Abstract. Immunoglobulin A nephropathy (IgAN) is a kidney disease and one of the commonest forms of glomerulonephritis worldwide. The present study investigated the role of dachshund family transcription factor 1 (DACH1) in IgAN and identified one of its binding microRNAs (miRNAs). The expression of DACH1 in human mesangial cells (HMCs) incubated with polymeric IgA (pIgA) isolated and purified from the serum of patients with IgAN or healthy individuals was evaluated by reverse transcription-quantitative (RT-q) PCR and western blotting. Cell proliferation and cell cycle assays were performed in DACH1-overexpressing HMCs to identify the role of DACH1 in IgAN and enzyme-linked immunosorbent assay was carried out to verify the release of inflammatory factors from HMCs. The target miRNAs of DACH1 were predicted using bioinformatics software and miR-140-3p was identified as a target of DACH1 by luciferase report assay, RT-qPCR and western blotting. The results demonstrated that DACH1 was downregulated in HMCs cultured with pIgA-IgAN at both mRNA and protein levels. Overexpression of DACH1 suppressed HMC growth and inhibited inflammatory cytokine release from HMCs cultured with pIgA-IgAN. The expression of DACH1 was negatively regulated by miR-140-3p in IgAN and miR-140-3p inhibition suppressed HMC growth and inhibited inflammatory cytokine release from HMCs cultured with pIgA-IgAN. The findings of the present study demonstrated that DACH1 decreased HMC growth and the release of inflammatory cytokines from HMCs may be targeted by miR-140-3p. The results suggested that DACH1 could be associated with the progression of IgAN and provide a potential target for further studies related to the mechanism of IgAN.

Introduction

Immunoglobulin A nephropathy (IgAN) is an immune complex glomerulonephritis characterized by IgA deposition in the mesangial area, abnormal proliferation of mesangial cells and excessive expansion of the mesangial matrix (1,2). It is one of the commonest causes of end-stage kidney failure and there is at present no specific treatment targeting IgAN (3). Thus, research has focused on exploring the pathogenesis of IgAN and developing effective treatment against it. As IgA deposition in the mesangial region is a key step in the development of IgAN (4) and the etiology and pathogenesis of this disease are unclear, the pathogenesis of IgAN can be discussed from the perspective of IgA deposition in the mesangial region.

Dachshund family transcription factor 1 (DACH1) is a key component of the retinal determination gene network family that has been shown to be closely associated with organogenesis and tumorigenesis (5,6). DACH1 participates in cell differentiation and proliferation in renal development (7) and decreased expression of DACH1 is associated with the progression and severity of glomerulopathy (8). DACH1 disorder has been reported in a variety of human malignancies such as breast cancer (9), ovarian cancer (10), renal carcinoma (11) and gastric cancer (12). It acts as a cell-fate determination factor that regulates cell growth and development (13,14), but the mechanism and function of DACH1 dysregulation in IgAN has yet to be explored.

Abnormal cell proliferation is a sign of cancer transformation (15) and abnormal expression of genes in cancer cells can be directly involved in the regulation of cell growth and cell cycle progression (16,17). Malfunctions in the cell cycle usually result in uncontrolled cell growth characteristics, allowing
cancer cells to proliferate excessively and eventually leading to the tumorigenesis (18). DACH1 inhibits cell proliferation and enhance cell cycle arrest. For example, Chen et al (19) demonstrated that DACH1 participates in p53-mediated p21 induction and cell cycle arrest in non-small-cell lung cancer. Wu et al (10) revealed that low expression of DACH1 in breast cancer cells promotes tumor growth by downregulating Nanog and Sox2. Kalousova et al (20) confirmed that DACH1 can bind to the promoter of the cell cycle inhibitor p27 to inhibit the proliferation and cell cycle progression of insulin-producing cells. The present study explored the role of DACH1 in IgAN in terms of cell proliferation and cell cycle progression.

MicroRNAs (miRNAs) are small non-coding RNAs that consist of ~22 nucleotides (21). miRNAs can degrade target mRNAs or inhibit their translation by binding to the 3'-untranslated region (3'UTR) of the target mRNAs to negatively regulate gene expression (22). Studies have shown that miRNAs are involved in the regulation of cell proliferation, differentiation, cell cycle, apoptosis and inflammation (22-25). The present study evaluated the expression and function of DACH1 in human mesangial cells (HMCs) cultured with polymeric IgA (pIgA) that was isolated and purified from the serum of patients with IgAN or healthy individuals. Bioinformatics analysis was used to screen for candidate miRNAs associated with DACH1 expression. Among the predicted miRNAs, miRNA (miR)-140-3p was selected and its expression and effect on cell proliferation and cell cycle progression in IgAN were investigated. It was found that miR-140-3p directly suppressed the expression of DACH1 and promoted the proliferation and cell cycle progression of HMCs in IgAN. The findings of the present study may provide a theoretical basis for the study of the mechanism of IgAN.

Materials and methods

Human IgAN samples. The serum of 30 patients with IgAN (men=23; women=7; age, 20-30 years n=19 and >30 years n=11) and 30 normal individuals was collected. Inclusion criteria included cases of IgA nephropathy and exclusion criteria included other nephropathies. The recruitment of patients was conducted at Shanghai Tenth People’s Hospital between January 2020 and April 2020. The serum collection was approved by the patients and all provided written informed consent. All blood samples (each sample ~5 ml) were anonymized. The present study was approved by the clinical research ethics committee of Shanghai Tenth People’s Hospital (approval no. SHSY-IEC-4.1/20-117-01).

Extraction of polymeric IgA (pIgA). The plgA was isolated and purified from the serum of patients with IgAN or healthy individuals using a jacalin-agarose column as previously described (26,27). The extracted plgA was stored at -80°C until use. The final concentration of plgA was 0.5 mg/ml.

Cell lines and culture. Human mesangial cells (HMCs) and 293T (cat. no. KCB200744YJ) cells were purchased from the Conservation Genetics CAS Kunming Cell Bank. HMCs were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and 80 U/ml penicillin and 0.1 mg/ml streptomycin (Beyotime Institute of Biotechnology). HMCs and 293T were cultured at 37°C in an atmosphere of 5% CO2.

Plasmids. Lentiviral vector plasmid pCDH-CMV-MCS-puro (pCDH) and recombinant plasmid pCDH-CMV-DACH1-puro (pDACH1) were purchased from GenScript for the transfection and production of lentiviruses. The pGL3-Control plasmid and pGL3-DACH1 3'UTR luciferase reporter plasmid were purchased from GenScript for use in detection of luciferase activity following transfection.

Lentivirus transduction and production. Lentivirus packaging vector psPAX2 (5 µg) and envelope vector pMD2.G (10 µg) were co-transfected with lentivirus plasmid pCDH (5 µg) or pDACH1 (5 µg) into 293T cells using Lipofectamine 2000 (50 µl) (Thermo Fisher Scientific, Inc.) to produce the corresponding lentivirus solution. The culture supernatant was collected 48-72 h after transfection. The supernatant contained the corresponding virus and was used for subsequent cell infection experiments.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from 5x10⁶ cells using TRIzol® reagent kit according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.) and reverse transcribed into single-stranded cDNA according to the instructions of the Reverse Transcription kit (Vazyme Biotech Co., Ltd.). The expression of mRNA was detected by RT-qPCR using the Rotor-Gene Q detection system (Qiagen GmbH) and the reaction volume was 20 μl, with the following cycling conditions: 95°C for 5 min, then 45 cycles of 95°C for 10 sec and 60°C for 30 sec. The expression of miRNA or mRNA was calculated using the 2ΔΔCq method (28). The mRNA expression level was normalized to that of GAPDH and the expression level of miRNA was normalized to U6 RNA. The primer sequences used were: IL-6 forward, 5'-AAC TCTTCTTCCACAAGCGCTT-3' and reverse, 5'-GTCATACTCGTTGTGAGAGTT-3'; IL-8 forward, 5'-GGCTCACTCGTTGTGAGAGTT-3' and reverse, 5'-GTCATACTCGTTGTGAGAGTT-3'; chemokine cc-motif ligand 1 (CXCL1) forward, 5'-CTC CAA CAT CA-3' and reverse, 5'-CCA CCT CGA TTT TGG TGT CTT TGA ATA T-3'; IL-13 forward, 5'-TGA GGA GCT GGT ACC TCC CAA CTG C-3' and reverse, 5'-TCA GCT CGA ACA CAT CTA C-3'; IL-1β forward, 5'-AAC TCTTCTTCCACAAGCGCTT-3' and reverse, 5'-GTCATACTCGTTGTGAGAGTT-3'; IL-4 forward, 5'-GGCTCACTCGTTGTGAGAGTT-3' and reverse, 5'-GTCATACTCGTTGTGAGAGTT-3'; and IL-8 forward, 5'-GGCTCACTCGTTGTGAGAGTT-3' and reverse, 5'-GTCATACTCGTTGTGAGAGTT-3'. The experiment was repeated three times.

Western blot analysis. The total protein of each group was harvested and collected using RIPA lysis buffer (Thermo Fisher Scientific, Inc.). Protein concentration was measured with a BCA Protein Assay kit (Beyoyte Institute of Biotechnology). Equal amounts of protein (30 µg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore). The membrane was blocked by incubation with 5% non-fat milk for 1 h at 24°C and then with primary antibodies overnight at 4°C. The primary antibodies used were: Anti-DACH1 (cat. no. ab176718; Abcam; 1:1,000), Anti-GAPDH (cat. no. 10494-2-AP; Kangchen; 1:10,000), and Anti-β-Tubulin (cat. no. ab59671; Abcam; 1:10,000).
anti-Cyclin D1 (cat. no. 55506; Cell Signaling Technology, Inc.; 1:1,000), anti-Cyclin A (cat. no. 91500; Cell Signaling Technology, Inc.; 1:1,000) and anti-P21 (cat. no. 2947; Cell Signaling Technology, Inc.; 1:1,000), anti-P53 (cat. no. 2527; Cell Signaling Technology, Inc.; 1:1,000). After washing three times with Tris buffered saline-Tween (TBS-T; 50 mM Tris, 150 mM NaCl, 0.05% Tween-20) the membranes were incubated with goat anti-rabbit (cat. no. ab6721; Abcam; 1:5,000) or anti-mouse (cat. no. ab205719; Abcam; 1:5,000) IgG/horseradish peroxidase secondary antibody at room temperature for 1 h. Protein expression was detected using a chemiluminescence detection system (Tanon 4600SF; Tanon Science and Technology Co., Ltd.).

**Cell proliferation assay.** HMCs of each group were seeded into 96 well plates at 1x10^4 cells/well one day prior to cell viability measurement at day 1, with additional measurements taken on days 3 and 5. Cell proliferation assay was determined using the CCK-8 cell count kit (cat. no. E606335, Sangon Biotech Co., Ltd.). According to the manufacturer's instructions, CCK-8 was mixed with DMEM at 1:10 v/v, then added to 96 well plates and incubated at 37°C for 2 h. The optical density (OD) values were measured at a wavelength of 562 nm using a multimode reader (BioTek Instruments, Inc.)

**Flow cytometry.** Cell cycle distribution was detected by Annexin/propidium iodide (PI) single staining (cat. no. P4170, Sigma-Aldrich; Merck KGaA). HMCs of each group were simultaneously inoculated into 6-well plates at 2x10^5 cells/well. After 72 h, the cells were collected and detached with 0.25% trypsin solution. The concentration of cells was adjusted to 1x10^6 cells/ml, with 1 ml of cells then centrifuged at 500 x g for 10 min at 4°C. The supernatant was discarded and the cells were collected. The cell suspension (1 ml) was added to 2 ml of PBS. This was regarded as the template for further use. The cells were centrifuged (500 x g, 10 min, 4°C) again and the supernatant removed. Next, the cells were reacted with precooled 70% (v/v) ethanol solution at 4°C overnight. The fixed cells were rinsed twice with PBS and then 100 µl of the cell suspension containing at least 1x10^6 cells/ml was stained with 1 ml of 50 mg/l PI dye solution containing RNAase (20 µg/ml) for 30 min at 25°C in darkness and then filtered. The cell cycle was detected by flow cytometry (BD AccuriC6; BD Accuri C6 Software; BD Biosciences).

**Enzyme-linked immunosorbent assay (ELISA).** HMCs of each group were simultaneously seeded into 6-well plates at 2x10^5 cells/well. After 72 h, the levels of several inflammatory cytokines including CXCL1 (cat. no. AD10945Hu), IL-6 (cat. no. AD11099Hu), IL-8 (cat. no. AD11098Hu) and IL-13 (cat. no. AD11110Hu) in HMC supernatants were determined using ELISA kits (Andy Gene Biotechnology Co., Ltd.) according to the manufacturer's instructions.

**Luciferase reporter assay.** miR-140-3p mimic, miR-140-3p mutant, other miRNAs and negative control was co-transfected into 293T cells together with DACH1 3'UTR luciferase reporter plasmid (GenScript). After 28 h of transfection, the cells were harvested using Dual-Lumi™ II Luciferase Reporter Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions and the luciferase activity was detected using a multimode reader (BioTek Instruments, Inc.). The Firefly luciferase activity was normalized to the Renilla luciferase activity.

**Target prediction.** miRTar (miRTar, developed by Dr. Hsien-Da Huang, http://mirtar.mbc.nctu.edu.tw/human/) was used to predict binding sequences of miR-140-3p in the 3'UTR of DACH1.

**miRNA mimics, mutation and inhibition.** Synthetic miR-140-3p mimic, mutation, inhibitor, other miRNAs and negative control were obtained from GenScript. The miR-140-3p mutant sequence was 5'-UACCACAGGGUAGAACCACGG-3', the miR-140-3p mutant sequence was 5'-AUGCACAGGGUAGACCAACGCG-3' and the negative control sequence was 5'-UCCGAAGUGUGACGUTT-3'. RT-qPCR results confirmed that miR-140-3p mimic, mutation and inhibitor were successfully transfected into HMCs or 293 (Fig. S1).

**Statistical analysis.** Quantitative data are presented as the mean ± standard deviation. The Tukey multiple comparisons test was used for statistical analysis, a one-way ANOVA was performed before Tukey's test. P>0.05 was considered no statistical significance (ns), P<0.05 was considered to indicate a statistically significant difference. All graphs were generated using GraphPad Prism 8.0 (GraphPad Software, Inc.). The experiment was repeated three times.

**Results**

**Exogenous DACH1 suppresses the proliferation and altered the cell cycle of HMCs.** A previous study has shown that DACH1 is expressed at low levels in IgAN and is associated with the progression and severity of glomerulopathy (8). However, its role in the development of human IgAN remains to be elucidated. The endogenous expression of DACH1 in HMCs that were cultured with plgA-IgAN remains to be elucidated. The endogenous expression of DACH1 in HMCs cultured with plgA-IgAN compared with cells cultured with plgA-control. To investigate the role of DACH1 in IgAN, HMCs were transfected with lentiviral DACH1 vectors. DACH1-transfected HMCs overexpressed DACH1 at the mRNA (Fig. S2B) and protein levels (Fig. 1B). The effect of DACH1 on cell proliferation and cell cycle progression was then examined. The cell proliferation assay demonstrated that DACH1 overexpression reversed the ability of plgA-IgAN to promote HMC proliferation (Fig. 1C). The cell cycle assay demonstrated that DACH1 enhanced plgA-IgAN-induced G2 phase arrest in HMCs (Fig. 1D and E). The expression of proteins closely associated with the cell cycle were detected by western blot. As shown in Fig. 1F, the expression levels of Cyclin D1 and Cyclin A were upregulated in the presence of plgA-IgAN but were downregulated by DACH1 and the expression levels of p21 and p53 were downregulated in the presence of plgA-IgAN but were upregulated by DACH1.
Exogenous DACH1 regulates inflammatory response in HMCs. To further investigate the function of DACH1 in IgAN, the release of inflammatory cytokines from HMCs that were stably transfected with DACH1 was measured. The expression of inflammatory factors (IL-6, IL-8, IL-13 and CXCL1) in the supernatant of DACH1-transfected HMCs was detected by ELISA. As shown in Fig. 2A, the concentrations of IL-6, IL-8, IL-13 and CXCL1 in HMCs cultured with plgA-IgAN were higher compared with the control group, whereas DACH1 reversed this phenomenon by reducing the plgA-IgAN-induced expression of IL-6, IL-8, IL-13 and CXCL1. In addition, the mRNA expression levels of IL-6, IL-8, IL-13 and CXCL1 in HMCs incubated with 0.5 mg/ml plgA-IgAN were higher compared with the control group. Similarly, DACH1 down-regulated the expression of IL-6, IL-8, IL-13 and CXCL1 (Fig. 2B).

Cellular miR-140-3p directly targets DACH1 3′UTR. To assess the mechanism mediating DACH1-induced inhibition of cell proliferation and inflammatory response, the present
study screened for miRNAs that may interact with DACH1 using bioinformatics software. A total of 10 miRNAs were selected, synthesized and transfected into HMCs. The mRNA and protein expression of DACH1 were detected by RT-qPCR and western blot, respectively. As shown in Figs. 3A and S2C, when miR-140-3p was transfected into HMCs, the mRNA and protein expression of DACH1 was decreased. To further verify whether DACH1 is regulated by miR-140-3p, the luciferase reporter plasmids containing the full-length sequence of DACH1 3’UTR region with 10 miRNAs we co-transfected into 293 cells. In the luciferase reporter assay, miR-140-3p reduced the luciferase activity of the 3’UTR of DACH1 (Fig. 3B). Subsequently, miR-140-3p mimics were transfected into HMCs and the expression of DACH1 detected by RT-qPCR and western blot. As shown in Fig. 3C, the expression of DACH1 was decreased by increasing the expression of miR-140-3p in a dose-dependent manner. miR-140-3p mimics and mutants were then transfected into 293 cells to examine the expression of DACH1. As shown in Fig. 3E-G, miR-140-3p mimics inhibited the expression of DACH1 and the luciferase activity of the 3’UTR of DACH1, while the miR-140-3p mutant had no
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These data suggested that DACH1 is a direct target of, and is regulated by, miR-140-3p.

miR-140-3p inhibition reverses the effect of pIgA-IgAN induced cell proliferation and cell cycle changes. Next, cell proliferation and cell cycle assays were performed to determine the functional role of miR-140-3p in IgAN. First, the expression of miR-140-3p was detected in HMCs that were cultured with pIgA-IgAN or pIgA-control. As shown in Fig. 4A, the expression of miR-140-3p was significantly higher in HMCs cultured with pIgA-IgAN compared with cells cultured with pIgA-control. Cell proliferation and cell cycle assays were performed in HMCs transfected with miR-140-3p inhibitors or negative controls in the presence of pIgA-IgAN. The cell cycle assay demonstrated that miR-140-3p enhanced pIgA-IgAN-induced G2 phase arrest and inhibited G1 phase activation in HMCs (Fig. 4B and C). The cell proliferation assay revealed that inhibition of miR-140-3p reversed the ability of
pIgA-IgAN to promote HMC proliferation (Fig. 4D). Upon miR-140-3p inhibition, cyclin D1 and cyclin A were down-regulated whereas p21 and p53 were upregulated (Fig. 4E).

miR-140-3p also regulates inflammatory response in HMCs. To further evaluate the function of miR-140-3p in IgAN, the release of inflammatory cytokines from HMCs transfected with miR-140-3p inhibitors was detected. The supernatant was collected after transfection and the expression of IL-6, IL-8, IL-13 and CXCL1 was detected by ELISA. As shown in Fig. 5A and B, miR-140-3p inhibition reversed the pIgA-IgAN-induced expression of IL-6, IL-8, IL-13 and CXCL1 in HMCs at the supernatant and at the mRNA level.

Discussion
IgAN is one of the common type of glomerulonephritis worldwide (29). According to statistics, ~40% of patients with IgAN...
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progress to end-stage nephropathy within 20 years of disease onset, severely affecting the physical and mental health of patients (30). A typical feature of IgAN is IgA immune complex-mediated mesangial cell proliferation and the detection of IgA deposition in mesangial cells by renal biopsy and immunohistochemistry is a method of IgAN diagnosis (31,32). The pathogenesis of IgAN is unclear and it is important to explore the mechanism of IgAN.

DACH1 has been shown to act as a tumor suppressor gene in various malignancies (9-12) and participates in tumor cell proliferation and metabolism by regulating cell cycle-related proteins (20). Liu et al (8) suggested that DACH1 expression is decreased in IgAN and that DACH1 is involved in disease progression and severity by regulating cell cycle-related proteins. The present study hypothesized that DACH1 may be involved in regulating the cell cycle and proliferation of mesangial cells. In the present study, pIgA was isolated and purified from 30 patients with IgAN and 30 healthy individuals. HMCs were incubated with pIgA to simulate the cell model of IgA deposition in mesangial cells in IgAN (27,33). It was found that DACH1 was downregulated in HMCs incubated with pIgA isolated from patients with IgAN compared with that in cells cultured with pIgA isolated from healthy individuals. In addition, exogenous DACH1 inhibited the proliferation and enhanced cell cycle arrest in HMCs induced by pIgA from patients with IgAN.

Each phase of the cell cycle is controlled by specific regulatory proteins. Cyclin D1 mainly promotes G1-S phase transition and regulates the G1 phase reentry of stationary cells into the cell cycle in G1 (34,35). Cyclin A is essential for DNA synthesis and its expression peaks at the late G1 phase and S phase (36,37). p53 activation initiates cell cycle arrest at the G1/S phase (38) and p21 is involved in the regulation of G2/M transition (39). In the present study, DACH1 overexpression decreased the expression of cyclin D1 and cyclin A in HMCs that were upregulated by pIgA from patients with IgAN.

Figure 5. DACH1 decreases the release of inflammatory cytokines by positively regulating miRNA-140-3p expression. (A) Secretion of IL-6, IL-8, IL-13 and CXCL1 in the supernatant of HMCs transfected with the miR-140-3p inhibitor or NC when pIgA-IgAN was added or not. (B) Reverse transcription-quantitative PCR analyses of the mRNA levels of IL-6, IL-8, IL-13 and CXCL1 in HMCs transfected with the miR-140-3p inhibitor or NC when pIgA-IgAN was added or not. Data are expressed as mean ± standard deviation. *P<0.05, **P<0.01 and ****P<0.0001. CK, control check; DACH1, dachshund family transcription factor 1; miR, microRNA; NC, negative control; HMCs, human mesangial cells; pIgA, polymeric IgA.
According to previous study, abnormal miRNA expression is present in most patients with IgAN and is closely associated with the extent of the disease (40). miRNAs participate in almost every process involved in cancer occurrence, development and progression (22,41-43). The present study confirmed that miR-140-3p expression was upregulated in HMCs treated with pIgA-IgAN and that the upregulation of miR-140-3p mediated the expression of DACH1. The present study also demonstrated that miR-140-3p regulated cell growth, cell cycle progression and the release of inflammatory cytokines in HMCs. Although the findings of the present study indicated that DACH1 was probably involved in the regulation of IgAN and was regulated by miR-140-3p, a variety of other factors are involved in regulating the occurrence and development of IgAN and further studies on the pathogenesis of IgAN are warranted.

In summary, the present study demonstrated that DACH1 was downregulated in HMCs in the presence of pIgA-IgAN. Moreover, DACH1 regulated cell proliferation, cell cycle progression and the release of inflammatory cytokines in HMCs and these phenomena may be targeted by miR-140-3p. This novel pathway is therefore expected to provide a weak theoretical basis for IgAN research.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
XZ and YL designed the study and performed the experiments. XZ drafted the manuscript. PG and CZ were major contributors in the conception, design and reviewing of the manuscript. Based on their contributions, XZ was listed as the first author, while CZ was the author for correspondence. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All patients provided written informed consent and all serum samples were anonymized. The present study was approved by the clinical research ethics committee of Shanghai Tenth People's Hospital (approval no. SHSY-IEC-4.1/20-117-01).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References

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