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RESEARCH

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Association between the single-nucleotide polymorphism (rs1030868) variant in matrix metallopeptidase 2 gene and the development of lymphedema

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Abstract

Background Understanding the genetic and molecular pathophysiology of lymphedema contributes to the identification of the complex interaction of genes associated with lymphedema and provides a key therapeutic opportunity to restore lymphatic function. This study aims to answer whether the single-nucleotide polymorphism (SNP) rs1030868 in the MMP-2 gene could be an associated variant in lymphedema development.

Methods This study was carried out on 93 lymphedema cases (patients' group) and 187 healthy individuals (control group). Genotyping of MMP-2 (rs1030868) was done following the protocol of Custom TaqMan® SNP Genotyping Assays. TaqMan™ Fast Advanced Master Mix (Cat# 4448892) was used.

Results The risk alleles for MMP-2 SNP rs1030868 were A and G of lymphedema development. The GG genotype was associated with triple the risk of lymphedema (OR 3.2, 95% CI 1.2–6.5, $p = 0.019$), while the AA genotype was associated with 5.9 times the likelihood of lymphedema (OR 5.9, 95% CI 2.3–11.9, $p < 0.001$).

Conclusions We concluded that the single-nucleotide polymorphisms of rs1030868 in the matrix metallopeptidase 2 gene (allele A and allele G) could be associated variants with the development of lymphedema.

Keywords Lymphedema, MMP-2gene, rs1030868, Genotyping, Single-nucleotide polymorphisms

Background

Lymphedema is a common chronic debilitating swelling resulting from disruption of the lymphatic system. It is characterized by the progressive accumulation of protein-rich fluid within the interstitium, fibro adipose tissue deposition, chronic inflammation, and recurrent infections [1]. It affects about 250 million patients worldwide [2]. Lymphedema can be categorized as either primary or secondary. Primary lymphedema is caused by abnormal development of the lymphatic system. These developmental abnormalities may be related to genetic defects that either directly or indirectly regulate lymphatic differentiation and function [3].

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Secondary lymphedema is the most common cause of lymphedema and develops secondary to injury of the lymphatic system by surgery, radiation, trauma, or infection. Lymphatic filariasis is the most common cause of secondary lymphedema worldwide. It is caused by three species of nematodes transmitted by mosquitoes: *Wuchereria bancrofti, Brugia malayi, and Brugia timori* [4].

Different pathways contribute in the pathophysiology of lymphedema. Lymphatic injury promotes infiltration of T-helper cells, cytokines, growth factors, and collagen deposition, decreases collateral lymphatic formation, increases lymphatic leakiness, and impairs collecting lymphatic vessel pumping [5].

Understanding the molecular pathophysiology of lymphedema based on genetic information leads to the discovery of lymphatic markers and mechanisms that regulate lymphangiogenesis and the mutations in the coding or noncoding regions of genes associated with primary and secondary lymphedema [6].

The matrix metallopeptidase 2 gene is involved in the remodeling of the vasculature, angiogenesis, tissue repair, and inflammation. The polymorphisms of MMP-2 (rs1030868) can predispose to secondary lymphedema and/or modulate the clinical course of lymphedema. A higher risk of secondary lymphedema is associated with the A allele [7].

Angiogenic and lymphangiogenic molecules such as vascular endothelial growth factors (VEGFs) are elevated in the plasma of lymphedema patients. Polymorphism in FOXC-2 and FLT-4 genes have been identified to be involved in chronic lymphedema. Understanding lymphangiogenic mechanisms provide a therapeutic key for better interstitial fluid drainage, lipid absorption, and immune response [8].

Based on previous studies, we investigate the association between the single-nucleotide polymorphism (rs1030868) in the matrix metallopeptidase 2 (MMP-2) gene and the development of lymphedema.

Subjects and methods

This study was carried out on 93 lymphedema cases (patients' group) and 187 healthy individuals (control group). Lymphedema examination and staging were done [9].

Sampling

Two mL of EDTA peripheral blood samples were collected from patients and controls for the detection of circulating filarial antigen (CFA) and DNA extraction. Filariasis test strip (FTS) was used for the determination of circulating filarial antigen [10].

DNA extraction

Extraction of DNA was performed using the QIAamp® DNA Blood Mini Kit following the manufacturer's recommendations (QIAGEN's EMEA headquarters in Hilden, Germany). DNA was eluted in approximately 200 µL of buffer AE. The extracted DNA was stored frozen at − 20 °C. Quantification of isolated DNA was performed spectrophotometrically using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) and the samples were measured at 17–45 ng/µL.

SNP genotyping

Single-nucleotide polymorphism (SNP) is a location of genetic variation in the genome that varies between individuals. Each SNP represents a difference in a single DNA nucleotide. The variant rs1030868 (reference SNP cluster ID) is an intron variant of the matrix metallopeptidase 2 (MMP-2) gene on chromosome 16 [11].

Rotor-Gene Q 5plex HRM Platform (Real-time PCR cycler and High-Resolution Melt analyzer) was used. A High-Resolution Melt analyzer (HRM) characterizes double-stranded PCR products based on their dissociation (melting) behavior (QIAGEN's EMEA headquarters in Hilden, Germany). The operating software was Rotor-Gene Q 2.3.4.3—Windows platforms—(EN). The detection of rs1030868 followed the protocol of Custom TaqMan® SNP Genotyping Assays manufacturing kits (Thermo Fisher Scientific, USA). TaqMan® SNP Genotyping Assays allow for genotyping of nucleotide polymorphisms using the 5′-nuclease assay for amplifying and detecting specific alleles in purified genomic DNA samples.

Each TaqMan® Genotyping Assay contains two primers for amplifying the sequence of interest and two TaqMan® MGB probes for allele detection. The presence of two probes in each reaction allows for genotyping of two possible variant alleles at the polymorphic site in a DNA target sequence (Context Sequence [VIC/FAM]: TGATTG CTACAGCCTGCTTTGGTCA[A/G] TACTGTGCC ATCCTAATGTGGCTAA). The genotyping assay determines the presence or absence of polymorphism based on the change in fluorescence of the dyes associated with the Probes. The TaqMan® MGB Probes consist of target-specific oligonucleotides with a reporter dye linked to the 5′-end of each probe Fig. 1. The FAM™ dye is attached to the guanine (G) base and gives a fluorescence green color. The VIC® dye attaches to the adenine (A) base and gives yellow fluorescence color.

TaqMan™ Fast Advanced Master Mix (Life Technologies, Cat# 4448892) was used according to the manufacturer's instructions. The order of DNAs from cases and

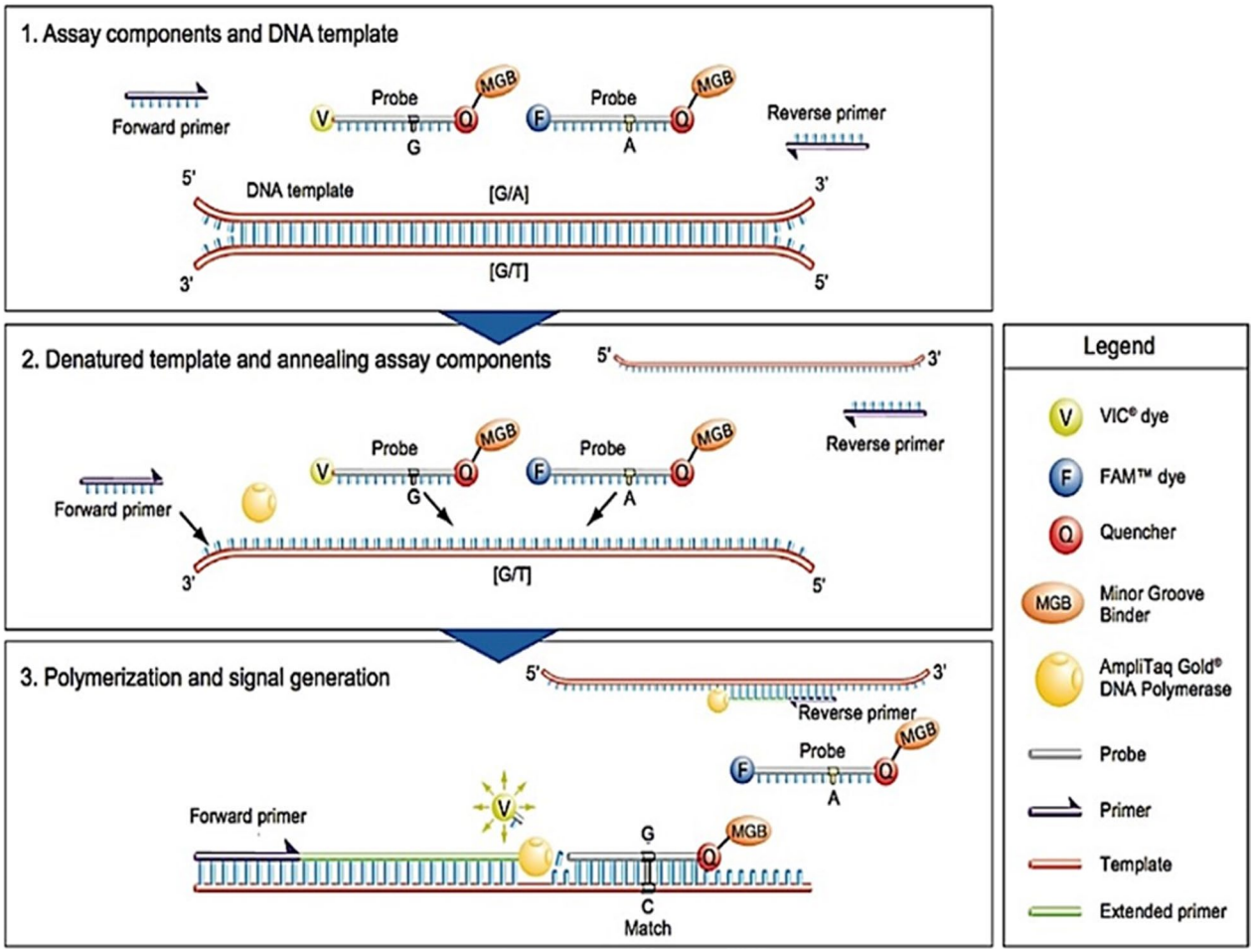

Fig. 1 Mechanism of the TaqMan SNP genotyping assay according to the manufacturing kits (Thermo Fisher Scientific, USA)

Table 1 Thermal cycler profile

Cycle	Cycle point
Hold	Hold @ 95 °C, 10 min 0 s
Cycling (40 repeats)	Step 1: Hold @ 95 °C, 15 s
	Step 2: Hold @ 60 °C, 60 s, acquiring to Cycling A([Green][1][1], [Yellow][2][2])

controls was arranged in a 72-well plate over six runs with duplication of four samples all over the runs for quality control purposes. The thermal cycler profile is shown in Table 1.

Statistical analysis

The data collected were tabulated and analyzed by statistical package for social science version 23.0 on IBM compatible computer. Frequency and percentage were used for the description of these qualitative data. The Chi-square test was used to measure the relationship between two or more qualitative variables. The results were considered significant when the *P* value was < 0.05.

Results

The patient's characteristics are shown in Table 2. In the patient's group, 61% of them were females and 39% were males. Of 93 patients complaining of lower limb lymphedema, seven cases were primary lymphedema and 86 cases were secondary lymphedema. All cases and controls were negative for circulating filarial antigen. In the patient's group, 26% were stage IV lymphedema and 20% were Stage VI.

The association between demographic characteristics and disease stage is presented in Table 2. There was a significant ($p < 0.001$) difference in the mean age between the various stages. For pairwise comparisons, patients of stage IV lymphedema ($n = 25$) were significantly ($p = 0.015$) older than those of stage VII ($n = 8$). There was a significant association between the disease stage

and sex of the patient ($p < 0.001$), i.e., sex distribution was as follows: 100% of stage I cases were females, equal distribution was observed in stage III and VII, stage II cases had almost equal sex distribution (7/6), in stage IV females represented 60%, stage V had the reverse (males represented about two-thirds), and most of stage VI cases were females (84%).

As shown in Table 3 and Fig. 2, compared with the wild genotype (GA), the GG genotype was associated with triple the risk of lymphedema (OR 3.2, 95% CI 1.2–6.5, $p = 0.019$) while the AA genotype was associated with 5.9 times the likelihood of lymphedema (OR 5.9, 95% CI 2.3–11.9, $p < 0.001$).

Table 2 Demographic and lymphedema stages of cases and control

Variable	Stage I (1) ($n = 5$)	Stage II (2) ($n = 13$)	Stage III (3) ($n = 10$)	Stage IV (4) ($n = 25$)	Stage V (5) ($n = 13$)	Stage VI (6) ($n = 19$)	Stage VII (7) ($n = 8$)	*P* value
Age/years	62.40 ± 3.6	58.85 ± 6.1	56.70 ± 7.3	41.56 ± 14.6	47.08 ± 9.8	46.01 ± 13.2	53.01 ± 6.1	< 0.001*
P value**	1 vs. 2 = 0.353	2 vs. 3 = 0.976	**3 vs. 4 = 0.001**	4 vs. 5 = 0.157	5 vs. 6 = 0.792	6 vs. 7 = 0.145	1 vs. 7 = 0.148	
	1 vs. 3 = 0.360	**2 vs. 4 < 0.001**	**3 vs. 5 = 0.046**	4 vs. 6 = 0.200	5 vs. 7 = 0.247	**1 vs. 6 = 0.005**	2 vs. 7 = 0.451	
	1 vs. 4 < 0.001	**2 vs. 5 = 0.030**	**3 vs. 6 = 0.017**	**4 vs. 7 = 0.015**	**1 vs. 5 = 0.012**	**2 vs. 6 = 0.009**	3 vs. 7 = 0.492	
Sex								
Female	5 (100%)	7 (53.8%)	5 (50%)	15 (60%)	5 (38.5%)	16 (84.2%)	4 (50%)	= 0.033***
Male	0 (0%)	6 (46.2%)	5 (50%)	10 (40%)	8 (61.5%)	3 (15.8%)	4 (50%)	

Bold values denote statistical significance at the $P < 0.05$ level

*One-way ANOVA test was used to compare means between groups

**Post hoc test was used for Pairwise Comparisons

***Chi-square test was used to compare the frequency distribution

Table 3 Genotype frequency and OR for SNP associated with disease status

Gene (db-SNP rs#)	Functional category of SNP	Genotyping	Control ($N = 187$)	Cases ($N = 93$)	*P* value	AOR (95% CI)*
MMP-2 (rs1030868)	Intron	GA	172 (92%)	67 (72%)	< 0.001	Reference
		GG	8 (4.3%)	10 (10.8%)	= 0.019	3.209 (1.215–6.497)
		AA	7 (3.7%)	16 (17.2%)	< 0.001	5.868 (2.311–11.901)

N number, % percentage, *G* guanine base, *A* adenine base

Significant association ($p < 0.05$)

*AOR = Adjusted Odds Ratio and 95% Confidence Interval of Risk of allele

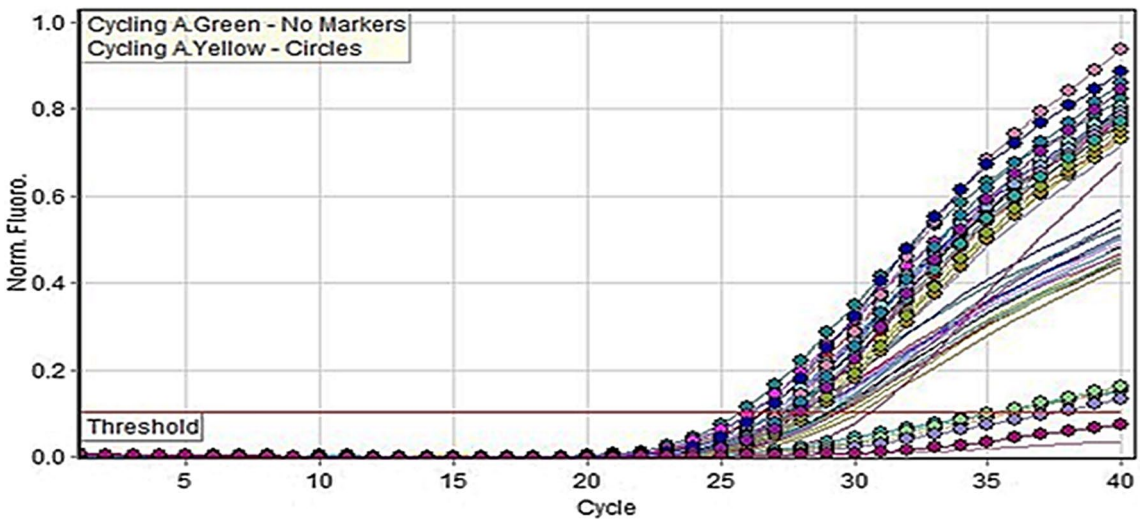

Fig. 2 VIC/FAM dye fluorescence on cycle number

Table 4 shows the distribution of genotype frequency according to disease determinants. The three genotype groups were matched for age and sex ($p=0.686$ and 0.575). For the stage of disease, there was a significant difference in genotype frequency for lymphedema staging ($p=0.007$). In other words, AA genotype frequency was higher in stage VI (56%), followed by stages IV and VII (19%), then stage V (6%) and 0% in stages I–III. For GA, the higher frequency was reported in stage IV (30%), stage II (16.5%), stage V (15%), stages III and VI (12%), and stages I and VII (7.5%). Regarding GG frequency, a higher percentage was observed in the stages from stage II to stage VI with 0% in stages I and VII.

Discussion

Studying the genetic basis of lymphedema could facilitate risk prediction susceptibility, understanding the host immunogenetics, and the development of effective drug therapy [12]. Host immune response plays an integral role in lymphedema molecular pathogenesis by inducing the expression of particular genes [13].

In this study, the genotyping for MMP-2 (rs1030868) was done to answer the question is there is an association between this SNP variant and lymphedema development.

According to National Center for Biotechnology Information (NCBI), MMP-2 is a member of the matrix metallopeptidase (MMP) gene family. It provides instructions for making enzymes called matrix metallopeptidase 2 which are zinc-dependent enzymes capable of cleaving components of the extracellular matrix and molecules involved in signal transduction. The protein encoded by this gene is a type IV collagenase and gelatinase A, which contains three fibronectin type II repeats in its catalytic site that allows the binding of denatured type IV and V collagen and elastin.

The matrix metallopeptidase 2 is an angiogenic factor involved in multiple physiological pathways including roles in the nervous system, endometrial menstrual breakdown, regulation of vascularization, tissue repair, and bone remodeling [14].

Although MMP gene expression is primarily regulated at the transcriptional level, posttranscriptional control of mRNA stability by cytokines, nitric oxide, or micro-RNA (miRNA) has been described as a significant contributing mechanism [15]. The matrix metallopeptidase 2 enzyme can be activated extracellularly by proteases or intracellularly by its S-glutathionylation with no requirement for proteolytical removal of the prodomain [16].

The MMP-2 expression and activity are tightly regulated via interactions between their activators and inhibitors. Imbalance among these factors results in dysregulated MMP-2 activity, which causes tissue destruction and functional alteration such as cardiovascular disease, neurodegenerative disease, Nodulosis–Arthropathy–Osteolysis (NAO) syndrome, inflammation as well as cancer, thus highlighting MMPs as promising therapeutic targets [17].

In the current study, the heterozygous wild-type GA was 72% (67 cases) in the patient's group and 92% (172 individuals) in the control group. MMP-2 (rs1030868)

Table 4 Relationship between genotype frequency and determinants

Variable	AA (1) ($n=23$)	GA (2) ($n=239$)	GG (3) ($n=18$)	*P* value
Age/years	42.39 ± 13.2	44.58 ± 15.1	46.33 ± 12.9	=0.686*
P value**	1 vs. 2 = 0.497	2 vs 3 = 0.628	1 vs. 3 = 0.397	
Sex				=0.575***
Female	16 (69.6%)	141 (59%)	10 (55.6%)	
Male	7 (30.4%)	98 (41%)	8 (44.4%)	
Severity (cases only)	($n=16$)	($n=67$)	($n=10$)	
Stage I	0 (0%)	5 (7.5%)	0 (0%)	=0.007$^{\$}$
Stage II	0 (0%)	11 (16.4%)	2 (20%)	
Stage III	0 (0%)	8 (11.9%)	2 (20%)	
Stage IV	3 (18.8%)	20 (29.9%)	2 (20%)	
Stage V	1 (6.3%)	10 (14.9%)	2 (20%)	
Stage VI	9 (56.3%)	8 (11.9%)	2 (20%)	
Stage VII	3 (18.8%)	5 (7.5%)	0 (0%)	

*One-way ANOVA test was used to compare means between groups

**Post hoc test was used for Pairwise Comparisons

***Chi-square test was used to compare the frequency distribution

$^{\$}$ Monte Carlo exact test was used to compare the frequency distribution

genotyping for filarial lymphedema in a cross-sectional study showed that the GA genotype was 47% in the patient group and 49% in the control group [18].

In our research, the patients with AA genotype (rs1030868) were associated with 5.9 times the likelihood of lymphedema (OR 5.9, 95% CI 2.3–11.9, $p < 0.001$). The GG genotype was associated with triple the risk of lymphedema (OR 3.2, 95% CI 1.2–6.5, $p = 0.019$).

In another study, the authors concluded that patients with MMP-2 (rs1030868) minor allele A SNP have a 1.3-fold risk of developing lymphedema than those who do not have these alleles [18].

MMPs participate in aging processes by destroying collagen in the dermis and breakdown of proteins in the ECM and the basement membrane. Metallopeptidase (MMP-2) also is involved in vascular remodeling and angiogenesis [19].

MMP-2 mRNA levels are known to be higher in lymphedematous specimens compared to non-lymphedematous specimens of progenitor cells [20] and a blockage or downregulation of this gene leads to reduced lymphangiogenesis, so the MMP-2 gene might have a role in lymphedema development [21].

The overexpression of MMP-2 alters and remodels the extracellular matrix which leads to disruption of the tight junctions with destruction and remodeling of the lymphatic vessel's architecture leading to lymphangiectasia, lymph stasis, and the lymph fluid entering the surrounding tissue causing lymphedema [18].

The filarial lymphedema with or without active infection is characterized by elevated levels of circulating MMPs. Altered ratios of matrix metallopeptidase (MMP)/tissue-inhibitors of metallopeptidase (TIMP) are an important underlying factor in the pathogenesis of tissue fibrosis in filarial lymphatic disease. There is an association between Type 2 cytokines, MMPs, and TIMPs in filarial infection which result in persistent and progressive tissue fibrosis [22]. There is an interaction between genes associated with lymphedema. There was a positive correlation between MMP-2 and VEGF expression; therefore, inhibition of MMP-2 may result in synergistic effects with other factors in this pathway [23].

The level of MMP-2 mRNA is higher in patients with disorders of the lymph flow of the extremities. Blocking or experimental reduction of MMP-2 gene activity leads to a decrease in lymphatic angiogenesis. The increased amount of MMP-2 may change and reconstruct the extracellular matrix around the vessels which promotes impaired vascular density and extravasation of lymph to the surrounding tissues [24].

Conclusion

The significant association of MMP-2 SNP rs1030868 is an indication that this gene might be involved in lymphedema development. There is a significant difference in genotype frequency for lymphedema staging (severity of lymphedema). The AA genotype frequency was higher in the advanced stages of lymphedema. The results of this study may contribute to understanding the genetic base of lymphedema. A large-scale study is needed to identify the complex interaction of genes associated with lymphedema.

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Author contributions
MY conceived and designed the research. SI, ME, MY, AM, and AO conducted the experiments. SI, AM, MY, AS, and AA analyzed the data and wrote the manuscript. All authors made critical reviews and approved the final version.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
The study was approved by the Research Ethical Committee of the Al-Azhar Faculty of Medicine. All patients signed informed consent before their inclusion in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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