Comprehensive Molecular Analysis of Serologically D-Negative and Weak/Partial D Phenotype in Thai Blood Donors

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Keywords

Blood group · Genotype · Phenotype · Rh · *RHD* variants

Abstract

Background: Molecular genetics of the Rh system has been extensively studied in Caucasians, Black Africans, East Asians, and Indians more recently. In this work, we sought to investigate the molecular basis of variant D expression in the Thai population, which remains unknown. **Materials and Methods:** Blood samples from 450 Thai donors showing the variant D phenotype were collected. The *RHD* gene was analyzed by quantitative multiplex polymerase chain reaction of short fluorescent fragments and/or Sanger sequencing. **Results:** The most frequent alleles in 200 D-negative and 121 DEL samples were the whole *RHD* gene deletion and the Asian DEL alleles, respectively. In 129 weak/partial D samples, 36 variant alleles were identified, including eight novel alleles. *RHD*06.03*, which is common in variant D samples from South China, is the most prevalent variant allele, followed by the recently reported Indian *RHD*01W.150* allele. **Discussion:** For the first time, a comprehensive overview of the nature and distribution of variant *RHD* alleles in Thailand is reported. It is a milestone to pave the way towards improvement of the current screening strategy to identify DEL donors accurately. The next step will be the design and implementation of a simple molecular test for screening the most frequent alleles, specifically in this population.

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Introduction

Antigen expression in the Rh system, which is the most complex and polymorphic blood group system, is driven by two homologous *RH* genes: *RHD* and *RHCE* [1]. The most clinically relevant antigen in this system, antigen D, is encoded by the former gene. Anti-D alloimmunization can occur in D-negative patients when exposed to D-positive red blood cells and may be responsible for hemolytic transfusion reaction and hemolytic disease of the fetus and newborn with severe clinical outcomes [2].

Variations in D phenotype expression in Caucasians, Black-Africans, and East-Asians, as well as the molecular genetics of *RH* genes, have been extensively studied and described, and the global distribution of variant *RH* alleles is well known in these populations [3–6]. In Western and East-Asian countries, molecular typing; i.e., genotyping, in addition to serological study, has become routine practice to assess the risk of alloimmunization [7, 8] and is therefore a critical step in the whole management of transfusion and pregnancy at risk.

A key point in molecular *RHD* typing is allele distribution, which is known to vary largely between ethnicities. The knowledge about the molecular background of

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the *RHD* gene on a regional/national basis is necessary to implement an efficient, dedicated genotyping strategy. For example, Caucasian-specific tests for routine diagnostics have been developed and implemented to identify preferentially the *RHD*weak D type 1*, *RHD*weak D type 2*, and *RHD*weak D type 3* alleles [9–12], which have long been known to be the most common variant alleles in serologic weak D individuals of Caucasian origin [3]. This latter strategy would be definitely inefficient for typing people of African or Asian origin. In Korea, Seo et al. [13] designed and implemented an effective diagnostic strategy for the detection of *RHD* variants in serologically D– Korean donors, including the typical Asian *DEL* allele characterized by the splice site variation c.1227G>A [14], which is the most prevalent *DEL* allele in the East-Asian population [6, 15, 16]. Extensive molecular data are available in Caucasians, Black Africans, East-Asians, and Indians since more recently. Conversely, populations of other origins, such as Thais, have been barely documented. It is worth mentioning that preliminary studies have been conducted to investigate the Rh blood group system in the Thai population. As early as in 2002, a large-scale study reported that 99.69, 0.30, and 0.01% of the Thai blood donors are D+, D–, and weak D, respectively [17]. Later, another investigation in samples with serological weak D expression showed that partial DVI, which is clinically relevant, is the most common phenotype (62/81, 76.5%) in this subset of samples [18]. In terms of molecular analysis, the Asian *DEL* allele was found in 18.9% of the serologically D– samples [19], and a recent study also reported preliminary *RH* genotyping data in a subset of nonselected donors from North and Central Thailand [20]. Overall, few studies have been carried out in Thailand, and the molecular determinants of Rh D variability, which is currently unknown in this population, remain to be described in detail at the genetic level. Here, we report for the first time a comprehensive study investigating the molecular basis of those variant D samples in the Thai population.

Materials and Methods

Blood Sample Collection and Serological Analysis

Blood samples from unrelated donors from Bangkok and suburban provinces were collected and provided by the National Blood Centre (NBC), Thai Red Cross Society (Bangkok, Thailand). Blood samples were analyzed routinely with automate analyzer PK7300 Microplate Agglutination Systems (Beckman Coulter) with monoclonal anti-D (clones LDM2 and RUM-1, NBC). Samples with inconclusive or negative results in D typing were retyped with two commercial blended IgM/IgG monoclonal anti-D reagents: IgM clone LDM2 and IgG clone LHM77 (NBC), IgM clone RUM-1 and IgG clone MS-26 (Plasmatec, Bridport, UK). Using conventional tube test at room temperature, 37 °C, and in-

direct antiglobulin test (IAT), 450 donor samples were classified either as weak/partial D or D negative. Weak/partial D samples showed either agglutination reaction $\leq 2+$ or IAT positive. Rh CcEe typing was carried out in all donor samples by using in-house anti-c and -E (NBC), and commercial anti-C and -e (DiaClon; DiaMed GmbH, Bio-Rad Laboratories) monoclonal IgM antibodies.

IAT-negative samples were subsequently screened for DEL by the adsorption-elution technique with monoclonal anti-D reagent (NBC). Briefly, 200 μ L of donor red blood cells were washed with normal saline solution (0.9%) and mixed with equal volume of anti-D reagent. The mixture was incubated at 37 °C for 1 h and then washed thoroughly with normal saline solution. The eluate was prepared by the in-house 0.1 M glycine-HCl buffer/ 10% EDTA (V/V: 4:1) technique as previously described [21]. Eluate and last-washed supernatant were used for IAT (conventional tube test) against D-positive and D-negative control cells (NBC).

DNA Extraction and Quantification

Genomic DNA was extracted from whole blood by using commercial kits (BioFact Genomic DNA Prep Kit; BioFact, Celbridge, Ireland; PureDireX Genomic DNA Isolation Kit; Bio-Helix, Keelung City, Taiwan; or PureLink Genomic DNA Mini Kit; Thermo Fisher Scientific, Bangkok, Thailand). DNA concentration and purity (A_{260}/A_{280}) were measured with a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific).

RHD Genotyping

Analysis by quantitative multiplex polymerase chain reaction (PCR) of short fluorescent fragments (QMPSF) was carried out to investigate potential exon copy number variations/rearrangements in the *RHD* gene by using the *F9* and *HFE* genes for positive amplification control and copy number normalization in conditions previously described [22]. Basically, QMPSF is a simple multiplex, fluorescent PCR-based method, which aim is here to specifically identify *RHD* exon markers to quantitatively assess exon copy number variations, which are frequently encountered at the *RHD* locus, and to easily identify variant alleles, including hybrid *D-CE* genes. Respective fragment length and peak areas were analyzed by GeneMapper v4.0 software (Applied Biosystems, Villebon-sur-Yvette, France). Exon 3 duplication allele was confirmed and screened in weak D samples by a previously reported PCR-based method [23]. Finally, when necessary, all ten *RHD* exons were amplified and directly sequenced, and sequencing data were analyzed by Sequencher v5.0 software (Gene Codes Corporation, Ann Arbor, MI, USA) [11]. Genotypes were deduced from both QMPSF and sequencing data on the basis of the most likely combination of variant *RHD* alleles.

Minigene Splicing Assay

Minigene splicing assay was carried out to investigate the effect of an intronic variation on splicing as previously described [11, 23]. Briefly, both wild-type and mutated regions of *RHD* exon 8 and flanking intronic domains were PCR amplified. PCR products were inserted into the minigene to generate recombinant vectors that were subsequently transfected into HEK293 cells (ATCC Number CRL-1573). Cells were harvested 48 h after transfection. Total RNA was extracted and purified by the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) and served as a template for reverse-transcription (RT)-PCR with the QIAGEN OneStep RT-PCR Kit (Qiagen). Finally, RT-PCR products were visualized on a 2% agarose gel containing ethidium bromide and directly sequenced after gel extraction.

Table 1. Serologic and *RHD* genotyping data in 200 D-negative Thai blood donors

Genotype ^a	Mechanism	Exon	CCee	Ccee	CcEe	ccEe	ccee	Occurrence	%
<i>RHD*01N.01</i>	Large deletion	1–10	6	39	1	4	100	150	75.0
<i>RHD*01N.03</i>	Hybrid gene	3–9	2	21	–	–	–	23	11.5
<i>RHD*01EL.01</i>	Splicing	9	10	11	–	–	–	21	10.5
<i>RHD*01N.05</i>	Hybrid gene	3–8	–	2	–	–	–	2	1.0
<i>RHD*01N.61</i>	Nonsense	7	–	–	–	1	–	1	0.5
<i>RHD*01N.83</i>	Frameshift	5	–	–	–	1	–	1	0.5
<i>RHD-CE(3-10)</i>	Hybrid gene	3–9	–	1	–	–	–	1	0.5
<i>RHD*05.04</i>	Missense	5	–	1	–	–	–	1	0.5
Total			18	75	1	6	100	200	100.0

^a Second allele is *RHD*01N.01*. *RHD*01N.01*: whole *RHD* gene deletion; *RHD*01N.03*: *RHD-CE(3-9)-D*; *RHD*01EL.01*: *RHD(c.1227G>A)*; *RHD*01N.05*: *RHD-CE(3-8)-D*; *RHD*01N.61*: *RHD(c.952C>T)*; *RHD*01N.61*: *RHD(c.702delG)*; *RHD*05.04*: *RHD(c.697G>C)*.

Table 2. Serologic and *RHD* genotyping data in 121 DEL Thai blood donors

Genotype ^a	Exon	CCee	Ccee	CCEe	CcEe	ccee	Occurrence	%
<i>RHD*01EL.01</i>	9	24	82	–	–	1	107	88.4
<i>RHD*01N.01</i>	1–10	–	4	–	–	7	11	9.1
<i>RHD*01N.03</i>	3–9	–	1	–	–	–	1	0.8
<i>RHD*01N.16/RHD*01EL.01</i>	3–8, 9	–	–	1	–	–	1	0.8
<i>RHD*15/RHD*01N.03</i>	3–9	–	–	–	1	–	1	0.8
Total		24	87	1	1	8	121	100.0

^a Second allele is *RHD*01N.01*, if not mentioned. *RHD*01EL.01*: *RHD(c.1227G>A)*; *RHD*01N.01*: whole *RHD* gene deletion; *RHD*01N.03*: *RHD-CE(3-9)-D*; *RHD*01N.16*: *RHD(c.711delC)*; *RHD*15*: *RHD(c.845G>A)*.

Results

Phenotypic Characterization of Thai Blood Donors with Variant D Phenotype

In the 450 Thai blood donors, 129 and 321 samples were classified as weak/partial D and D negative by routine testing, respectively. Subsequent test by adsorption-elution in the 321 latter samples actually identified 121 DEL samples, while the other 200 samples were D negative. The *RHD* gene was then genotyped in all samples to identify the respective molecular bases of the variant D phenotype.

Molecular Analysis of Serological D-Negative Samples

Samples typed as D negative by serological analysis ($n = 200$) were first investigated by *RHD* QMPF, which has proven useful for genotyping such samples. As commonly observed, whole deletion of the *RHD* gene at the homozygous state is the most frequent genotype (Table 1; 150/200, 75.0%) notably found in all 100 ccee samples, followed by hybrid genes in 26 samples. *RHD* gene exons were subsequently sequenced in the remain-

ing 24 samples. Interestingly, the Asian DEL allele; i.e., *RHD(c.1227G>A)*, was found at the hemizygous state in 21 samples, while the other three samples carry single-nucleotide variations (Table 1).

Molecular Analysis of Serological DEL Samples

All 121 serological DEL samples were first screened in *RHD* exon 9. The Asian DEL allele was identified in 108 samples (Table 2, 89.2%). QMPF analysis and direct sequencing resolved twelve samples carrying negative *RHD* alleles, and are thus supposed to be D negative, while a single sample was found to carry the *RHD*weak-D type 15* allele (Table 2).

Molecular Analysis of Weak-/Partial-D Samples

The 129 weak/partial D samples were investigated by *RHD* QMPF and/or direct sequencing. This analysis showed a large heterogeneity in terms of genotypes in the Thai population (Table 3). Indeed, as many as 52 allele combinations, including 33 observed only once, were found in those samples. The most common weak/partial D allele is partial DVI type 3 (or *RHD*06.03*; al-

Table 3. Serologic and RHD genotyping data in 129 weak/partial D Thai blood donors

RHD allele 1	RHD allele 2	CCee	Ccee	CcEe	ccEe	ccee	Occurrence	%
RHD*06.03	RHD*01N.01	3	17	–	–	–	20	15.5
RHD*06.03	RHD*01EL.01	6	1	–	–	–	7	5.4
RHD*06.03	RHD*01N.03	2	–	–	–	–	2	1.6
RHD*18	RHD*01N.01	–	4	1	–	–	5	3.9
RHD*18	RHD*18	1	–	–	–	–	1	0.8
RHD*18	RHD*01EL.01	1	–	–	–	–	1	0.8
RHD*01W.150	RHD*01N.01	–	6	–	–	–	6	4.7
RHD*01W.150	RHD*01EL.01	1	1	–	–	–	2	1.6
RHD*05.04	RHD*01N.01	–	5	–	–	–	5	3.9
RHD*0W.122	RHD*01N.01	1	3	–	–	–	4	3.1
RHD*0W.122	RHD*01EL.01	1	–	–	–	–	1	0.8
RHD(R10Q,V174M) ^a	RHD*01N.01	1	3	–	–	–	4	3.1
RHD(S75N) ^a	RHD*01N.01	–	3	–	–	–	3	2.3
RHD(Q89P) ^a	RHD*01N.01	1	2	–	–	–	3	2.3
RHD*08.01	RHD*01N.01	3	–	–	–	–	3	2.3
RHD*15	RHD*01N.01	–	–	–	2	–	2	1.6
RHD*15	RHD*01EL.01	–	–	1	–	–	1	0.8
RHD*01W.33	RHD*01N.01	1	1	–	–	–	2	1.6
RHD*01W.33	RHD*01N.03	1	–	–	–	–	1	0.8
RHD*01W.33	RHD*06.03	1	–	–	–	–	1	0.8
RHD*01W.25	RHD*01N.01	–	–	–	2	–	2	1.6
RHD*01W.152	RHD*01N.01	–	1	–	–	–	1	0.8
RHD*01W.152	RHD*01EL.01	–	1	–	–	–	1	0.8
RHD*01W.152	RHD*06.03	1	–	–	–	–	1	0.8
RHD(G307E) ^a	RHD*01N.01	–	–	–	–	1	1	0.8
RHD(G307E) ^a	RHD*01N.03	–	1	–	–	–	1	0.8
RHD*01W.6	RHD*01N.01	1	–	–	–	–	1	0.8
RHD*01W.31	RHD*01N.01	–	–	–	1	–	1	0.8
RHD*01W.72	RHD*01N.01	–	1	–	–	–	1	0.8
RHD*01W.100	RHD*01N.01	–	–	–	1	–	1	0.8
RHD*01W.119	RHD*01N.01	–	1	–	–	–	1	0.8
RHD*01W.128	RHD*01N.01	–	–	–	–	1	1	0.8
RHD*01W.154	RHD*01N.01	1	–	–	–	–	1	0.8
RHD(L20Q) ^a	RHD*01N.01	1	–	–	–	–	1	0.8
RHD(L110P,F223V) ^a	RHD*01N.01	1	–	–	–	–	1	0.8
RHD(S230N)	RHD*01N.01	–	–	–	1	–	1	0.8
RHD(K394T) ^a	RHD*01N.01	1	–	–	–	–	1	0.8
RHD*05.01	RHD*01N.01	–	1	–	–	–	1	0.8
RHD*06.02	RHD*01N.01	–	–	1	–	–	1	0.8
RHD*14.02	RHD*01N.01	–	1	–	–	–	1	0.8
RHD*01W.10	RHD*01EL.01	1	–	–	–	–	1	0.8
RHD(P221L) ^a	RHD*01EL.01	–	–	1	–	–	1	0.8
RHD(c.960G>A)	RHD*01EL.01	1	–	–	–	–	1	0.8
RHD*09.02.01	RHD*01EL.01	–	1	–	–	–	1	0.8
RHD*17.04	RHD*01EL.01	1	–	–	–	–	1	0.8
RHD*c.1153+6T>C	RHD*01EL.01	1	–	–	–	–	1	0.8
RHD*01EL.01	RHD*01	3	–	–	–	–	3	2.3
RHD*01EL.01	RHD*01N.01	–	1	–	–	–	1	0.8
RHD*01EL.01	RHD*01N.03	1	–	–	–	–	1	0.8
RHD*01	RHD*01N.01	6	5	–	–	1	12	9.3
RHD*01	RHD*01	6	1	1	–	–	8	6.2
RHD*01N.01	RHD*01N.01	–	–	–	–	3	3	2.3
Total		50	61	5	7	6	129	100.0

^a Novel alleles. Refer to The Human RhesusBase v2.3 (URL: www.rhesusbase.info/) for details about the respective alleles.

Table 4. Novel *RHD* alleles identified in the Thai population

Designation	Nucleotide change (exon)	Amino acid change (position ^a)	Most likely haplotype	GenBank Accession No.
<i>RHD</i> (R10Q,V174M)	c.29G>A (1), c.520G>A (4)	p.Arg10Gln (ICL1), p.Val174Met (M5)	<i>CDe</i>	MK086934
<i>RHD</i> (L110P,F223V)	c.329T>C (2), c.667T>G (5)	p.Leu110Pro (M3), p.Phe223Val (M6)	<i>CDe</i>	MK086935
<i>RHD</i> (L20Q)	c.59T>A (1)	p.Leu20Gln (M0)	<i>CDe</i>	MK086936
<i>RHD</i> (S75N)	c.224G>A (2)	p.Ser75Asn (M2)	<i>CDe</i>	MK086937
<i>RHD</i> (Q89P)	c.266A>C (2)	p.Gln89Pro (M2)	<i>CDe</i>	MK086938
<i>RHD</i> (P221L)	c.662C>T (5)	p.Pro221Leu (M6)	<i>cDe</i>	MK086939
<i>RHD</i> (G307E)	c.920G>A (6)	p.Gly307Glu (M9)	<i>cDe</i>	MK086940
<i>RHD</i> (K394T)	c.1181A>C (9)	p.Lys394Thr (ICL7)	<i>CDe</i>	MK086941

^a Position in the RhD protein indicated in accordance with reference [24]. ICL: intracellular loop; M: transmembrane domain.

Fig. 1. Functional analysis of variant c.1153 + 6T>C by minigene splicing assay. **A** RT-PCR profiles and **B** direct sequencing patterns showing (1) inclusion and (2) full skipping of *RHD* exon 8. 100 to 500: size in base pairs; Empty: empty vector (no insert); H₂O: no template control.

lele frequency = 0.120). Interestingly, the most frequent weak *D* allele (*RHD**weak *D* type 150) recently reported in the Indian population, which involves a duplication of a ~12 kilobase-region including *RHD* exon 3, was observed in 8 samples (6.2%). Eight novel alleles (Table 4) were found in 16 donors, suggesting that the Thai population is heterogeneous in terms of *RHD* molecular genetics. Of those, both *RHD*(R10Q,V174M) and *RHD*(L110P,F223V) involve two variations already reported separately. The other six novel alleles involve single missense variations.

We next paid attention to a variant in intron 8, c.1153+6T>C (or IVS8+6T>C), which was previously reported (GenBank Accession No.: JX114750). To get insight into the functional defect induced by the intronic variant, we took advantage of our previously reported minigene assay, which has proven useful for the characterization of variants affecting splicing [11, 23]. Our test clearly demonstrated that c.1153+6T>C significantly al-

ters normal splicing by promoting *RHD* exon 8 exclusion from the mature transcript (Fig. 1A). A “normal” full-length transcript including exon 8 was though identified by sequencing analysis (Fig. 1B). Overall, this assay suggests that c.1153+6T>C is a splicing variant affecting D-antigen expression quantitatively, but not qualitatively, and therefore is not of clinical importance.

Discussion

The nature and distribution of variant *RHD* alleles are fundamental data for the Public Healthcare system to implement efficient and cost-effective genotyping tests and to guide population-specific transfusion practice and obstetrical management [24]. In order to get insights into the molecular determinants of variant *D* expression, we sought to investigate the *RHD* gene in the Thai population, which has remained globally unexplored so far. For

the first time, we report a comprehensive overview of the variant *RHD* alleles in this population.

As expected, whole *RHD* gene deletion at the homozygous state is the most common *D*-negative genotype in serological *D*-negative Thai blood donors (Table 1, 75.0%). When considering only the subset of *C/E*+ samples ($n = 100$), frequency of this genotype is in the range of what has been reported before in other Asian populations (36.4–63.1%) [15, 16, 25, 26]. Interestingly, as many as 21 samples out of 200 were found to carry the Asian *DEL* allele. This result suggests that either another variation in *RHD*, which has not been identified yet, impairs RhD protein expression, or reagents and/or conditions for identifying *DEL* samples are not optimal, thus yielding false-negative results. In this more likely latter hypothesis, *DEL* screening methodology should be reviewed urgently in a large-scale study including genotyped samples. Unexpectedly, one sample found to be *D* negative serologically was genotyped as *RHD**05.04. This finding may be due to either the absence of some *D* epitopes recognized by the antibodies used in this study as previously reported [27], leading to a “false” apparent *D*-negative phenotype, or another unidentified deleterious variation in the *RHD* gene that impairs *D* antigen expression.

As also expected, *RHD*(c.1227G>A) is the most frequent *DEL* allele found in ~90% of the samples (Table 2). Based on the genotyping data, in this group of serologic *DEL* samples, the percentage of false-positive results is significant (Table 2; 12/121, 9.9%), confirming that the methodology for *DEL* sample identification should be revised.

Finally, as many as 36 variant *RHD* alleles were found in 129 serological weak/partial *D* samples, highlighting the large variability of the *RHD* gene in the Thai population. Eight novel alleles were found, thus contributing to extend the molecular catalogue of *RHD* polymorphism. It also suggests that many other novel alleles are very likely to be found in future studies in the Thai population. The most frequent allele is *RHD**06.03, which was also reported to be the most frequent in *D* variant donors from South China (28/62, 45.2%) [28]. Very interestingly, the second most common variant allele is *RHD**01W.150, which was recently reported in the Indian population [29]. Taken together, besides the population-specific pattern observed in Thais, those data highlight the mixed nature of this population and, in parallel with the geographical context, provide data to bridge the molecular gap between South China and India. Replication studies in other regions of Thailand will definitely be of interest to investigate potential region-specific patterns of variant *RHD* allele distribution.

It is important to pay attention to the last six genotypes reported in Table 3, including a total of 28 samples. Indeed, an apparent wild-type *RHD* allele was found in

23/129 samples (17.8%), while they were found to be weak *D* serologically. It is interesting to compare these data with those reported recently in the Indian population, where 37/223 serologically weak *D* samples (16.6%) were found to carry at least one apparent wild-type *RHD* gene copy [29]. On the basis of these observations and by considering that replication studies in both populations yield comparable results, we suggest that more complete *RHD* gene investigation, including yet unexplored regions of regulation and introns, should be carried out to further identify potential functional variations altering *D* antigen expression. Discrepancy in the genotype-phenotype correlation in the remaining five samples (i.e., *RHD**01EL.01/*RHD**01N.01 [$n = 1$]; *RHD**01EL.01/*RHD**01N.03 [$n = 1$], and *RHD**01N.01/*RHD**01N.01 [$n = 3$]) remains to be elucidated by additional serological testing in the same samples first.

Overall, we report the molecular basis of variant *D* expression in Thai blood donors for the first time. Beyond the fundamental interest of this study in terms of population genetics and molecular epidemiology of the Rh blood group system, the next step will be the design and implementation of a population-specific screening test based on our data to help clinicians in their current practice to further improve blood transfusion safety.

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Statement of Ethics

The project was approved by Mahidol University Central Institutional Review Board (MU-CIRB 2018/104.1805) and Research Ethics Committee, NBC, Thai Red Cross Society (COA No. NBC 13/2018). All participants provided their informed consent.

Disclosure Statement

The authors declare no conflicts of interest.

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