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A cross-sectional pilot study to estimate the frequency of minor blood group alleles and phenotypes in RhD-negative North Indian blood donors by DNA microarray analysis

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Abstract:

INTRODUCTION: There are scarce data on Indian blood donors with respect to blood group phenotypes using molecular diagnostic modalities. Hence, we planned to estimate frequencies of blood group alleles/phenotypes using DNA microarray analysis in the north Indian RhD-negative blood donor population. With this initial pilot study, we plan to expand it to our entire donor population.

METHODOLOGY: The cross-sectional prospective study was conducted on 50 Indian blood donors (O RhD negative), to study the blood group genotype frequency. Genotyping for the most relevant red blood cell antigens (Rh, Kell, Duffy, Kidd, MNS, Lutheran, and Dombrock) was done using Bioarray Precise Type^{HM} Human Erythrocyte Antigen BeadChip kit containing probes directed to polymorphic sites.

RESULTS: In the Rh system, the most common alleles were *RHCE**e/*RHCE*e* (98%) and *RHCE**c/*RHCE*c* (80%). Phenotype K-k+ (genotype- *KEL*02/KEL*02*) was seen in 98% of samples, Js(a-b+) (*KEL**02*.07/KEL*02.07*) was detected in 98% (49/50) of the samples tested. Jk(a + b+) (*JK**01*/JK*02*) was the most common phenotype (48%) in the Kidd blood group system. In MNSs system, M+N+ **(***GYPA**01*/GYPA*02*) 44% and S+s+U+ **(***GYPB******03*/GYPB*04*) 34% were the most common phenotypes detected.

CONCLUSION: This pilot study shows the feasibility of genotyping a Northern Indian donor population. To the best of our knowledge, it is the first study on molecular blood grouping in Indian blood donors using the Bioarray platform.

Keywords:

Blood group frequency, molecular blood group, rare donor registry

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Introduction

Blood transfusion, pregnancy, and organ transplant can lead to alloimmunization due to the introduction of foreign red blood cell (RBC) antigens in the recipient lacking these antigens.[1] In multitransfused patients who are chronically dependent on transfusion support like thalassemics and hemato-oncology patients, it is important to provide extended phenotyped matched red cells to prevent alloimmunization. Alloimmunization can pose difficulties in compatibility testing and can predispose the recipients to the risk of adverse transfusion reactions.[2] In such situations, the specificity of the antibodies to red cell antigens should

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be identified to determine their clinical relevance. Antibody screening and identification involve testing with reagent RBCs expressing the common polymorphic antigens including low- and high-prevalence antigens. There are currently 45 recognized blood group systems containing 360 red cell antigens (International Society of Blood Transfusion [ISBT], July 2023).[3] The 45 systems are genetically determined by 50 genes, governed by a single gene, or two or three closely-linked homologous genes. Most of the published literature from different parts of India on the frequency of minor blood group phenotypes in blood donors is based on the use of serotyping.[4,5] DNA-based techniques provide assured results for both patients and donors in situations where serological techniques pose some restraints as where antisera are not available, and patients with recent transfusion, to confirm weak and variant antigens, and to resolve ABO discrepancies.[6] In addition, blood group genotyping is reliable in direct antiglobulin test-positive blood donors.

Most of the studies on molecular blood grouping are from the western countries, but blood group genotyping data from the Indian blood donor population are lacking. This data are essential to prevent alloimmunization in transfusion-dependent patients by minimizing the differences in patient and donor red cell antigens. The situation becomes more complex when a particular antigen-matched blood is needed for a RhD-negative patient as the number of Rh-negative red cell units is always limited in the inventory. The prevalence of RhD-negative blood group in the Indian blood donor population is 5%–6%.[4,7] Hence, we planned to estimate frequencies of blood group alleles/phenotypes using the DNA microarray analysis in a north Indian RhD-negative blood donor population. This study can ease the complexity of the alloimmunization in the case of Rh-negative patients as it will help in tracing antigen-negative units among a lesser number of available Rh-negative blood units. The data after blood group genotyping can be used to prepare dedicated blood group donor registries and as a tool for donor recruitment and retention. This is the first Indian study to report molecular blood grouping in blood donors (Rh negative) by microarray-based genotyping platform (Beadchip Bioarray technique).

Methodology

The cross-sectional prospective study was conducted in the Department of Transfusion Medicine at a tertiary care referral and teaching institute after ethical approvals from the institutional ethics committee (AIIMS/IEC/18/365). The blood donors were selected per the criteria mandated by the Drugs and Cosmetics Act and Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India.[8,9]

We selected DNA samples from 50 consecutive Indian blood donors (O RhD negative), to study the blood group antigens genotypic frequency in the given population.

Inclusion criteria

A healthy individual, fulfilling the criteria for blood donation, after he was declared fit after a detailed history and a brief medical examination, for donating blood, with RhD-negative blood group.

Exclusion criteria

An individual who did not fulfill the criteria for blood donation.

- A blood donor with RhD-negative blood group but weak D-testing positive.

Blood sample collection

Five milliliter ethylenediaminetetraacetate (EDTA) blood sample was collected during the sampling done routinely at the end of phlebotomy in pilot tubes for testing from 50 blood donors from North India with O RhD-Negative blood group in the Department of Transfusion Medicine, after taking informed consent from them. The RhD-negative blood group was ascertained from the donor's history and was further screened by grouping by slide method. Blood grouping for RhD antigen was done using anti-D antisera from two different manufacturers (Span Diagnostics, Venette France; Tulip Diagnostics, Goa India) or the same manufacturer with different batches of manufacturing. Weak D status was tested by microcolumn agglutination system (Make-Ortho Biovue, Pencoed UK).

Procedural Details

Genotyping for the most relevant RBC antigens and three phenotypic variants from the blood group systems (Rh, Kell, Duffy, Kidd, MNS, Lutheran, Dombrock) was done using Bioarray Precise Type^{HM} human erythrocyte antigen (HEA) BeadChip kit (Immucor) containing probes directed to polymorphic sites.

PreciseType HEA BeadChip detects single nucleotide variants (SNV) responsible for 35 RBC antigens from 11 blood group systems according to Table 1.

However, in our analysis, results could not be obtained for Landsteiner–Wiener, Diego, Colton, and Scianna blood group systems as the results were inconclusive. Hence, these systems were not included in the analysis.

Molecular BeadChip Test (PreciseType HEA Immucor) was used as per the manufacturer's directions to predict antigen phenotypes in blood group systems. There are four major steps involved in running the BeadChip test.

Table 1: Single nucleotide polymorphisms in red cell antigens detected by PreciseType BeadChip human erythrocyte antigen array (Immucor)

Blood group	Red blood cell antigens
Rh	C (RH2), c (RH4), E (RH3), e (RH5), V (RH10), VS (RH20)
Kell	K (KEL1), k (KEL2), Kp^a (KEL3), Kp^b (KEL4), Js^a (KEL6), Js^b (KEL7)
Duffy	Fy^a (FY1), Fy^b (FY2), GATA (FY-2), Fy^x (FY2W)
Kidd	Jk^a (JK1), Jk^b (JK2)
MNS	M (MNS1), N (MNS2), S (MNS3), s (MNS4), Uvar (MNS-3,5W), Uneg (MNS-3,-4,-5)
Lutheran	Lu^a (LU1), Lu^b (LU2)
Dombrock	Do^a (DO1), Do^b (DO2), Hy (DO4), Jo^a (DO5)
Landsteiner-Wiener	LW^a (LW5), LW^b (LW7)
Diego	Di^a (DI1), Di^b (DI2)
Colton	Co^a (CO1), Co^b (CO2)
Scianna	Sc^1 (SC1), Sc^2 (SC2)

1. Genomic DNA extraction (1 h):

Genomic DNA was isolated in the department of Biochemistry, of the institute.

DNA preparation

The genomic DNA was extracted from 200 µl aliquots of EDTA blood by a manual spin vortex separation (QIAmp, Qiagen, Valencia, CA, USA), as per the instructions of the manufacturer and eluted into 200 µl of the buffer at 56°C for 10 min. The DNA aliquot of 8 µl, containing ~10 ng of genomic DNA, was transferred to the polymerase chain reaction (PCR).

2. Multiplex PCR (2 h). BioArray BeadChips uses multiplex PCR which amplifies several different target regions of extracted genomic DNA in a single process
   PCR was done within the thermal cycler to amplify target regions of DNA that contain the polymorphisms determining blood group antigens. PCR was done using oligonucleotide primers (which are complementary to DNA sequences) to identify the target regions, dNTPs (DNA building blocks), and an enzyme-Taq DNA Polymerase.
3. Post PCR processing (2 h) – After the Multiplex PCR process, the products were processed to remove residual primers and dNTP with a clean-up Reagent. Single-stranded DNA was generated using lambda exonuclease to selectively destroy one strand of each amplicon. It involved incubation at 37°C and inactivation at 80°C
4. BeadChip analysis (1 h) – Single-stranded DNA amplicons were mixed with Elongation Reagent which contains fluorescently labeled nucleotides and DNA polymerase. The mixture was transferred onto BeadChip and incubated. During BeadChip incubation, DNA amplicons bind to complementary DNA probe sequences or the corresponding beads. If the sequence of the amplified DNA perfectly matches to the 3′ end base, the probe is elongated, incorporating the fluorescent tag. If the amplified DNA does not perfectly match the probe, the DNA probe is not elongated, so the fluorescent tag is not incorporated. The Array Imaging System (AIS400) acquires an image of the chip fluorescent (positive) beads and nonfluorescent (negative) beads. Data were analyzed, and results were interpreted and reported using the BASIS software. BASIS (BioArray Information System) superimposes the assay image over the "decoding" image (provided with the kit on CD) to map the corresponding blood group genes, per individual bead type. This mapping occurs at each of the 4000 beads on the BeadChip. Intensities were determined and normalized, a discrimination ratio was generated, and an algorithm was used to predict allele type.

The genotypes and the predicted phenotype frequencies were expressed in numerical values of the total (*n*) and finally as percentages.

Results

Table 2 shows the frequencies of different minor blood group genotypes and their predicted phenotypes studied in 50 O RhD-negative blood donors. In the Rh system, the most common genotypes were *RHCE**e/*RHCE***e* (98%) and *RHCE**c/*RHCE***c* (80%) with their corresponding predicted phenotypes as E-e+ and C-c+. Phenotypes E+e-, E-e-, C+c-, C-c-, and C^w were not detected in the study population. In the Kell blood group system, phenotype K-k+ (genotype- *KEL*02/KEL*02*) was seen in 98% of the samples; phenotype K+k-, K+k+, and K-k-could not be found. Kp(a-b+) (*KEL**02.*04/KEL*02.04*) and Kp(a+b+) (*KEL**02.*03/KEL*02.04*) were seen in 98% and 2% of the RhD-negative donor samples, respectively. Js(a-b+) (*KEL**02.*07/KEL*02.07*) was detected in 98% of the samples tested. In the Duffy blood group system, Fy(a+b-) (*FY**01/*FY*01*) was the most common phenotype predicted (40%), followed by Fy(a+b+) (*FY**01/*FY*02*) (34%). Fy(a-b-) was not seen in any of the samples. Jk(a+b+) (*JK**01/*JK*02*) was the most common phenotype (48%) and Jk(a-b-) was not detected in the Kidd blood group system. In MNSs system, M+N+ (*GYPA**01/*GYPA*02*) 44% and S+s+U+ (*GYPB**03/*GYPB*04*) 34% were the most common phenotypes detected. M-N- and S-s- were not found in the study population of Rh-negative donors. In the Lutheran blood group system, Lu(a-b+) (*LU**02/*LU*02*) was seen in 98% of the samples. In Dombrock system, Do(a+b+) (*DO**01/*DO*02*) was seen in 40% samples; followed by Do(a-b+) (*DO**02/*DO*02*) 34%. Lu(a+b-), Lu(a-b-), and Do(a-b-) had nil frequency.

Table 2: Frequencies of different minor blood group phenotypes

Blood group system	Genotype	Genotype (ISBT)	Predicted phenotypes	*n*=50	Percentage (*n*)	Remarks
Rh	*GG*‡	*RHCE*e/RHCE*e*‡	E−e+	49	98% (49/50)	
			E−e−	0		
	CC‡	*RHCE*c/RHCE*c*‡	C−c+	40	80% (40/50)	
			C−c−	0		
	CT‡	*RHCE*C/RHCE*c*‡	C+c+	7	14% (7/50)	
	TT‡		C+c−	0		
	CG‡	*RHCE*e/RHCE*E or RHCE*01/RHCE*03*	E+e+	1	2% (1/50)	
			E+e−	0		
	AA‡ *CC*‡ *TT*‡	-	Cw	0	0% (0/50)	NA-12, OUT-1
KELL	*CC*‡	*KEL*02/KEL*02*‡	K−k+	49	98% (49/50)	
			K+k+	0		
			K+k−	0		
			K−k−	0		
	CC‡	*KEL*02.04/KEL*02.04*	Kp(a−b+)	49	98% (49/50)	
			Kp(a−b−)	0		
			Js(a+b+)	0		OUT-1
			Js(a+b−)	0		
	CT‡	*KEL*02.03/ KEL*02.04*‡	Kp(a+b+)	1	2% (1/50)	
			Kp(a+b−)	0	-	
	TT‡	*KEL*02.07/KEL*02.07*	Js(a−b+)	49	98% (49/50)	
			Js(a−b−)	0	-	
DUFFY	*AG*‡ *TT*‡ *CC*‡	*FY*01/FY*02*‡	Fy(a+b+)	17	34% (17/50)	NA and OUT-2
	GG‡ *TT*‡ *CC*‡	*FY*01/FY*01*‡	Fy(a+b−)	20	40% (20/50)	
	AA‡ *CT*‡ *CC*‡	*FY*02/FY*02N.01*‡	Fy(a−b+)	11	22% (11/50)	c. 125G/A, c.-67T/C
			Fy(a−b−)	0	-	
KIDD	*AG*‡	*JK*01/JK*02*‡	Jk(a+b+)	24	48% (24/50)	NA-1
	GG‡	*JK*01/JK*01*‡	Jk(a+b−)	17	34% (17/50)	
	AA‡	*JK*02/JK*02*‡	Jk(a−b+)	8	16% (8/50)	
			Jk(a−b−)	0	-	
MNSs	*CT*‡ *GT*‡	*GYPA*01/GYPA*02*‡	M+N+	22	44% (22/50)	NA-4
	CC‡ *TT*‡	*GYPA*01/GYPA*01*‡	M+N−	16	32% (16/50)	
	TT‡ *GG*‡	*GYPA*02/GYPA*02*‡	M−N+	8	18% (8/50)	
			M−N−	0	-	
	TC‡ *CC*‡	*GYPB*03/GYPB*04*‡	S+s+U+	17	34% (17/50)	OUT-16, NA-5, UNX-1
	CC‡ *CC*‡ *GG*‡	*GYPB*04/GYPB*04*‡	S−s+U+	8	16% (8/50)	
			S−s−	0	-	
	TT‡	*GYPB*03/GYPB*03*	S+s − U+	3	6% (3/50)	
LUTHERAN	*GG*‡	*LU*02/LU*02*‡	Lu(a−b+)	49	98% (49/50)	
			Lu(a−b−)	0	-	
	GA‡	*LU*01/LU*02*‡	Lu(a+b+)	1	2% (1/50)	
			Lu(a+b−)	0	-	
DOMBROCK	*AG*‡	*DO*01/DO*02*‡	Do(a+b+)	20	40% (20/50)	OUT-1, NA-1
	AA‡	*DO*02/DO*02*‡	Do(a−b+)	17	34% (17/50)	
	AA‡	*DO*01/DO*01*‡	Do(a+b−)	11	22% (11/50)	
			Do(a−b−)	0	0	

UNX=Unidentified, NA=Not applicable, OUT=Not found in sample data, ISBT=International Society of Blood Transfusion

Discussion

The blood group antigens are governed by alleles of a single locus or very close loci. Biochemically, red cell blood group antigens are glycoproteins or glycolipids in nature. ISBT has defined 45 blood group systems and over 360 blood group antigens.[3] Disparity between blood group antigenic expression in donor and recipient can lead to alloimmunization, the risk is higher in multitransfused patients dependent on chronic transfusion therapy. Alloantibodies can complicate the transfusion management of the patient, posing difficulties with compatibility testing and increasing the risk of adverse transfusion reactions.

An individualized transfusion therapy that is phenotyping the blood donors to provide a better match for a typed recipient is a practical approach to improve transfusion safety. Maintaining an extended RBC antigen phenotyped blood donor pool is optimum to support patients who are long-term transfusion dependent. The hemagglutination-based technique is a standard method for RBC antigen phenotyping but has some limitations for which DNA-based testing modalities provide an alternative.[10]

Molecular genotyping is a viable method for donor centers to determine red cell units with rare antigen phenotype profiles to meet the demands for antigen-negative units. Literature from Western countries shows studies for validation of different platforms for extended blood group genotyping in blood donors. In a study by Nathalang *et al*., Duffy blood group genotyping was done on 500 Thai blood donors. In this study, the probability of obtaining Fy antigen-negative red cell units was calculated in concordance with Hardy–Weinberg equilibrium according to the estimated Fy allele frequencies. It was observed that the Fy genotyping was in 100% concordance with phenotyping results.[11] Ba *et al*. determined the frequencies of clinically relevant blood group alleles in 300 blood donors from Mali by reverse sequence-specific oligonucleotide (PCR-RSSO).[12] Schoeman *et al*. studied the targeted exome sequencing for 28 protein-based blood group systems in 28 blood donor samples and concluded that the approach is economical compared with whole-genome sequencing and is suitable for a RBC reference laboratory setting.[13] Hong *et al*. established a Korean rare blood donor registry by performing RBC genotyping in 419 blood donors.[14]

Very few studies have been published in India on molecular genotyping.[15,16] In a study on ABO blood group alleles in the Indian population from Mumbai, seven common ABO alleles with 19 different genotypes were found and two rare alleles were also identified. Another study on molecular genotyping of clinically important blood group antigens in thalassemic patients and their regular blood donors compared the serological phenotyping of clinically significant blood group antigens with molecular genotyping. As genotyping data from the Indian blood donor population are lacking and there is no study to date in blood donors from North India, so through this study, we aim to determine the different blood group system allele frequencies in our blood donor population.

Various platforms are available for RBC blood group genotyping varying in their principle, accuracy, and complexity. Most semi-automated RBC molecular typing platforms are DNA-based assays with the principle of multiplex PCR amplification of genes encoding various blood group antigens. It involves fluorescence-based hybridization to RBC antigen allele-specific oligonucleotide probes embedded on glass slides, silica beads, or fluidic bead suspensions.[17,18] In our study, molecular blood grouping was done by the HEA BeadChip microarray kit. PreciseType BeadChip HEA technique is based on the principle of allele-specific hybridization between complementary probes and labeled DNA fragments from the sample, which is measured to determine genotype. PreciseType™ HEA test is a FDA-approved *in vitro* diagnostic multiplexed molecular assay that generates detailed molecular information from patient and donor samples, rapidly detecting genotypes for accurate prediction of phenotypes. The test results have a high level of reliability and require no confirmation with antisera – which saves time and reduces costs. It simplifies the identification of rare antigens, enables characterization of donor units for the most relevant antigens, and is not affected by autoantibodies or differences in reagent reactivity.

DNA analysis to predict RBC antigen expression has been incorporated in reference immunohematology laboratories, but there are scarce data from India with respect to blood donors. BeadChip DNA analysis, including automated array imaging with computerized data management, has made possible the mass screening of donors for several antigens and to maintain a diverse inventory of well-characterized donors. In a study by Kappler–Gratias using BeadChip to test RBC panel donors, a predicted phenotype was obtained for 99.74% of the 12,104 markers tested.[19] A practical approach that can be adopted by blood centers is to phenotype selected donors based on their preferences (such as age, ABO blood group, ethnicity, and frequency of donations). It would be optimum to maintain an inventory of predetermined blood group phenotypes of younger regular repeat voluntary donors preferably the ones who have responded to calls during emergencies, rather than first-time/replacement donors who have little intentions to return. Travali *et al*. and Chou *et al*.

transfused antigen-matched RBCs based on BioArray™ HEA Beadchip™ to thalassemia major and sickle cell disease patients with optimum results.[20,21]

In our study, among 50 RhD-negative blood donors, we found that E, Kp^a, and Lu^a (detected in 2% of the study population) were the least common antigens present in the given population whereas c, e, k, Kp^b, and Js^b, were the most common antigens present. In India, various serological studies have been carried out to study the prevalence of blood group phenotypes over a period of time.[22-25] The prevalence of RhD-negative group varies from 6% to 7% in India.[18,25] The frequency of various Rh system antigens as C, E, c, and e in the present study was 14%, 2%, 100%, and 98%, respectively, which is comparable to those in studies in North Indian blood donors by Thakral *et al*. (8.54%, 3.66%, 100%, and 100%) and Lamba *et al*. (10%, 4.3%, 98.6%, and 100%). In a study in the donor population from South India, the corresponding figures are 5.43%, 1.28%, 99.68%, and 100%, respectively. In a study from Western India, the prevalence of C, E, c, and e antigens in RhD-negative donors was reported as 16.67%, 16.67%, 88.89%, and 100%. It is difficult to compare the prevalence of other minor blood group antigens with the abovementioned serological studies as the donor population in our study was all RhD Negative, whereas these studies involved both RhD-positive and negative donors.

Molecular blood group genotyping with microarray technology offers many advantages over standard serological or basic PCR-based tests as it gives detailed information about blood group systems which can be objectively interpreted into a genotype and a predicted phenotype. The other advantage concerning blood donors is that it is effective in detecting RHD variants. Current high throughput blood group genotyping platforms are rapid and reliable methods to enable large-scale screening of blood donors for rare blood group phenotypes.[26]

The main challenge to current DNA-based molecular platforms is their limitation to detect unknown single nucleotide variants (SNV). Any novel mutations, molecular variants, large deletions, hybrid alleles, and complex genes such as ABO and Rh are less likely to be detected as only the alleles incorporated in the microarray kit are identified. Other limitations are that a genotype may be falsely interpreted as a positive phenotype in case a null allele is not included in the assay. Designing optimal primers or probes for some blood groups is also a challenge.[10,27] The challenges faced by us in conducting the study were financial constraints, logistics required to perform the tests as infrastructure, dediacted molecular laboratory, trained staff, and support from the reference laboratory. The results of some of the blood group systems could not be obtained as the alleles could not be amplified.

This pilot study was conducted in a single institute with a small sample size due to financial constraints. As there are negligible data on molecular genotyping in Indian blood donors, we initiated this pilot study on 50 blood donors. As the prevalence of Rh negative blood group in the Indian population is 5%–6%, so Rh-negative blood group donors were selected which justified the small study population. In addition, serological studies could not be done due to a limited budget.

As donor blood group genotyping will identify donors with rare blood groups and RBC phenotypes negative for specific multiple antigens, this policy should be adopted by blood centers in India. It will facilitate the formation of comprehensive rare blood donor registries, and sharing the databases between different transfusion centers throughout the country will improve the transfusion services. It could help to provide "personalized" red cell units for the recipient.[19] It will in addition help to streamline donor management by identifying and maximizing the use of RBC units containing rare antigens and facilitating targeted donor recruitment.

Conclusion

This pilot study shows the feasibility of genotyping a Northern Indian donor population. In our study, we found that E, Kpa , and Lua (detected in 2% of the study population) were the least common antigens present in the given population, whereas c, e, k, Kpb , and Jsb were the most common antigens present. To the best of our knowledge, it is the first study on molecular blood grouping in Indian blood donors using the Bioarray platform. With this initial pilot study, we plan to expand it to our entire donor population.

Author contributions

GN – Study concept and design, acquisition, analysis, or interpretation of data, critical revision of the manuscript for important intellectual content, and statistical analysis.

SM – Study design, acquisition, analysis, or interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, administrative, technical, or material support, and study supervision.

BG – Study design, acquisition, analysis, or interpretation of data, critical revision of the manuscript for important intellectual content, administrative, technical, or material support, and study supervision.

PKS – Study design, acquisition, analysis, or interpretation of data, critical revision of the manuscript for important intellectual content, administrative, technical, or material support, and study supervision.

AAM – Study design, acquisition, analysis, or interpretation of data, critical revision of the manuscript for important intellectual content, administrative, technical, or material support, and study supervision.

Data availability statement

Data will be available from the first/corresponding author for academic reasons upon reasonable request.

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Conflicts of interest

There are no conflicts of interest.

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