Rapid detection of *FMO3* single nucleotide polymorphisms using a pyrosequencing method

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Abstract. The present study aimed to develop a reliable pyrosequencing method to detect four single nucleotide polymorphisms (SNPs) of the flavin-containing monooxygenase 3 (*FMO3*) gene and to compare the ethnic differences in their allelic frequencies. The pyrosequencing method was used to detect four *FMO3* SNPs, namely, c.855C>T (N285N, rs909530), c.441C>T (S147S, rs1800822), c.923A>G (E308G, rs2266780) and c.472G>A (E158K, rs2266782). The allelic frequencies of these SNPs in 122 unrelated Korean subjects were as follows: i) 44.7% for c.855C>T; ii) 23.4% for c.441C>T; iii) 23.0% for c.923A>G; and iv) 27.1% for c.472G>A. Linkage disequilibrium (LD) analysis revealed that the SNPs c.923A>G and c.472G>A exhibited a strong LD (D'=0.8289, r^2=0.5332). In conclusion, the pyrosequencing method developed in this study was successfully applied to detect the c.855C>T, c.441C>T, c.923A>G and c.472G>A SNPs of *FMO3*.

Introduction

Flavin-containing monooxygenases (FMOs) form a family of microsomal antioxidant defense enzymes responsible for nicotinamide adenine dinucleotide phosphate-dependent oxygenation of soft nucleophiles (1,2). Five functional isoforms of FMO have been identified in humans (FMO1-5) (2). *FMO3*, primarily located in the liver, is the second most common FMO that metabolizes various nitrogen- and sulfur-containing drugs and exhibits a broad range of substrates (3-5). The *FMO3* gene is clustered on chromosome 1 (q24.3) and contains nine exons ranging from 80 to 705 bp (2). Several genetic polymorphisms have been identified in this region (2). Moreover, previous studies have reported genetic polymorphisms of *FMO3* that affect the enzyme activity and plasma concentrations of certain medications, and diseases such as trimethylaminuria (6-8). Of these polymorphisms, the c.855C>T (N285N, rs909530), c.441C>T (S147S, rs1800822), c.923A>G (E308G, rs2266780) and c.472G>A (E158K, rs2266782) mutations are commonly detected single nucleotide polymorphisms (SNPs) in East Asian populations (9-12). Considering their clinical importance and prevalence, there is a need to investigate the differences in the allelic frequencies of these polymorphisms between various ethnic groups and develop a reliable method for such analysis, which could be applied for optimal subject group targeting in clinical practice (8).

In the present study, a rapid and reliable pyrosequencing method was developed to detect SNPs of the *FMO3* gene, including two synonymous (c.855C>T and c.441C>T) and two non-synonymous (c.923A>G and c.472G>A) variants, all of which are clinically important and common in the Korean population (13,14). Additionally, this study aimed to compare the allelic frequencies of these SNPs in a Korean population with those reported in other ethnic groups.

Materials and methods

Subjects and methods. This study was conducted in Korea University Anam Hospital (Seoul, Korea) between April 2017 and February 2020. Genomic DNA was extracted from the blood samples of 122 unrelated healthy Korean subjects (age: 20-45, all male participants) who provided written informed consent to participate in this study. The protocol for this assay was approved by the institutional review board of Anam Hospital, Korea University Medical Center (IRB approval no. 2017AN0117, Seoul, South Korea).

Polymerase chain reaction (PCR) conditions and FMO3 genotyping using pyrosequencing. Genomic DNA was extracted from peripheral blood leukocytes as previously described (15). GeneAll® Exgene Blood SV kit (GeneAll) was used according to the manufacturer's instructions. DNA quantification was processed by using Biospec-Nano (Shimadzu, Kyoto, Japan). A pyrosequencing method was developed to detect the func-

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tional SNPs of the *FMO3* gene: c.855C>T, c.441C>T, c.923A>G and c.472G>A. PCR primers used for *FMO3* genotyping and pyrosequencing are listed in Table I. PCR was performed to amplify the specific sequences and detect each SNP of *FMO3* using the newly developed primer sets after tagging the 5' end of each forward (or reverse) primer with biotin using the PSQ Assay Design software (version 2.0; Qiagen GmbH).

The PCR mixture (30 μl) comprised genomic DNA (30 ng), 10X PCR buffer (Intron Biotechnology, Inc.), dNTPs (0.25 mM), 10 pmol primers (1 μl each) and 5 units Taq polymerase (Intron Biotechnology, Inc.). PCR was performed with an initial denaturation step at 95˚C for 3 min, followed by 45 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec. The final termination step was performed at 72˚C for 5 min. For pyrosequencing reactions, 25 μl PCR template in a single well was immobilized by incubation (with continuous shaking at 1,400 rpm for 10 min at room temperature) with a mixture of 5 μl streptavidin beads (Streptavidin Sepharose™ High Performance; Cytiva) and 40 μl annealing buffer containing 0.4 μM sequencing primer incorporated into each well. For strand separation, the liquid component was removed using a vacuum prep workstation (Qiagen GmbH). The beads captured on the probes were treated in 70% ethanol, and the solution was passed through a filter for 5 sec. The beads were then treated with a denaturing solution (0.2 M NaOH), and the solution was passed through a filter for 5 sec. Thereafter, a wash buffer (10 mM Tris-acetate, pH 7.6) was used to rinse the beads for 5 sec. The liquid component was completely removed from the probes, and the beads were placed into a PSQ 96 Plate Low (Pyrosequencing AB) containing the sequencing primer. The prepared PSQ 96 Plate Low was heated at 85˚C for 2 min, and the reactions were allowed to cool to room temperature. The resulting mixture was analyzed using the PSQ 96MA pyrosequencer (Pyrosequencing AB). The accuracy of pyrosequencing was validated by direct DNA sequencing of randomly selected samples using the same genomic DNA. The analyzed allelic frequencies were then compared with those of other ethnic groups and those reported in the HapMap database (https://www.ncbi.nlm.nih.gov/snp).

Statistical analysis. Genetic equilibrium and linkage disequilibrium (LD) were tested according to the Hardy-Weinberg equation (HWE) (16) using SNPalyzer software (version 9.0; DYNACOM Co., Ltd.). A chi-square test was performed to assess the deviation of the pyrosequencing results from the HWE. The detected genotype frequencies were then compared to the expected frequencies. $P<0.05$ (two-tailed) was considered to indicate a statistically significant difference. D' and r^2 are standard measurements for the LD (17). D' values were calculated as D/D_{max}, where D is the coefficient of LD ranging from -0.25 to 0.25. In general, the standardized value of D' is preferred because D is often affected by allelic frequencies (18).

Results

Each FMO3 SNP, including c.855C>T, c.441C>T, c.923A>G and c.472G>A, was successfully detected, as shown in the predicted pyrosequencing histogram (Fig. 1). Representative peaks for each SNP are shown in Fig. 2. The sequenced data obtained using the pyrosequencing method were randomly selected and validated by direct DNA sequencing. The results were 100% concordant with the pyrosequencing data, indicating 100% specificity and sensitivity (data not shown).

The allelic frequencies of *FMO3* SNPs in the Korean population obtained using our pyrosequencing method were as follows: i) 44.7% for c.855C>T; ii) 23.4% for c.441C>T; iii) 23.0% for c.923A>G; and iv) 27.1% for c.472G>A (Table II). The allelic frequencies obtained in these genetic analyses did not deviate from the Hardy-Weinberg equilibrium (χ^2=0.1843, 0.1201, 0.0318 and 0.4729 for c.855C>T, c.441C>T, c.923A>G and c.472G>A, respectively; P=0.6677, 0.7290, 0.8584 and 0.4917 for c.855C>T, c.441C>T, c.923A>G and c.472G>A, respectively); however, the LD analysis revealed that c.923A>G and c.472G>A exhibited strong LD (D'=0.8289, r^2=0.5332; Table SI).

The ethnic differences of the SNPs were described in Table III. Although the data were limited, particularly for the European and African populations; however, the trend of the allelic frequencies for *FMO3* SNPs obtained in the present study was similar to that previously reported in a Japanese population (12). In particular, the allelic frequencies of c.923A>G and c.472G>A appeared to be similar to those in the Chinese population (3). The SNP c.923A>G frequency exhibited some similarity to the minor allele frequency (MAF) of the HapMap data of Europeans (Utah residents with Northern and Western European ancestry from the CEPH collection reported by the National Center for Biotechnology Information SNP database; HapMap-CEU; https://www.ncbi.nlm.nih.gov/snp), whereas the frequencies of other SNPs exhibited remarkable differences from the MAF of this population.

Discussion

The results of the present study indicated that this newly developed rapid pyrosequencing method for analyzing the c.855C>T, c.441C>T, c.923A>G and c.472G>A SNPs of the *FMO3* gene was a reliable and accurate technique. The allelic frequencies obtained in 122 Korean subjects using this method revealed that these frequencies were most similar to those reported in the Japanese population (12). To the best of our knowledge, this was the first study to analyze *FMO3* SNPs using a pyrosequencing method.

Various methods have been proposed to analyze the targeted SNPs. For example, *FMO3*-related SNPs have been detected by using PCR-restriction fragment length polymorphism analysis (19), real-time PCR (20) and direct sequencing methods (21). Sequencing technology was first conceptualized and developed in the 1970s by Sanger *et al* (22). The principle of this method is based on the use of dideoxynucleotide triphosphates for DNA sequence termination. The pyrosequencing method that was designed to analyze *FMO3* SNPs in the current study was based on the solution-based pyrosequencing method suggested by Ronaghi *et al* (23) in 1998. This is a simple method that is suitable for automation as it uses apyrase, DNA polymerase and luciferase, which eventually detect light emission through pyrophosphate production during DNA synthesis (23). The major advantages of this method are its simplicity, reliability, high sensitivity and specificity compared

Table I. Oligonucleotide primers used for PCR and pyrosequencing to detect *FMO3* SNPs.

SNP	Primer	Sequences	Size, bp	PCR Tm, ˚C
FMO3 c.855C>T (rs909530)	Forward	B 5'-TTGGGTCATTTTTTCCTTCCTTAT-3'	261	60
	Reverse	5'-ACCCTGTTGCAAAGATTACACAGT-3'		
	Sequencing	5'-TTGCTGGGAGCTCAT-3'		
FMO3 c.441C>T (rs1800822)	Forward	B 5'-CCACTGAAAGGGATGGTAAAAA-3'	125	60
	Reverse	5'-AGCAGCTTAAATTTTGGCCTTAC-3'		
	Sequencing	5'-TGGGATACACATGATGTC-3'		
FMO3 c.923A>G (rs2266780)	Forward	5'-AGCATTCTGTGTGGCATTGT-3'	144	60
	Reverse	B 5'-AAGGAAGGGGTAGGCAAAACTAT-3'		
	Sequencing	5'-CGTGAAGGAATTCACAG-3'		
FMO3 c.472G>A (rs2266782)	Forward	B 5'-ATGGTAAAAAAGAATCGGCTGTC-3'	132	60
	Reverse	5'-TTTTGTCAGTTATGTGGCTAGCAG-3'		
	Sequencing	5'-GCCTTACCTGGAAAGGACT-3'		

FMO3, flavin-containing monooxygenase 3; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; B, biotinylated at the end of the primer; Tm, melting temperature.

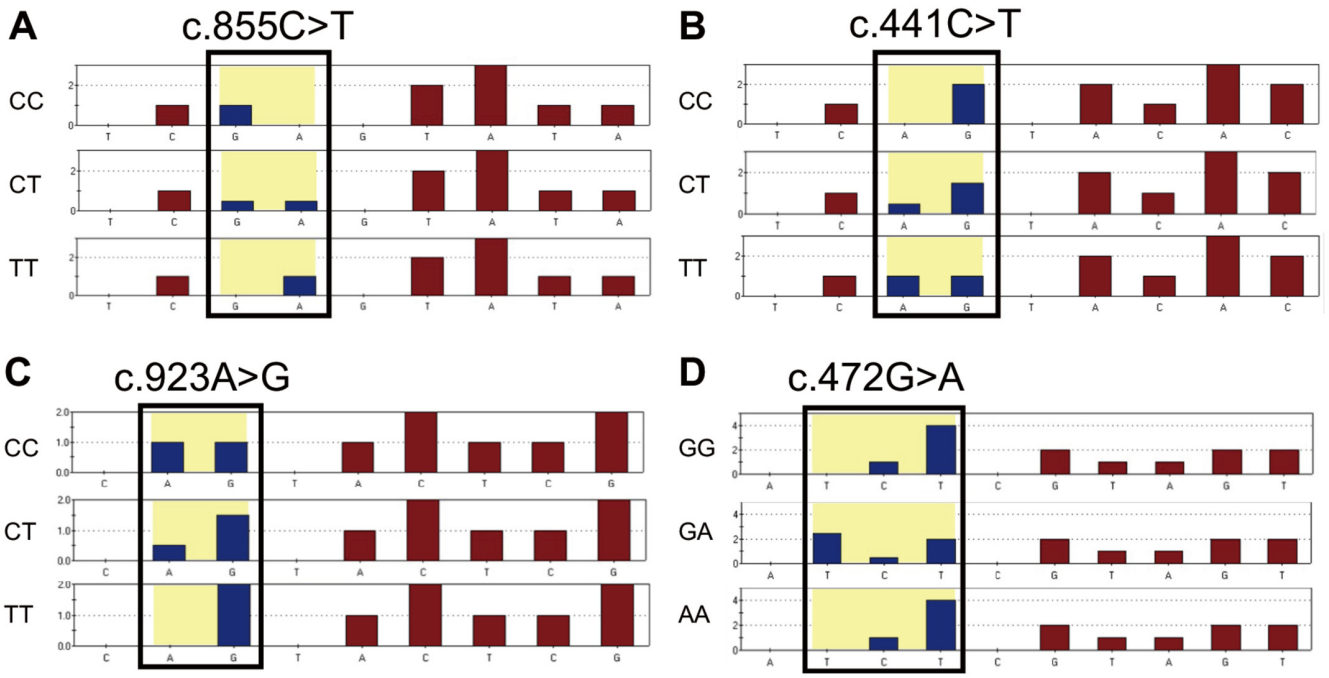

Figure 1. Predesigned predicted histograms of *FMO3* SNPs generated using the pyrosequencing software. (A) c.855C>T, (B) c.441C>T, (C) c.923A>G and (D) c.472G>A SNPs of the *FMO3* gene. Area under the black box indicates the detected polymorphism site. *FMO3*, flavin-containing monooxygenase 3; SNPs, single nucleotide polymorphisms.

with conventional sequencing systems (24). Therefore, it was speculated that the method described in the present study could be suitable for precise, rapid and cost-effective assessment of SNP frequencies in a relatively large sample set.

SNPs are the most frequently occurring sequence variations in the human genome and often vary among different ethnic groups (1,2). The allelic frequencies of selected *FMO3* SNPs observed in this study were comparable to those reported in the Japanese population (9), whereas the frequency of each genotype in the Chinese population was generally lower than that in the Korean or Japanese populations (3,12). *FMO3* c.855C>T was the most commonly detected SNP in the current study; this result was consistent with that previously reported in a smaller Korean population previously (n=41, MAF=0.329) (13). The frequencies of the c.855C>T and c.472G>A SNPs were higher in the African population (HapMap-YRI database; https://www.ncbi.nlm.nih.gov/snp,32) than in the Asian populations; however, the frequencies of c.441C>T and c.923A>G in the African population were markedly lower (<5%) (HapMap-YRI database; https://www.ncbi.nlm.nih.gov/snp). Therefore, *FMO3* appears to exhibit a large interethnic difference (3,9,13).

Table II. Genotyping and allelic frequencies of *FMO3* SNPs identified in this study.

A, c.855C>T

Genotype	Counts	Genotyping frequency	Allele	Allelic frequency	χ^2	P-value
G/G	36	0.2951	G	0.5533	0.1843	0.6677
G/A	63	0.5164	A	0.4467		
A/A	23	0.1885				

B, c.441C>T

Genotype	Counts	Genotyping frequency	Allele	Allelic frequency	χ^2	P-value
G/G	70	0.5738	G	0.7664	0.1201	0.7290
G/A	47	0.3852	A	0.2336		
A/A	5	0.0410				

C, c.923A>G

Genotype	Counts	Genotyping frequency	Allele	Allelic frequency	χ^2	P-value
A/A	72	0.5901	A	0.7705	0.0318	0.8584
A/G	44	0.3607	G	0.2295		
G/G	6	0.0492				

D, c.472G>A

Genotype	Counts	Genotyping frequency	Allele	Allelic frequency	χ^2	P-value
C/C	63	0.5164	C	0.7295	0.4729	0.4917
C/T	52	0.4262	T	0.2705		
T/T	7	0.0574				

The expected and observed frequencies were compared using the Hardy-Weinberg equation. FMO3, flavin-containing monooxygenase 3; SNP, single nucleotide polymorphism.

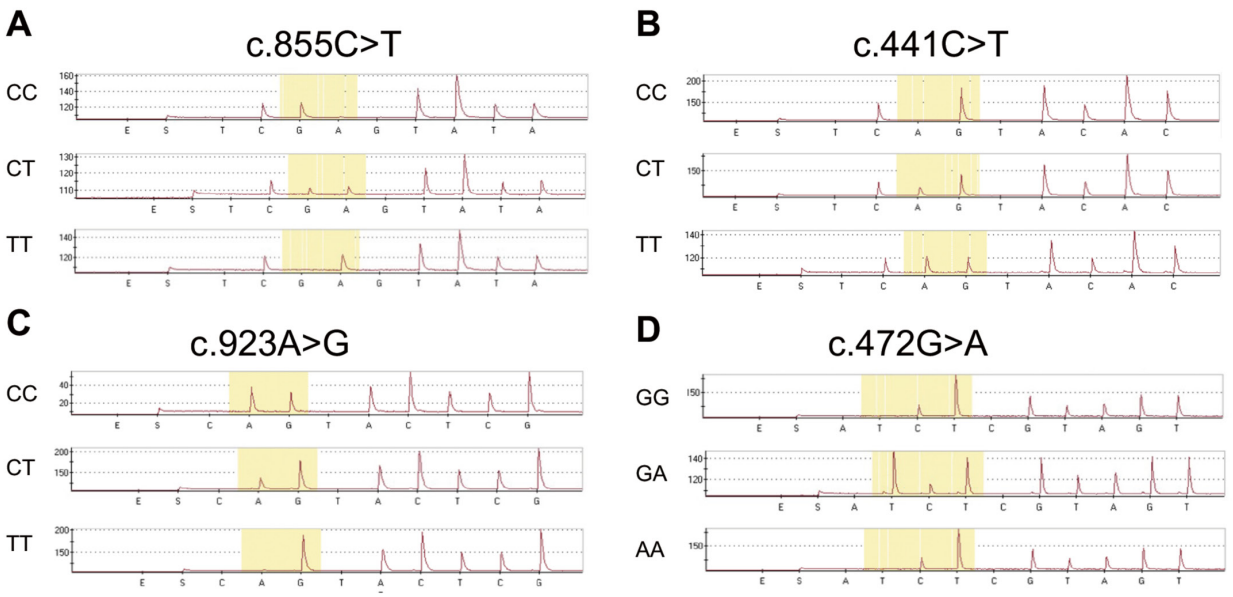

Figure 2. Representative pyrograms of flavin-containing monooxygenase 3 single nucleotide polymorphisms. Yellow highlights show (A) c.855C>T, (B) c.441C>T, (C) c.923A>G and (D) c.472G>A identified using the established pyrosequencing method.

Table III. Comparisons between *FMO3* allele frequencies obtained in this study and those in other ethnic groups.

A, c.855C>T

Population	Number, n	MAF, %	Refs.
Korean	122	44.7	Present study
Japanese	3,552	38.8	(12)
Chinese	285	26.1	(3)
European	226	27.9	HapMap-CEU database
Sub-Saharan African	226	54.0	HapMap-YRI database

B, c.441C>T

Population	Number, n	MAF, %	Refs.
Korean	122	23.4	Present study
Japanese	3,552	19.9	(12)
Chinese	285	5.8	(3)
European	226	6.6	HapMap-CEU database
Sub-Saharan African	226	3.1	HapMap-YRI database

C, c.923A>G

Population	Number, n	MAF, %	Refs.
Korean	122	23.0	Present study
Japanese	3,552	19.8	(12)
Chinese	285	19.8	(3)
European	170	35.9	(31)
Sub-Saharan African	226	1.3	HapMap-YRI database

D, c.472G>A

Population	Number, n	MAF, %	Refs.
Korean	122	27.1	Present study
Japanese	3,552	21.0	(12)
Chinese	285	16.5	(3)
European	224	42.0	HapMap-CEU database
African-American	133	41.9	(32)

FMO3, flavin-containing monooxygenase 3; MAF, minor allele frequency; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; YRI, Yoruba in Ibadan, Nigeria.

FMO3 genetic polymorphisms have been the focus of considerable interest in research; these findings can be applied to various studies on the pharmacokinetics of various medications, including anti-diabetics (e.g., teneligliptin) (5,6), antibiotics (e.g., voriconazole) (20,25) and non-steroidal anti-inflammatory drugs (e.g., sulindac) (4,13,14), as well as human diseases, such as cardiovascular disorders (2,7). FMO3 increases plasma trimethylamine N-oxide (TMAO) levels by catalyzing the conversion of trimethylamine (TMA) derived from the gut microbiome (26,27). Therefore, SNPs responsible for FMO3 loss-of-function seem to result in increased plasma TMA levels (9). At a clinical level, TMAO is associated with atherosclerosis (28), and a recent study demonstrated that higher plasma TMAO levels were associated with poor cardiovascular outcomes, while the *FMO3* SNP (c.472G>A) has been shown to reduce TMAO levels in the Asian population (7).

FMO3 also affects the levels of several clinically important medications, and its polymorphisms are associated with drug toxicity (25,29,30). The c.923A>G SNP has been shown to increase voriconazole concentrations by reducing FMO3 enzyme activity (25), while c.855C>T SNP can increase the concentration of teneligliptin (6). *FMO3* c.441C>T and

c.855C>T have been associated with fast tacrolimus elimination in Chinese patients (30). Studies by Park *et al* (13) and Sung *et al* (14) demonstrated that the SNPs c.855C>T and c.472G>A affected the pharmacokinetics of sulindac in women who underwent preterm labor. Febrile neutropenia, myelosuppression and agranulocytosis related to these SNPs have also been reported previously (25,29,30).

Considering the relatively high frequency of *FMO3* genetic polymorphisms in the population, the functional defects in FMO3 enzymes associated with these SNPs may have notable clinical implications, such as the variations in drug exposure followed by toxicity or delayed elimination of toxic substances. Therefore, the development of a faster and more precise method to identify *FMO3* SNPs could be clinically beneficial when purposed for optimal treatment (e.g., suggesting lower dosage in the patients with *FMO3* genetic polymorphism to reduce the drug toxicity and adverse events). However, evidence should be accumulated through clinical studies.

The ethnic and interindividual differences in SNPs and their suspected clinical manifestations, personalized dosing, pharmacokinetics and pharmacodynamics studies of drugs based on *FMO3* SNPs may present a novel research direction. Thus, the pyrosequencing method developed in this study could be applied directly to analyze individual *FMO3* SNPs for research in this domain.

In conclusion, the pyrosequencing method developed in the present study was successfully applied to detect the SNPs c.855C>T, c.441C>T, c.923A>G and c.472G>A of the *FMO3* gene. In Korean subjects, c.855C>T was the most frequent among the four *FMO3* SNPs.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to information that could compromise the privacy of research participants, but are available from the corresponding author upon reasonable request.

Authors' contributions

JWP was responsible for data acquisition, analysis and interpretation, and drafting of the article. JYP conceptualized and co-designed the study, critically screened the revised article for important intellectual content, and provided final approval of the submitted manuscript. KAK designed the study, and performed data analysis and interpretation. IHP, JMK and JHN were responsible for data acquisition and analysis. JWP and JYP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The protocol for this assay was approved by the Institutional Review Board of Anam Hospital, Korea University Medical Center (Seoul, South Korea). Subjects provided written informed consent to participate in this study.

Patients consent for publication

Not applicable.

Competing interests

The author declare that they have no competing interests.

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