

# Combined effects of hyperphosphatemia and hyperglycemia on the calcification of cultured human aortic smooth muscle cells

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**Abstract.** Vascular calcification (VC) is common in patients with diabetes and/or chronic kidney disease (CKD). It is strongly associated with cardiovascular morbidity and mortality. Hyperphosphatemia caused by CKD induces the transformation of vascular smooth muscle cells (VSMCs) into chondrocytes or osteoblast-like cells. Hyperglycemia may also accelerate VC. However, the exact mechanisms of this remain unclear. The effects of simultaneous hyperphosphatemia and hyperglycemia require investigation. CKD rat models are typically used to study VC, which are far removed from the clinical situations of patients with CKD. The present study cultured human aortic smooth muscle cells (HASMCs) in normal, hyperphosphatemic and/or hyperglycemic conditions for 14 days. Alizarin red staining, calcification content, VSMC differentiation marker gene expression, phenotypic osteoblast gene expression and type III sodium-dependent phosphate cotransporter-1 (Pit-1) protein expression was examined. Hyperphosphatemia and hyperglycemia had combined effects in promoting calcification, phenotypic transition and Pit-1 expression in cultured HASMCs. In the present study, the combined effects of hyperphosphatemia and hyperglycemia on the calcification and phenotypic transition of HASMCs were demonstrated. Hyperphosphatemia combined with hyperglycemia medium should be considered an appropriate experimental model to study VC in diabetic kidney disease (DKD). Pit-1 should be considered as a promising index of VC.

## Introduction

Vascular calcification (VC) is a common event in patients with diabetes and/or chronic kidney disease (CKD) (1,2). It is strongly associated with cardiovascular morbidity and mortality (3,4). Previous studies have revealed that hyperphosphatemia caused by CKD triggers the transformation of vascular smooth muscle cells (VSMCs) into chondrocytes or osteoblast-like cells, and induces medial calcification deposits (5-7). It has also been demonstrated that hyperglycemia induces VC (8,9). The expression of VSMC differentiation marker genes, including smooth muscle 22 $\alpha$  (SM22 $\alpha$ ) and smooth muscle  $\alpha$ -actin (SM $\alpha$ -actin), decrease in smooth muscle cell phenotypic transition (10). Expression of the phenotypic osteoblast gene, runt-related transcription factor 2 (Runx2), also known as core binding factor alpha-1, is increased in VC (11,12). Phosphate transport into cells is primarily mediated by sodium-phosphate (NaPi) cotransporters, of which there are 3 types. Type III sodium-dependent phosphate cotransporter-1 (Pit-1) is the predominant NaPi cotransporter in human VSMCs (13). It has been identified as a pivotal transporter in phosphate-induced VSMC calcification. Pit-1 may promote vascular calcification via modulation of anti-calcification proteins (such as matrix Gla protein) or modification of kinases that phosphorylate secreted matrix proteins (such as osteopontin) (14). Pit-1 has been a focus of previous VC research, and its regulation serves a significant role in the pathogenesis of VC (15,16). However, the exact mechanisms of VC remain unclear.

Two experimental CKD rat models are typically used to research VC: An adenine-induced CKD model and a partial nephrectomy model. However, these are not the predominant causes of CKD in patients (17,18). Research into the interactions between hyperphosphatemia and hyperglycemia in VC and the underlying mechanisms is limited. In the present study, the effects of hyperphosphatemia and hyperglycemia on the phenotypic transition and calcification of cultured human aortic smooth muscle cells (HASMCs) were investigated, and the associated mechanisms were examined.

## Materials and methods

**Cell culture and calcification model.** HASMCs were purchased from Procell Life Science and Technology Co., Ltd. (Wuhan,

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China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% (v/v) fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% streptomycin/penicillin in 5% (v/v) CO<sub>2</sub> at 37°C in a humidified atmosphere. Cells at passages 4–6 were used for further experimentation. When 80% confluence was reached, cells were incubated in calcifying media containing 2.5 mM Pi (inorganic phosphorus) and/or 30 mM glucose for up to 14 days to induce calcification. Medium was replaced every 3 days. Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O and/or glucose were added to the serum-supplemented DMEM to create various high phosphate and glucose environments, according to our experimental groups, with a pH between 7.2 and 7.4. There were four experimental groups (n=9 per group): i) Control (CNT), normal Pi (0.9 mM) and glucose (5.5 mM); ii) HPi, high Pi (2.5 mM) and normal glucose; iii) HG, normal Pi and high glucose (30 mM); and iv) HGHPi: High glucose (30 mM) and high Pi (2.5 mM).

**Quantification of HASMC calcification.** HASMCs were grown in six-well plates and treated with growth or calcifying medium. On days 2, 8 and 14, the culture medium in several six-well plates was removed and washed with PBS, and cells were subsequently treated with 0.6N HCl overnight at 4°C. Calcium concentration in the supernatant was determined by the o-cresolphthalein complexone method, using a Calcium Assay kit (Nanjing Jiancheng Bioengineering Institute Co., Ltd., Nanjing, China). A Bicinchoninic Acid (BCA) Protein Assay kit (Aspen Biotechnology Co., Ltd., Wuhan, China) was used to evaluate protein concentration, in order to normalize the calcium concentration.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from HASMCs with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA USA), according to the manufacturer's instructions. cDNA was generated using a PrimeScript™ RT reagent kit with gDNA eraser (Takara Bio, Inc., Otsu, Japan). qPCR was performed on a StepOne™ Real-Time PCR system (Thermo Fisher Scientific, Inc.) with the SYBR® Premix Ex Taq™ reagent kit (Takara Bio, Inc.). The PCR conditions were as follows: 95°C initial denaturation for 1 min., 40 cycles with 95°C denaturation for 15 sec, 58°C annealing for 20 sec, 72°C elongation for 45 sec. The program for analytic melting was followed by an increase in temperature from 60°C to 95°C with a 0.05°C/sec ramp rate. GAPDH was used as the reference gene. PCR primers were purchased from Genecreate Bioengineering Co., Ltd. (Wuhan, China) and the sequences were as follows: Runx2, 5'-TACTCTGCCGAGCTACGA AATG-3' (forward), 5'-TGAAACTCTTGCCTCGTCCG-3' (reverse); SM22α, 5'-ATCCAAGCCAGTGAAGGTGC-3' (forward), 5'-ACTCCCTCTTATGCTCCTGGG-3' (reverse); SMα-actin, 5'-GTGACGAGGACGAGACCACC-3' (forward), 5'-GGGTCAGGATACCTCGCTTG-3' (reverse); GAPDH, 5'-CGCTAACATCAAATGGGGTG-3' (forward), 5'-TTG CTGACAATCTTGAGGGAG-3' (reverse). The specificity of the PCR products was confirmed by melting curve analysis. Relative expression levels were determined using the 2<sup>-ΔΔC<sub>q</sub></sup> method (19).

**Western blotting.** HASMCs were lysed using radioimmunoprecipitation assay buffer (Aspen Biotechnology Co., Ltd.) and protein concentrations were measured with a BCA protein assay. Total protein (40 μg/lane) was separated by 8 or 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Following blocking in 5% non-fat milk in Tris-buffered saline and Tween 20 for 1 h at room temperature, membranes were incubated with the following primary antibodies overnight at 4°C: Rabbit anti-GAPDH (dilution 1:10,000; cat. no. ab37168; Abcam, Cambridge, UK) and rabbit anti-Pit-1 (dilution 1:2,000; cat. no. ab177147; Abcam). Membranes were then incubated with horseradish peroxidase-labeled secondary antibody for 30 min at room temperature (dilution 1:10,000; cat. no. AS1107; Aspen Biotechnology Co., Ltd.). Immunoreactive proteins were detected using enhanced chemiluminescent reagents (Aspen Biotechnology Co., Ltd.). Quantitative densitometry analysis was performed using AlphaEaseFC software Version 3.3.0 (ProteinSimple, San Jose, CA, USA).

**Alizarin red staining.** HASMCs in six-well plates were washed three times with PBS and fixed with 10% (v/v) formaldehyde for 10 min at room temperature. The slides of cells were subsequently washed with PBS three times. Cells were exposed to 1% (w/v) Alizarin red for 30 min at 37°C and then washed with 0.2% (v/v) acetic acid. Red indicated positive staining of calcium nodules using a light microscope (Olympus Corporation, Tokyo, Japan; magnification, x200).

**Statistical analysis.** All numerical data are expressed as the mean ± standard deviation. The mixed-effects model of repeated measures (MMRM) was used to analyze every parameter. The model included group, time point and group-by-time point interaction as fixed factors. The restricted maximum likelihood (REML) was used. The covariance structure to model the within-sample errors was unstructured. The Kenward-Roger method was used to estimate the denominator degrees of freedom. Type III tests for the least-squares means were used for statistical comparisons. Comparisons between each group at every time point were reported. PROC MIXED in SAS Version 9.2 (SAS Institute, Cary, North Carolina, USA) was used for MMRM. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effects of hyperphosphatemia and hyperglycemia on calcification.** To determine the effects of hyperphosphatemia and hyperglycemia on the calcification of cultured HASMCs, Alizarin red staining intensities (Fig. 1A) and Ca concentrations (Fig. 1B) were determined on days 2, 8 and 14. The results revealed that no calcification occurred under normal conditions. Significant calcification was present in hyperphosphatemia or hyperglycemia medium on days 8 and 14, compared with the CNT (P<0.01), and calcification in HG media was more severe than in HPi media (P<0.01). When cultured in both hyperphosphatemia and hyperglycemia media, the most severe calcification occurred as early as day 2 compared with CNT, HPi and HG (P<0.01).

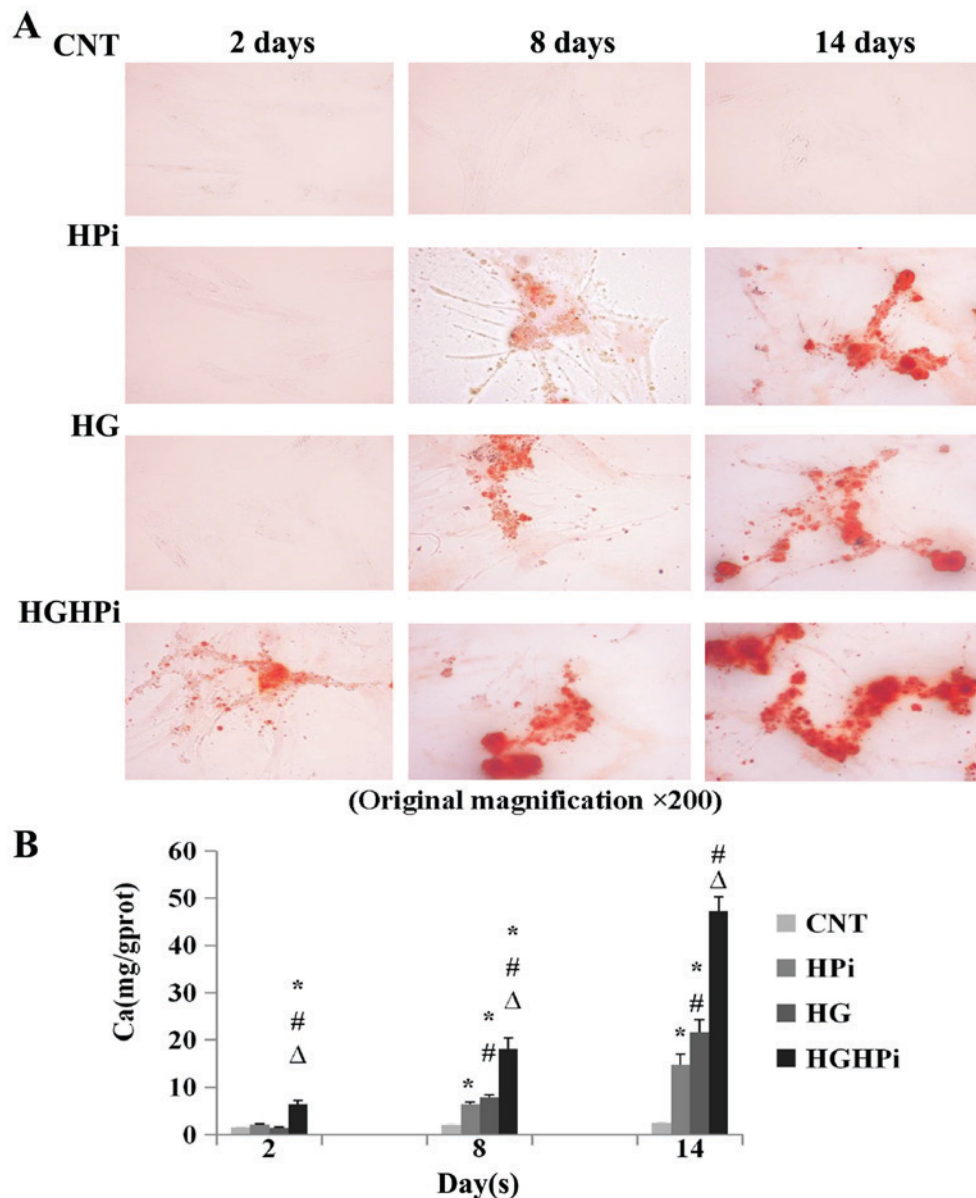


Figure 1. (A) Images of Alizarin red staining sections (original magnification,  $\times 200$ ). HASMCs were cultured in Dulbecco's modified Eagle's medium supplemented with CNT (0.9 mM Pi, 5.5 mM glucose), HPi (2.5 mM Pi) and/or HG (30 mM glucose) for 14 days to induce calcification. Sections exhibited no staining when cultured in normal medium, and significant calcification was observed in HPi or HG medium. The most severe calcification was observed in the HGHPi group. (B) Quantification of HASMC calcification induced by HPi and/or HG. Calcium content was normalized to total cellular protein, as determined by a bicinchoninic acid protein assay. The combined effects of hyperphosphatemia and hyperglycemia on HASMC calcification were observed. Values are expressed as the mean  $\pm$  standard deviation ( $n=9$ ). \* $P<0.01$  vs. CNT; # $P<0.01$  vs. HPi;  $\Delta P<0.01$  vs. HG. HASMC, human aortic smooth muscle cell; Pi, inorganic phosphorus; CNT, control; HPi, high Pi; HG, high glucose; HGHPi, high Pi and glucose.

Thus, it was concluded that there were combined effects of hyperglycemia and hyperphosphatemia on the calcification of HASMCs.

**Effects of hyperphosphatemia and hyperglycemia on the expression of Pit-1 proteins.** Pit-1 protein expression on day 2, 8 and 14 was detected by western blot analysis (Fig. 2). The results demonstrated that HPi and HG elevated the expression of Pit-1 on days 8 and 14, compared with the CNT ( $P<0.01$ ). The combined effects of hyperphosphatemia and hyperglycemia on Pit-1 expression were also observed: HASMCs cultured in hyperphosphatemia and hyperglycemia medium expressed the most Pit-1 protein from day 2, compared with that in the CNT, HPi and HG groups ( $P<0.01$ ).

**Effects of hyperphosphatemia and hyperglycemia on phenotypic transition.** The relative expression of Runx2, SM22 $\alpha$  and S $\alpha$ -actin mRNA in cultured HASMCs was evaluated by RT-qPCR analysis (Fig. 3). Upregulation of Runx2, as well as downregulation of SM22 $\alpha$  and S $\alpha$ -actin was observed in the HPi, HG and HGHPi groups, compared with the CNT. The differences in the expression of SM22 $\alpha$  and Runx2 mRNAs on day 14 were significant between the HGHPi and HPi or HG groups ( $P<0.01$ ). The differences in the expression of S $\alpha$ -actin mRNA on day 14 between the HGHPi and HG groups were not significant ( $P=0.106$ ), but both groups exhibited significantly decreased expression compared with the HPi group ( $P<0.01$ ). Therefore, it was concluded that there were combined effects of

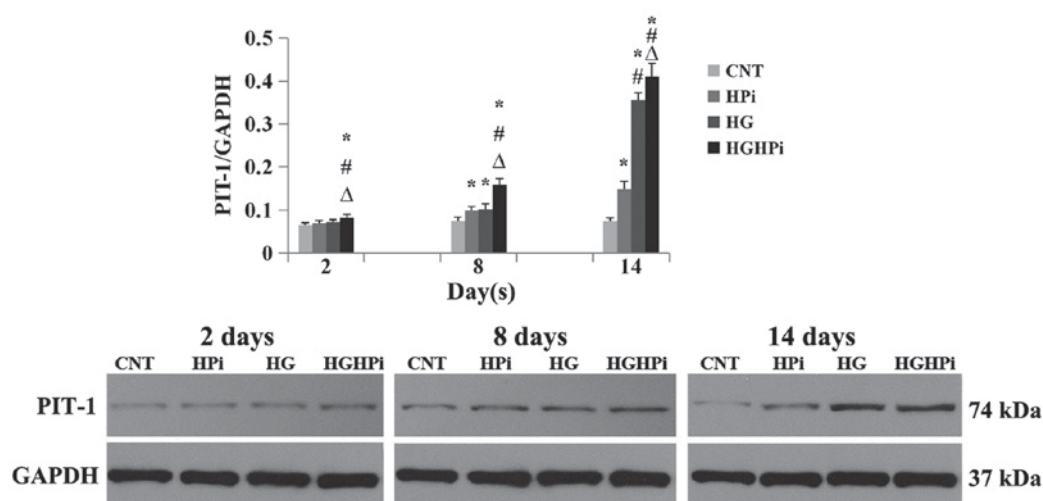


Figure 2. Expression of Pit-1 protein in human aortic smooth muscle cells cultured with normal, HPi and/or HG medium. Pit-1 protein expression was increased by HPi or HG. The combined effects of hyperphosphatemia and hyperglycemia on Pit-1 expression were also observed. Values are expressed as the mean  $\pm$  standard deviation (n=9). \*P<0.01 vs. CNT; #P<0.01 vs. HPi;  $\Delta$ P<0.01 vs. HG. Pi, inorganic phosphorus; CNT, control; HPi, high Pi; HG, high glucose; HGHPi, high Pi and glucose.

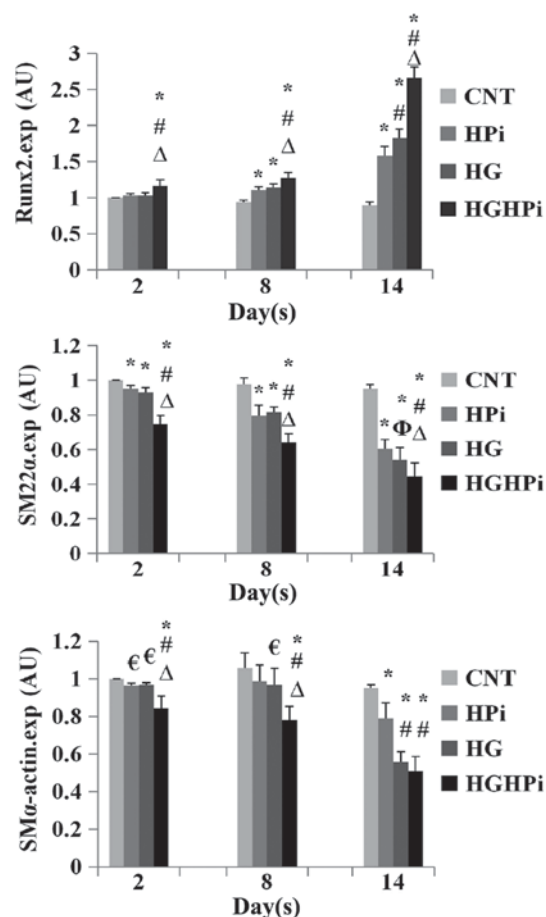


Figure 3. Relative expression of Runx2, SM22 $\alpha$  and SM $\alpha$ -actin mRNA of HASMCs cultured in normal, HPi and/or HG medium. Upregulation of Runx2 and downregulation of SM22 $\alpha$  and SM $\alpha$ -actin was observed in the HPi, HG and HGHPi groups. The combined effects of HPi and HG on HASMC phenotypic transition were also observed. Values are expressed as the mean  $\pm$  standard deviation (n=9). \*P<0.05 vs. CNT;  $\epsilon$ P<0.01 vs. CNT;  $\phi$ P<0.05 vs. HPi; #P<0.01 vs. HPi;  $\Delta$ P<0.01 vs. HG. AU, arbitrary units; HASMC, human aortic smooth muscle cell; Pi, inorganic phosphorus; CNT, control; HPi, high Pi; HG, high glucose; HGHPi, high Pi and glucose; Runx2, runt-related transcription factor 2; SM22 $\alpha$ , smooth muscle 22 $\alpha$ ; SM $\alpha$ -actin, smooth muscle  $\alpha$ -actin.

hyperglycemia and hyperphosphatemia on the phenotypic transition of HASMCs from vascular smooth muscle cells to osteoblast-like cells.

## Discussion

Clinically, hyperphosphatemia and hyperglycemia are common comorbidities of patients with CKD. The results of the present study indicated that both hyperphosphatemia and hyperglycemia induced HASMC calcification and the phenotypic transition from vascular smooth muscle cells to osteoblast-like cells. These results are consistent with previous reports (10,20). It is unclear what occurs when hyperphosphatemia and hyperglycemia are present at the same time; however, these are likely to be the conditions that occur in patients with diabetic kidney disease (DKD) (17). It is well established that CKD patients with hyperglycemia have a shorter survival time and/or higher mortality rate (21,22). Yoshida *et al* (23) incubated rat and human aortic SMCs with various concentrations of phosphate and glucose, and demonstrated that calcium accumulation is increased by high phosphate concentration in a dose-dependent manner, but not by high glucose concentration. However, this was inconsistent with the findings of the present study and others (9,24). The exact reasons for these differences are unclear; they may be due to the complicated mechanisms of VC, or perhaps there were some overlooked details in these previous experiments, which resulted in insufficient induction of VC by hyperglycemia. Rat aortic vascular rings were cultured in hyperphosphatemia and hyperglycemia in our previous work, and these results also supported the findings of the present study (unpublished data). Hence, it is reasonable to propose that there are combined effects of hyperglycemia and hyperphosphatemia on VC, which may contribute to the mortality of CKD patients with hyperglycemia. The current results provide experimental evidence for this hypothesis and suggest that it would be beneficial to advocate stricter control on the serum phosphorus and glucose levels of patients with DKD. In addition, conditions



of hyperphosphatemia combined with hyperglycemia should be considered an appropriate experimental model to study VC in DKD.

There are three types of NaPi cotransporter in humans, and Pit-1 is the predominant type in VSMCs (15,25). In recent decades, an increasing number of studies have demonstrated that Pit-1 regulation serves a significant role in the pathogenesis of VC (16,26,27). The present study suggested that Pit-1 expression was upregulated not only by hyperphosphatemia but also by hyperglycemia, and the level of expression appeared to be associated with Ca deposition in calcified HASMCs. Hence, Pit-1 may be a promising index for VC.

However, the present study had certain limitations. The model did not represent the structure and/or matrix of a vessel, and HASMCs were cultured *in vitro*. Thus, the results may differ *in vivo*. More detailed research, in both animals and clinical trials, will be required in order to verify the underlying mechanisms of and effective control strategies for VC.

In conclusion, it was observed that there were combined effects of hyperphosphatemia and hyperglycemia on HASMC calcification. Hyperphosphatemia medium combined with hyperglycemia medium should be considered an appropriate experimental model to study VC in DKD. Pit-1 may be a promising index for VC. These findings may aid in making clinical decisions for patients with CKD.

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## Availability of data and materials

All data generated and analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

All authors conceived and designed the research. PW and PZ performed the experiments and drafted the manuscript. DP analyzed the data and revised the manuscript. WC reviewed the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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