	(rs1128503)	C (189)	78	80	31				0.179	236 (0.62)	142 (0.38)			
		OC (128)	68	50	10				0.848	186 (0.7)	70 (0.27)			
	e21/G2677T/A		AA	AG	AT	GG	GT	TT		A	G	T	0.919	–
	(rs2032582)	C (189)	2	25	11	42	72	37	0.486	40 (0.11)	181 (0.48)	157 (0.42)		
		OC (128)	0	15	9	28	51	25	0.768	24 (0.09)	122 (0.48)	110 (0.43)		
	e26/C3435T		CC	CT	TT					C	T		0.889	1.02 (0.74–1.42)
	(rs1045642)	C (189)	71	86	32				0.496	228 (0.60)	150 (0.40)			
		OC (128)	49	55	24				0.228	153 (0.60)	103 (0.40)			
(B) Mothers of oral cleft probands vs controls														
ABCB1	e12/C1236T		TT	TC	CC					T	C		0.772	1.05 (0.76–1.46)
	(rs1128503)	C (189)	78	80	31				0.180	236 (0.62)	142 (0.38)			
		M (129)	53	58	18				0.739	164 (0.64)	94 (0.36)			
	e21/G2677T/A		AA	AG	AT	GG	GT	TT		A	G	T	0.189	–
	(rs2032582)	C (189)	2	25	11	42	72	37	0.486	40 (0.11)	181 (0.48)	157 (0.42)		
		M (129)	2	20	9	33	50	15	0.932	33 (0.13)	136 (0.53)	89 (0.35)		
	e26/C3435T		CC	CT	TT					C	T		0.054	1.39 (0.99–1.94)
	(rs1045642)	C (189)	71	86	32				0.496	228 (0.60)	150 (0.40)			
		M (129)	61	53	15				0.506	175 (0.68)	83 (0.32)			

Abbreviations: C, control; CI, confidence interval; HWE, Hardy–Weinberg equilibrium; M, mothers; OC, oral clefts; OR, odds ratio.
P-value of <0.05 highlighted in bold.
[a]Bonferroni corrected.

Bonferroni correction would result in an overly conservative correction. In the case–control study between Chinese NSOC probands and ethnic-matched controls, *ABCB1* rs1128503 was confirmed to be significantly associated with NSOC by both allelic and genotypic analyses, despite our modest sample size and limited power (<60%) in case–control analysis.[11] There was a high risk for developing NSOC in carriers of at least one T-allele of rs1128503 compared with those with the CC genotype. This observation is also consistent with the results of our family-based study, which showed preferential transmission of the T-allele to NSOC probands compared with the C-allele. A joint analysis of these data from the family and case–control studies for *ABCB1* rs1128503 revealed a high effect size for the role of this polymorphism in susceptibility to NSOC. In another comparison between Chinese mothers of NSOC probands and ethnic-matched healthy controls, no significant association was observed between the *ABCB1* SNPs and NSOC, suggesting the status of *ABCB1* polymorphism in fetus, and not in mother, is the determinant for conferring risk for NSOC.

In our family-based study, SNP allele analysis also revealed rs2032582 T-allele had significantly higher transmission to NSOC probands compared with the A- and G- alleles. In addition, rs1045642 T-allele was observed to have higher transmission to affected offspring compared with the C-allele. These findings are consistent with previous studies reporting the rs2032582 and rs1045642 T-alleles were associated with lower expression of ABCB1 in term placentas.[27–29] In contrast, other studies have reported higher levels of ABCB1 mRNA expression in kidney[30] and heart[31] tissues of carriers of rs2032582 T- and A-alleles compared with G-allele carriers. However, regulation of ABCB1 expression may differ significantly from tissue to tissue. Moreover, linkage studies in Chinese, Malays, and Indians have disclosed that the T-allele of SNPs rs1128503, rs2032582, and rs1045642 are in strong linkage disequilibrium.[14] Hence, the observed preferential transmission of the T-alleles of these SNPs could be due to a haplotype effect that needs to be further evaluated.

Although rs1128503 is a silent/synonymous SNP, a potentially functional role for this polymorphism has been observed by others.[32] It has been observed in 3T3 isogenic fibroblasts, mutation at e12/1236 (rs1128503) can significantly alter ABCB1 expression, regardless of additional mutations at positions e21/2677 (rs2032582) and e26/3435 (rs1045642).[33] Another study has reported effects of *ABCB1* polymorphisms at e21/2677 and e26/3435 on expression of ABCB1 in term placentas.[27] Our observation of a higher risk for NSOC in carriers of rs1128503 T-allele is also in accord with a previous report that the T-variant significantly alters ABCB1 expression in breast tumors.[34] We speculate that fetal genotypes at *ABCB1* carrying the rs1128503 T-allele are more vulnerable to NSOC, especially under situations of maternal exposure to non-genetic risk factors for NSOC such as smoking or pharmacotherapy. It is also possible rs1128503 is not a causal polymorphism but is merely in strong linkage disequilibrium with an unobserved causal SNP.

ABCB1 expression has been reported to be affected by both genetic polymorphisms[27] and environmental factors, such as diet and drugs.[32] However, one study reported no significant difference in placental ABCB1 activity or expression between smokers and non-smokers.[24] In our current study, the genetic marker was considered as the main determinant for expression of ABCB1 in the placenta, and the effect of non-genetic factors such as maternal smoking and medication in pregnancy was assumed to be minimal. However, it is possible even in the presence of wild-type placental *ABCB1* genotype, fetuses exposed to potentially toxic xenobiotics are also at higher risk for NSOC. This

is because the concurrent exposure to multiple substrates of ABCB1, or an ABCB1 inhibitor, may impair placental ABCB1 activity.[35]

In conclusion, findings from our family-based and case–control studies of nine potentially functional polymorphisms within *ABCB1*, *ABCC1*, *ABCC2*, and *ABCG2* provide strong support for the role of *ABCB1* polymorphisms in conferring risk for NSOC. Our results also suggest pharmacogenetic testing of *ABC*-transporter genes should also involve fetal DNA testing to estimate the risk of maternal medications on fetal development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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