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*SMIM*1 intron 2 gene variations leading to variability in Vel antigen expression among Brazilian blood donors

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ABSTRACT

Background: There is a significant inter-individual heterogeneity of Vel antigen expression which can lead to inaccuracies on Vel phenotyping of blood donors and, potentially, to hemolytic post-transfusion reactions. Our aim was to evaluate the impact of genetic variants in the *SMIM*1 intron 2 on the expression of Vel antigen among Brazilian blood donors harboring the *c.64_80del17* deletion in heterozygosity.

Methods: Donors presenting the *SMIM1 c.64_80del17* in heterozygosity were included in the study and subjected to *SMIM1* intron 2 direct sequencing aiming to genotype the following polymorphisms: rs143702418, rs1181893, rs191041962, rs6673829, rs1175550 and rs9424296.

Results: SMIM1 intron 2 sequencing was performed on two hundred donors presenting one *c.64_80del17* allele. The rs1175550 polymorphism significantly impacted on Vel antigen expression. Variations in the strength of agglutination on Vel phenotyping were also observed according to the rs6673829 genotype, but this difference did not persist with statistical relevance after multivariate analysis.

Conclusion: The presence of the rs1175550A allele of *SMIM1* is significantly and independently associated with a decrease in Vel antigen expression. Even though the population in Brazil is intensely mixed, the allele frequencies obtained in the current study were very similar to that reported for Europeans.

1. Introduction

The molecular basis of the Vel blood group system was recently elucidated [1–3]. The rare Vel− phenotype is inherited as a 17 nucleotide- deletion (hg19, chr.1: *g.3691998_3692014delGTCAGCCTAGG GGCTGT*) in homozygosity in the *SMIM*1 exon 3 [1–3], whose frequency varies from 0 to 0,057% depending on the ethnic background of the studied population [1–6]. There is a significant variability in the Vel antigen expression among Vel+ individuals, which is partly explained by the presence of one *SMIM1***64_80del* allele [2,3]. This inter-individual expression variability is of great importance for transfusion practice, because it may result in false negative results on Vel antigen phenotyping of blood donors, potentially causing hemolytic transfusion reactions in patients sensitized with anti-Vel [4].

It has been demonstrated that the *SMIM*1 intron 2 is an important regulatory region for Vel antigen expression [2,4,7]. A strong correlation was observed between the expression of Vel antigen and the presence of the *SMIM*1 variant rs1175550, whose major allele rs1175550A was associated with significant decrease in *SMIM*1 expression in a gene expression quantitative trait locus (e-QTL) study [8]. The role played by rs1175550A on reducing Vel expression was reproduced by many other studies [4,7,9]. Another gene variation of *SMIM*1 intron 2, rs143702418 (C > CGCA), was also associated with a small but independent effect on Vel expression [4]. This variant is in linkage

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disequilibrium with rs1175550 in Caucasians, but has separate effects in African Americans [4]. Recently, one study suggested that the *SMIM*1 intron 2 variant rs6673829 may also be related to the reduction of Vel antigen expression [10], but it was not confirmed by other studies.

Characterization of the *SMIM*1 intron 2 has already been performed in different populations [11]. However, information about the distribution of SMIM1 intron2 polymorphisms in admixed population such as Brazilian is still incomplete. In this context, our aim was to characterize genetic variations in *SMIM*1 intron 2 among Brazilian blood donors presenting the *c.64_80del17* in heterozygosity correlating to the Vel antigen expression.

2. Materials and methods

2.1. Overall description of the study

A total of 208 blood donors presenting one *SMIM1*64_80del* allele (WT/Δ) from Fundação Pró-Sangue/Hemocentro de São Paulo, Brazil, were selected for the study between August 2014, and July 2018. These donors were genotyped using an *in house* validated TaqMan protocol, which was described in details elsewhere [12]. Among all WT/Δ donors selected for the study ($n = 208$), serological Vel phenotyping was performed in 116 participants and conventional PCR to check for Vel zygosity together with *SMIM*1 intron 2 direct sequencing in 200 participants, as described below. The protocol was approved by the ethical committee from the Reference and Training Center in DST/AIDS, São Paulo, Brazil (CAAE 45481715.6.0000.5375).

2.2. Nucleic acid purification

Buffy-coat DNA was individually extracted from all selected samples using the PureLink Genomic Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Purity and concentration of the material were evaluated by spectrophotometry (Nanodrop 1000 Spectrophotometer, Wilmington, DE, USA). DNA samples were diluted to a final concentration of 100 ng/μL for genotyping.

2.3. Determination of Vel zygosity

All donors carrying the *SMIM1*64_80del17* allele were subjected to conventional PCR to check for Vel zygosity using a PCR-restriction enzyme protocol, as previously described [1,3]. PCR conditions are described in the Supplementary data.

2.4. Serological testing

The expression of Vel antigen was quantified through serological phenotyping using gel-method. A polyclonal human antiserum of blood group B containing immune anti-Vel and anti-K was used for the tests. It is important to highlight, at this point, that no standard reagent antibody exists for Vel phenotyping and that Vel phenotyping results will depend on the specific laboratory single source antibody used. In brief, 25 μL of the anti-serum was added to DG Gel anti-IgG cards (Grifols, Barcelona, Spain) together with a 1% suspension of the red blood cells (RBCs). After incubation at 37 °C and centrifugation at 990 rpm (6220.4 rad·min^{-1}) for 9 min, the reactivity was graded as negative, weak, 1+, 2+ or 3+ [13]. Antibody screening was performed on plasma from all samples in which the deletion was detected.

2.5. SMIM1 Intron 2 sequencing

All samples of blood donors classified as WT/Δ were subjected to *SMIM*1 intron 2 direct sequencing through Sanger method. Initially, conventional PCR was performed using the following primers: *SMIM*1_128_F/*SMIM*1_472_R and INTRON_2_VEL_F/INTRON_2_VEL_R (Supplementary data), designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). PCR conditions are described in the Supplementary data.

2.6. Statistical analysis

Allelic and genotypic frequencies of the *SMIM*1 intron 2 gene in donors with deletion *c.64_80del17* in homozygous and heterozygous were determined by direct counting. Differences between allelic frequencies among samples with distinct antigen strengths were compared by using Mann-Whitney *U* test. Chi-square test or Fisher's exact test were used to compare the distribution among different genotypes of *SMIM*1 intron 2 gene. Unadjusted and adjusted analyses were conducted by using Logistic Regression (LR) models to determine the association of covariates with binary outcome. The logistic regression results were presented as odds ratios (OR) with Wald 95% confidence interval (CI) and *p*-values. All statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS software, Chicago, IL, USA) version 22.0 and GraphPad Prism software, version 8.00 (GraphPad software, San Diego CA, USA). A p-value of < 0.05 was considered to be statistically significant, and all reported *p*-values were two-sided. The Hardy-Weinberg equilibrium and linkage disequilibrium analysis for all polymorphisms were determined through Haploview (https://www.broadinstitute.org/haploview/downloads).

3. Results

3.1. Study population

Two hundred and eight blood donors presenting the *SMIM*1 *c.64_80del17* in heterozygosity (*WT/Δ*) were selected for the study. These donors were identified within a group of > 25,000 donors, as published elsewhere [5]. *SMIM1* intron 2 was genotyped through direct sequencing for 200 of the *WT/Δ* samples, as in eight samples there was no DNA left for the tests. The main demographic and serological characteristics of this studied donor population is described in Table 1.

3.2. Serological analysis

Vel antigen phenotyping was performed using polyclonal anti-Vel (human origin) in 116 samples exhibiting the *c.64_80del17* in heterozygosity, but *SMIM1* intron2 genotyping was performed in only 112 phenotyped donors because no DNA was available for the tests of the

Table 1
Demographic and serological characteristics of the studied blood donors.

Variables	No./total no. (%)
Self-declared race	
White	70/208 (33.7)
Black	62/208 (29.8)
Ignored	76/208 (36.5)
ABO type	
O	100/206 (48.5)
A	80/206 (38.8)
B	20/206 (9.6)
AB	6/206 (2.9)
K1 phenotype	
Negative	145/154 (94.2)
Positive	9/154 (5.8)
Vel phenotype	
Negative	46/116 (39.7)
Positive	70/116 (60.3)
Vel phenotyping reactivity	
Negative	46/116 (39.7)
Weak	22/116 (19.0)
1+	22/116 (19.0)
2+	24/116 (20.7)
3+	2/116 (1.7)

Table 2
Analysis of the linkage disequilibrium of the studied SMIM1 intron2 polymorphisms.

rs143702418, rs1181893, rs6673829, rs1175550 and rs9424296			
Haplotype associations	Frequency	Chi square	*p* value
CCGAC	0.477	1.033	0.3095
GCGGC	0.197	0.672	0.4124
GCGAC	0.143	0.788	0.3748
CCAAC	0.100	0.221	0.6383
CCGAA	0.038	8.197	0.0042
CCGGC	0.028	0.51	0.4749
CAGAC	0.018	5.598	0.018

Table 3
Frequency *SMIM*1 Intron 2 genotype (n = 200).

Variables	No. (%)
rs143702418	
CC	132 (66.0)
CGCA/CGCA	68 (34)
rs1181893	
CC	194 (97.0)
AC	5 (2.5)
AA	1 (0.5)
rs191041962	
CC	200 (100.0)
rs6673829	
GG	165 (82.5)
GA	30 (15.0)
AA	5 (2.5)
rs1175550	
AA	149 (74.5)
AG	12 (6.0)
GG	39 (19.5)
rs9424296	
CC	186 (93.0)
CA	13 (6.5)
AA	(0.5)

remaining four. Phenotyping resulted negative in 46 donors (39.7%) and positive in the remainder 70 donors (60.3%), with strength of agglutination varying from weak to 3+. It was not possible to perform Vel phenotyping in 86 donors because RBCs were ABO-incompatible with the selected anti-Vel serum and in 6 donors because they phenotyped as K+ and the selected serum had anti-K. All donors exhibiting *c.64_80del17* in homozygosity (n = 2) phenotyped as Vel− and one of them had anti-Vel (titer = 32/score = 43).

3.3. SMIM1 intron 2 genotyping

3.3.1. Linkage disequilibrium and haplotype frequency analysis

The genotypic and allele distribution of the polymorphisms rs1181893, rs191041962, rs6673829 and rs9424296 were in agreement with *Hardy-Weinberg* equilibrium (HWE). Rs143702418 and rs1175550 were in disequilibrium (HW*p*val < 0.05). Haplotype linkage disequilibrium analysis (Fig. 2, Table 2) showed the following situations of strong linkage disequilibrium: rs143702418 and rs6673829 (LD = 100); rs143702418 and rs1175550 (LD = 81); rs143702418 and rs9424296 (LD = 100); rs6673829 and rs1175550 (LD = 100). The data were processed in Haploview (https://www.broadinstitute.org/haploview/downloads).

3.3.2. Correlation between SMIM1 intron 2 genotype and phenotype data

Two hundred donors presenting one *c.64_80del17* allele were genotyped for the following SNPs: rs143702418, rs1181893, rs191041962, rs6673829, rs1175550 and rs9424296, all located in the intron 2 of *SMIM*1.

3.3.3. rs1175550

Among the two hundred genotyped donors, 149 (74,5%) presented the AA genotype, 39 (19,5%) the GG genotype and 12 (6%) the AG genotype (Table 3). Among the donors with AA genotype (n = 149), 86 were phenotyped for Vel antigen. Of these, 41 (47.7%) were phenotypically Vel− and 45 (52.3%) typed as Vel-positive with the following strength of agglutination: weak (n = 19), 1 + (n = 16) and 2+ (n = 10). Among the donors with GG genotype (n = 39), 20 were

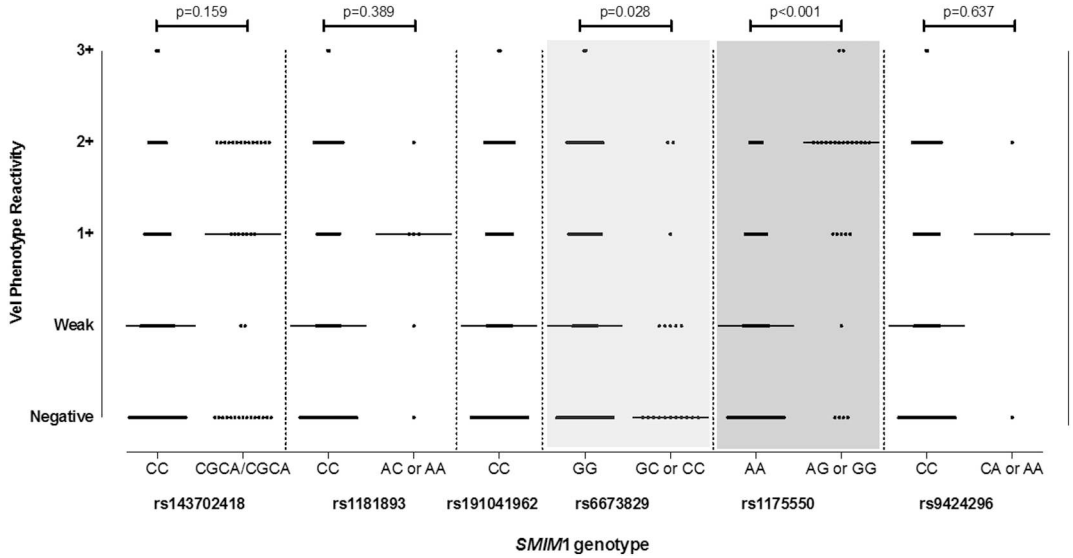

Fig. 1. Correlation between the levels of Vel antigen expression and *SMIM1* intron 2 genotypes (rs143702418, rs1181893, rs191041962, rs6673829, rs1175550 e rs9424296) in 112 blood donors with the presence of the *c.64_80del17* deletion. The vertical axis of the graph shows the levels of Vel antigen expression and the horizontal axis shows the genotype of the rs variants in *SMIM1* intron 2. In the individual analysis of variants rs6673829 (light gray quadrant) and rs1175550 (dark gray quadrant) the *p*-value was significant. Mann-Whitney *U* test was used to compare the means of Vel phenotype reactivity between the *SMIM1* genotypes.

Table 4
Logistic regression analyses predicting association of Vel reactivity in blood donors with *SMIM*1 Intron 2 genotype (n = 112).

	Vel reactivity		Unadjusted		Adjusted[a]	
SNP	Negative (n = 45)	Positive (n = 67)	OR (CI95%)	p	OR (CI95%)	p
rs143702418 - no. (%)						
CC	30 (40.5)	44 (59.5)	1.0 (ref.)			
CGCA/CGCA	15 (39.5)	23 (60.5)	1.045 (0.470–2.324)	0.913		
rs1181893 - no. (%)						
CC	44 (41.5)	62 (58.5)	1.0 (ref.)			
AC/AA	1 (16.7)	5 (83.3)	3.548 (0.401–31.438)	0.255		
rs191041962 - no. (%)						
CC	45 (40.2)	67 (59.8)	NA	NA		
rs6673829 - no. (%)						
GG	34 (36.6)	59 (63.4)	1.0 (ref.)			
GA/AA	11 (57.9)	8(42.1)	0.419 (0.154–1.144)	0.089	0.591 (0.210–1.661)	0.319
rs1175550 - no. (%)						
AA	41 (47.7)	45 (52.3)	1.0 (ref.)			
AG/GG	4 (15.4)	22 (84.6)	5.011 (1.593–15.768)	0.006	4.466 (1.385–14.396)	0.012
rs9424296 - no. (%)						
CC	44 (40.4)	65 (59.6)	1.0 (ref.)			
CA	1 (33.3)	2 (66.7)	1.354 (0.119–15.390)	0.807		

OR, odds ratio. CI, confidence interval.
[a] The adjustment was performed because the self-declared race of the included donors was not available and the distribution of ABO types could reflect the racial distribution of the studied population.

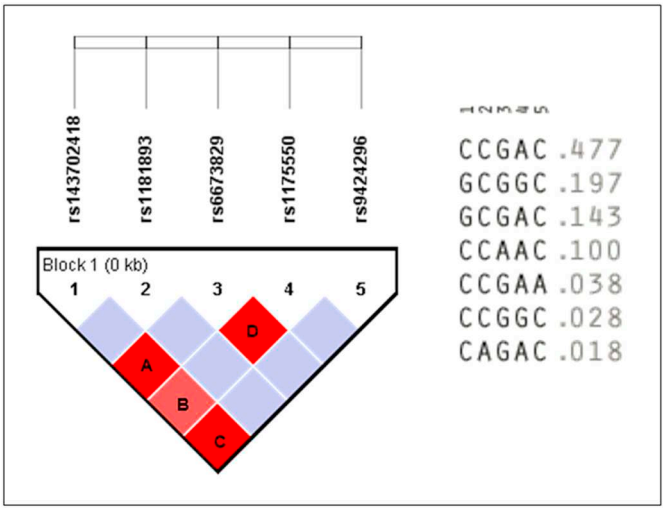

Fig. 2. Linkage disequilibrium (LD) and haplotype frequency analysis of the polymorphisms in *SMIM1* intron 2. The GCA insertion (rs143702418) is represented as the G nucleotide. Linkage disequilibrium was detected in four situations (red boxes): A. rs143702418 and rs6673829 (LD = 100), B. rs143702418 and rs1175550 (LD = 81), C. rs143702418 and rs9424296 (LD = 100) and D. rs6673829 and rs1175550 (LD = 100). Blue boxes represent situations of linkage equilibrium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phenotyped for Vel antigen. Of these, 4 (20%) typed as Vel-negative and 16 (80%) as Vel-positive with the following strength of agglutination: weak (n = 1), 1+ (n = 4) and 2+ (n = 11). In the group of donors with AG genotype (n = 12), 6 (50%) were phenotyped and the resulting Vel expression varied between 1+ and 3+. In the statistical analyses, the levels of Vel antigen expression were significantly lower in donors homozygotes for the A allele compared to donors heterozygous and homozygotes for the G allele ($p < 0.01$) in univariate analysis (Fig. 1) and the difference persisted in multivariate analysis (p = 0.012) (Table 4).

In the studied sample, 97.4% (38/39) of the donors with GG genotype and all donors with AG genotype had the GCA insertion in homozygosity (rs143702418). One donor with GG genotype was genotyped as CC for rs143702418.

3.3.4. rs6673829, rs143702418, rs1181893, rs9424296 and rs191041962

The genotype frequencies of rs6673829, rs143702418, rs1181893, rs9424296 and rs191041962 are summarized in Table 3.

Vel antigen expression was compared between the different genotypes of the studied SNPs (Fig. 1). Variations in the strength of agglutination were observed only for rs6673829, depending on the genotype. Vel antigen expression was significantly higher in donors homozygotes for the G allele compared to donors heterozygous and homozygotes for the A allele (p = 0.028). However, this difference did not persist with statistical relevance after multivariate analysis (p = 0.319).

Table 5
New polymorphism in *SMIM*1 intron 2.

ID	Vel phenotype	Vel genotype	g.1894_1899delGCCCCG	rs1175550	Self-declared race
	Reactivity	Storry et al.			
Donor1	Negative	*WT/Δ*	G/GCCCCG	AA	Black
Donor2	Not tested	*WT/Δ*	G/GCCCCG	AA	Black
Donor3	1+	*WT/Δ*	G/GCCCCG	AA	Black

WT - wild type.
Δ - deleted.

3.4. Never-described SMIM1 allele

A novel allele exhibiting the deletion of six nucleotides in the *SMIM*1 intron 2 (chr1:3691245_3691250delGCCCCG) was identified in three donors (Table 5, Supplementary data). Vel phenotyping was performed in two of these donors: one resulted negative and the other resulted positive (1 +).

4. Discussion

This study evaluated the impact of gene variations in the *SMIM*1 intron 2 on the expression of Vel antigen among donors harboring the *c.64_80del17* deletion in heterozygosity (WT/Δ). It was observed that the expression of the Vel antigen was significantly heterogeneous among WT/Δ individuals and that, in 39.7% of the cases (46/116), donors exhibited false-negative results on Vel phenotyping. The rs1175550 was independently associated with Vel antigen expression, which was significantly lower in donors homozygous for the A allele compared to donors heterozygous and homozygous for the G allele. The other studied gene variations (rs6673829, rs143702418, rs1181893, rs9424296 and rs191041962) did not correlate significantly with Vel antigen expression, except for rs6673829 and rs143702418, which were both in strong linkage disequilibrium with rs1175550. This is the first study that targeted the c.64_80del17 allele and selected a large group of WT/Δ donors to extensively investigate the *SMIM1* intron2. This allowed the study to evaluate the additional role played by intron2 polymorphisms on Vel expression, besides the effect of *SMIM1 c.64_80del17* in heterozygosity. Also, most of what has been previously published referring to the allele frequency of rs1175550 derived from Caucasian population and the present study focused on Brazilian population, which is highly admixed.

Our results confirm previous evidences that *SMIM*1 variant rs1175550 independently modulates Vel antigen expression [2,4,7]. The AA genotype of rs1175550 was significantly associated with lower strength of agglutination as well as with higher rates of false-negative results on Vel phenotyping of *WT/Δ* donors. There were 45 *WT/Δ* donors in the studied cohort presenting with Vel-negative phenotype. Of these, 41 (91.1%) were homozygous for the major allele rs1175550A. The other four donors had the GG genotype, but they also presented the GCA insertion (rs143702418) in homozygosis, what may have justified the low antigen density on erythroid membrane, based on the evidences described by Christophersen et al. [4] This author has evaluated the molecular basis of the inter-individual heterogeneity of Vel antigen expression stating that both rs1175550 and rs143702418 impact on Vel expression [4]. In the specific case of rs143702418, the linkage disequilibrium with rs1175550A is intense, especially among Caucasians [4]. This may explain why a statistically relevant association between rs143702418 and Vel expression could not be observed from the multivariate statistical analysis, as, in this study, a mixed population (of both Caucasian and African descent) was in focus.

We have found a significant association between Vel antigen expression and the rs6673829 genotype in univariate statistical analysis, in accordance to a previous Brazilian report [10]. However, all donors presenting the rs6673829 AA and GA genotypes, which were associated with lower strengths of agglutination on Vel phenotyping, also genotyped as AA for rs1175550. This confirmed the linkage disequilibrium between rs6673829 and rs1175550, weakening the hypothesis of an independent association between rs6673829 and Vel expression. The other studied polymorphisms of *SMIM*1 intron 2 (rs1181893, rs9424296, rs191041962) did not associate with Vel expression, also in accordance with previous literature reports [4]. A novel 6 bp-deletion in *SMIM1* intron 2 (chr1:*3691245_3691250delGCCCCG*) was detected in three WT/Δ donors with strengths of agglutination on Vel phenotyping varying from negative to 1 + . Further studies are needed in order to confirm if this variant decreases *SMIM1* transcript levels.

The allele frequencies of the studied gene variations, representing the Brazilian population, were compared to that of African, American, East Asian, European and South Asian populations (Supplementary Table S1). Even though the population in Brazil is intensely admixed, the allele frequencies obtained in the present study were very similar to that found in Europeans. The most important example is the rs1175550, in which the frequency of rs1175550G is 60% among Africans against 22% in our studied population. This may reflect the fact that the prevalence of people of Caucasian-descent among blood donors is higher than in general Brazilian population [10].

This study has some limitations. The fact that Vel phenotyping was performed only in part of the samples restricted the number of individuals available for statistical comparisons. Also, Vel antigen expression was not quantified through flow cytometry. However, the assessment using intensity of agglutination obtained from Vel phenotyping was suitable for the proposed statistical comparisons and the results obtained were in agreement with the supporting literature.

In conclusion, there is a wide variability of Vel antigen expression among individuals exhibiting the *c.64_80del17* deletion in heterozygosity and the presence of the rs1175550A allele of *SMIM1* intron 2 is significantly and independently associated with a decrease in this expression.

Author contribution

Designed the study: MRD, CLD, JEL, AMJ; Performed the experiments: MRD, CNG, IHR, VBO, MCAVC, MLPC, AFA, SRAB, NAS; Analyzed the data: MRD, TGMO, MLPC; Statistical analysis: MRD, ACN and CLD; Wrote the manuscript: MRD, ACN and CLD; Reviewed the data and the paper: JEK, ACP, ECS, VR, AMJ, CLD and JEL. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2019.03.006.

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