Introduction

Bone tissue engineering (BTE) aims to support the body’s own regenerative potential to restore structure and functionality of damaged bone tissue by means of progenitor cells, osteoconductive matrices, and growth factors. A promising cell candidate for this purpose is the abundant human adipose tissue–derived mesenchymal stem cell (hAD-MSC). Factors may be incorporated into a matrix in order to direct mesenchymal stem cells (MSCs) toward osteogenic differentiation and ensure differentiation following implantation. Previous studies have demonstrated enhanced osteogenic differentiation of MSCs when cultured in an extracellular matrix–like environment. Incorporation of factors into alginate hydrogel–coated titanium dioxide (TiO₂) scaffolds, like simvastatin, was recently demonstrated to enhance osteogenic differentiation of both human osteoblasts (hOBs) and hAD-MSCs. Also incorporation of proteins, like amelogenin, which is the major component of enamel matrix derivative, may have potential for local delivery of enamel matrix derivative and other stimulatory factors for use in bone tissue engineering.

Abstract

The purpose of bone tissue engineering is to employ scaffolds, cells, and growth factors to facilitate healing of bone defects. The aim of this study was to assess the viability and osteogenic differentiation of primary human osteoblasts and adipose tissue–derived mesenchymal stem cells from various donors on titanium dioxide (TiO₂) scaffolds coated with an alginate hydrogel enriched with enamel matrix derivative. Cells were harvested for quantitative reverse transcription polymerase chain reaction on days 14 and 21, and medium was collected on days 2, 14, and 21 for protein analyses. Neither coating with alginate hydrogel nor alginate hydrogel enriched with enamel matrix derivative induced a cytotoxic response. Enamel matrix derivative–enriched alginate hydrogel significantly increased the expression of osteoblast markers COL1A1, TNFRSF11B, and BGLAP and secretion of osteopontin in human osteoblasts, whereas osteogenic differentiation of human adipose tissue–derived mesenchymal stem cells seemed unaffected by enamel matrix derivative. The alginate hydrogel coating procedure may have potential for local delivery of enamel matrix derivative and other stimulatory factors for use in bone tissue engineering.

Keywords

Enamel matrix derivative, human adipose tissue–derived mesenchymal stem cells, primary human osteoblasts, alginate hydrogel, TiO₂ scaffold

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Alginete hydrogel enriched with enamel matrix derivative to target osteogenic cell differentiation in TiO₂ scaffolds
enamel matrix derivative (EMD), has been demonstrated to enhance osteogenic differentiation of bone marrow–derived MSCs.8 In this study, a TiO2 scaffold coated with an EMD-enriched alginate hydrogel was designed. Previous studies have reported the TiO2 scaffold to have properties which may have potential for BTE purposes, such as porosity, interconnectivity, and compressive strength.9 EMD is commercially available, and in clinical use as Emdogain® and contains extracellular matrix derivatives from embryonic porcine tooth enamel. It is used in periodontal regeneration where it facilitates formation of new cementum, periodontal ligament, and bone.10,11 Furthermore, it has been shown to enhance differentiation of osteoblasts.12,13 Hence, primary hOBs can be used for proof of concept, to verify the osteogenic effect of EMD. The hypothesis of this study was that cells seeded on TiO2 scaffolds would demonstrate osteogenic differentiation when EMD was added to the subsequent alginate hydrogel coating.

The aim of the study was to assess the viability and osteogenic differentiation of hAD-MSCs and primary hOBs seeded on porous TiO2 scaffolds subsequently coated with an alginate hydrogel enriched with EMD.

Materials and methods

Scaffold production

Porous TiO2 scaffolds, 4 mm in height and 9 mm in diameter, were produced by polymer sponge replication as previously described.9 The scaffolds were sterilized by autoclaving at 121°C for 20 min.

MSCs

The hAD-MSCs were isolated from liposuction material from abdominal regions of three healthy female donors (aged 36, 50, and 61 years). The donors provided informed consent and collection and storage of adipose tissue, and hAD-MSCs were approved by the regional ethics committee for medical research. The isolation and in vitro expansion of hAD-MSCs were carried out as previously described.14 The hAD-MSCs were cultured in medium consisting of Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco) supplemented with 10% human platelet lysate plasma (PLP),15 2 IU/mL heparin, and 1% penicillin/streptomycin, 10 mM β-glycerophosphate, 10 mM dexamethasone, and 150 µM L-ascorbic acid-2-phosphate. The medium was changed every 3–4 days. Cells were harvested after 21 days of culture for use in quantitative reverse transcription polymerase chain reaction (qRT-PCR) and staining. For alizarin red staining, the cells were washed with phosphate buffered saline (PBS), fixed for 1 h with 1% paraformaldehyde (PFA), and rinsed with PBS. Mineralization was observed by staining with 40 mM Alizarin Red S (pH 4.2) for 5 min. The qRT-PCR was done for RUNX2 and ALPL (Table 1).

Adipogenic differentiation

Adipogenic differentiation of hAD-MSCs was performed at passage 7. Cells were seeded at 3.5 × 104 cells per well in a 12-well plate and differentiated in a medium consisting of DMEM/F12 (Gibco) containing 10% PLP, 2 IU/mL heparin, and 1% penicillin/streptomycin, 10 mg/mL insulin (Novo Nordisk, Bagsvaerd, Denmark), 0.5 mM 1-methyl-3-isobutylxanthine, 1 µM dexamethasone, and 100 µM indomethacin (Dumex-Alpharma, Copenhagen, Denmark). The medium was changed every 3–4 days. Cells were harvested after 21 days of culture for use in qRT-PCR and Oil Red O staining. The qRT-PCR was done for PPARG (Table 1).

Chondrogenic differentiation

For chondrogenic differentiation, 1 × 106 cells were imbedded in 100 µL alginate.15 Chondrogenic differentiation was induced by DMEM/F12 (Gibco) supplemented with 2.8 g/L glucose, 1 mM sodium pyruvate (Gibco), 0.1 mM ascorbic acid-2-phosphate, 0.1 µM dexamethasone, 1% ITS (insulin 25 µg/mL, transferrin 25 µg/mL, and sodium selenite 25 ng/mL), 1.25 mg/mL human serum albumin (Octapharma, Jessheim, Norway), 500 ng/mL bone morphogenetic protein-2 (Wyeth Pharmaceuticals, Taplow, UK), 25 ng/mL recombinant human transforming growth factor-β1 (R&D Systems, Minneapolis, MN, USA), and 200 mg/mL insulin-like growth factor 1 (IGF1; Sigma–Aldrich, St. Louis, MO, USA). The following fluorochrome-conjugated antibodies were used for cell surface marker staining: CD14/PE (Diatec), CD19/APC (Diatec), CD45/PE (Southern Biotech), HLA-DR/APC (Diatec), IgG/PE (Diatec), CD34/APC (BD Biosciences), CD73/APC (Diatec), CD90/PE (BD Biosciences), and CD44/PE (BD Biosciences).
USA). Medium was changed every 3–4 days. Cells were harvested after 21 days of culture for use in qRT-PCR. The qRT-PCR was done for SOX9, COL2A1, and ACAN (Table 1).

**Alizarin red staining**

For alizarin red staining, the cells were washed with PBS, fixed for 1 h with 1% PFA, and rinsed with PBS. Mineralization was observed by staining with 40 mM alizarin red S (pH 4.2) for 5 min.

**Oil Red O staining**

For Oil Red O staining, the cells were washed with PBS, fixed for 15 min with 4% PFA, and rinsed with isopropanol. Lipid droplets were visualized by staining the cells for 10 min with Oil Red O, followed by washing with isopropanol.

**Osteoblasts**

The hOBs (Cambrex Bio Science, Walkersville, MD, USA) from three male donors (aged 10, 16, and 41 years), two from femur and one from tibia, were cultured in osteoblast culture medium supplemented with 10% fetal bovine serum, 0.1% gentamicin sulfate, amphotericin-B, and ascorbic acid (Lonza, Walkersville, MD, USA). The hOBs from the femur donors were subcultured till passages 6 and 8, and the hOBs from tibia were propagated till passage 9.

**Cell seeding and coating**

Cell seeding was performed using an agitated seeding method in order to ensure a homogeneous cell distribution throughout the scaffold.16 Scaffolds were precoated in culture medium and placed in 24-well plates, after which 1 mL cell suspension was added at a density of 2 × 10^5 cells/mL. Following agitation, the cell-seeded scaffolds were transferred to new culture plates in 1 mL culture medium and incubated overnight for 18 h at 37°C in humid conditions. Following agitation, the cell-seeded scaffolds were transferred to new culture plates in 1 mL culture medium and incubated overnight for 18 h at 37°C in a humidified atmosphere of 5% CO₂. The next day, the cell-seeded scaffolds were coated with EMD alginate (EMD group). The other control group was treated with an alginate coating in a self-gelling alginate system.17 Scaffolds were washed twice in 4.6% d-Mannitol solution and subsequently centrifuged at 300 × g for 1 min to remove excess alginate solution. The alginate-coated scaffolds were stabilized in a 50 mM CaCl₂ solution and transferred to new culture plates in 1 mL culture medium and maintained at 37°C in a humidified atmosphere of 5% CO₂ for up to 21 days. To assess the effect of EMD and alginate coating, two more groups were included. One group of scaffolds was coated with alginate without EMD (alginate group). The other control group was washed twice in 4.6% d-Mannitol, centrifuged at 300 × g for 1 min, as for the other groups, and subsequently transferred to new culture plates in 1 mL culture medium (control group). Triplicates of each donor, each treatment, and for two harvest time points were included, rendering a total of 108 cell-seeded scaffolds (54 hOBs and 54 hAD-MSCs). The culture medium was changed every second day and collected for analyses. The cells in scaffolds were harvested after 14 and 21 days of culture for use in qRT-PCR.

**Visualization of cells and alginate coating**

The alginate coating was visualized by periodic acid–Schiff (PAS) staining. Scaffolds were cut in half and collected for analyses. The cells in scaffolds were harvested after 14 and 21 days of culture for use in qRT-PCR.

**Table 1. List of TaqMan probes used in qRT-PCR.**

| Gene symbol | Gene name                                      | TaqMan assay no.        |
|-------------|------------------------------------------------|-------------------------|
| GAPDH       | Pyceraldehyde-3-phosphate dehydrogenase        | Hs99999905_m1           |
| COL1A1      | Collagen type I alpha 1                        | Hs00164004_m1           |
| TNFRSF11B   | Osteoprotegerin                                | Hs00900360_m1           |
| SP1         | Osteopontin                                    | Hs00959010_m1           |
| BGLAP       | Osteocalcin                                    | Hs01587814_g1           |
| PPARG       | Peroxisome proliferator-activated receptor gamma | Hs01115513_m1           |
| SOX9        | SRY (sex determining region Y)-box 9          | Hs00165814_m1           |
| COL2A1      | Collagen type II alpha 1                       | Hs00264051_m1           |
| ACAN        | Aggrecan                                       | Hs00202071_m1           |
| RUNX2       | Runt-related transcription factor 2            | Hs00231692_m1           |
| ALPL        | Alkaline phosphatase                           | Hs00758162_m1           |

qRT-PCR: quantitative reverse transcription polymerase chain reaction.
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anti-human Pan-Cadherin (1:2000, I-8H5; MP Biomedicals, Santa Ana, CA, USA) for 1 h at room temperature. After washing in PBS containing 2.5% BSA and 0.05% Tween 20, the scaffolds were incubated in secondary antibody, Alexa Fluor 488 rabbit anti-goat IgG (1:250) (Invitrogen Life Technologies, Paisley, UK), for 30 min at room temperature. Finally, scaffolds were incubated in PBS containing 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 5 mg/mL for nuclear labeling. Stained scaffolds were placed on a coverslip and covered with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Confocal laser scanning microscopy (CLSM) was performed on a FluoView 1000 (Olympus, Center Valley, PA, USA). Alexa Fluor 488 and DAPI were detected using specific filters for the respective fluorophores, and the PAS staining was detected with a filter for Alexa-546. For macroscopic evaluation of PAS staining, scaffolds were photographed using a SONY NEX-5N camera (Sony, Tokyo, Japan).

Lactate dehydrogenase activity in culture medium and acridine orange/ethidium bromide staining for viability assessment

The cytotoxicity was estimated based on the lactate dehydrogenase (LDH) activity in the culture medium collected every second day up to 14 days with a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. The absorbance was measured in a plate reader (Biochrom Asys Expert 96 Microplate Reader, Biochrom, Holliston, MA, USA). Viability of hAD-MSCs was evaluated by acridine orange/ethidium bromide (AO/EB) staining at day 2 after alginate coating. Scaffolds were cut in half to reveal the center of the scaffold and incubated with AO/EB staining solution in culture medium (1:10) for 5 min. Fluorescence images were captured using CLSM. The AO and EB were both excited using a 488-nm laser. The emitted fluorescence for AO was acquired at 500–560 nm, and for EB, the emission was acquired at 600–700 nm using a virtual channel setting. The scaffold surfaces were visualized using CLSM in reflection mode.

Analysis of messenger RNA expression

Total RNA was isolated from scaffolds using Qiagen RNA mini-kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocols. The complementary DNA (cDNA) was synthesized with RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) using random primers. The qRT-PCR was performed in the Applied Biosystems 7300 Real-Time System (Life Technologies) with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems, Paisley, UK). The qRT-PCR was done for GAPDH, COL1A1, TNFRSF11B, SPP1, and BGLAP. List of probes used in qRT-PCR is provided in Table 1. The qRT-PCR analysis was performed in duplicate for EMD and control groups at days 14 and 21. Relative messenger RNA (mRNA) levels were calculated by the comparative CT method.

Quantification of secreted proteins

Multianalyte profiling of protein levels in culture medium was performed on the Luminex 200 system (Luminex, Austin, TX, USA) employing xMAP technology. Acquired fluorescence data were analyzed by xPONENT 3.1 software (Luminex). The amount of osteoprotegerin (OPG), osteopontin (OPN), and osteocalcin (OC) in the culture medium was measured using the human bone panel kit (Millipore, Billerica, MA, USA) in triplicates after 2 and 14 days for hAD-MSCs and hOBs, and after 21 days in triplicates for hAD-MSCs and duplicates for hOBs. All analyses were performed according to the manufacturer’s protocols.

Statistics

The data obtained by gene expression and protein secretion analyses were compared groupwise using the Holm–Sidak test following a parametric one-way analysis of variance (ANOVA). Whenever the equal variance and/or the normality test failed, a Kruskal–Wallis one-way ANOVA on ranks was performed (SigmaPlot 12.0; Systat Software, San Jose, CA, USA). A probability of $\leq 0.05$ was considered significant.

Results

Characterization of hAD-MSCs

Surface antigen profiles obtained at passage 5 were concurrent with those of hAD-MSCs. Furthermore, cells differentiated into adipogenic and osteogenic lineages at passage 7 as verified by extensive staining of lipid droplets (Oil Red O) and calcium deposits (Alizarin Red) as well as by up-regulation of PPARg (adipogenic) and RUNX2 and ALPL (osteogenic) expression by real-time RT-PCR analysis. Cells also differentiated into the chondrogenic lineage with up-regulation of the chondrogenic markers (SOX9, COL2A1, ACAN) (Figure 1).

Characterization of cell-seeded and alginate-coated scaffolds

PAS/Pan-Cadherin double staining revealed that the alginate coating left an even distribution of alginate hydrogel throughout the scaffolds (Figure 2). Confocal microscopy further confirmed that cells as well as alginate were
attached to the struts leaving the pores in the scaffolds unplugged (Figure 2).

**Cell viability**

The LDH activity in the culture medium from hAD-MSC cultures did not change during the initial culture period for either EMD or alginate groups. However, a 10% higher activity was detected at day 12 in the medium from EMD group when compared to control group (Figure 2). In the medium from hOB-seeded scaffolds, a lower LDH activity was observed for both EMD and alginate groups compared to control group at days 2 and 4 (Figure 2). The low cytotoxicity was verified by live/dead staining demonstrating that the majority of cells were viable at day 2 after alginate coating (Figure 2).

![Figure 1](image-url)
Figure 2. PAS/Pan-cadherin double staining of (a) hAD-MSC-seeded uncoated and (b and c) alginate-coated scaffolds. The alginate is distributed throughout the scaffold as visualized by the PAS staining (red) depicted in the cross-sectional view (b). The alginate (arrowheads) coats the struts (S) without filling the pores (P), and pan-cadherin (yellow) stained cells (arrows) attach to the scaffold struts (S). The images are representative of the respective groups. (d) AO/EB staining of hAD-MSCs 2 days after alginate coating. The majority of cells are viable (green), and only few necrotic cells (N) and cells undergoing apoptosis (AP) are present. The image is representative of the respective group. LDH activity measured in culture medium from (e) hAD-MSCs and (f) hOBs cultured on (e) EMD (EMD) and alginate (Alginate) scaffolds. The result is presented in percentage of control, uncoated scaffold. Values represent the mean of three donors (six parallels per donor) + SD.
Figure 3. The effect of EMD on relative mRNA expression levels for COL1A1, TNFRSF11B, SPP1, and BGLAP after 14 and 21 days of culture for hAD-MSCs and hOBs on scaffolds. The result is presented in percentage of control, alginate group, normalized to reference gene GAPDH, and shown for each individual hAD-MSC and hOB donor, respectively (D1, D2, D3). Horizontal lines indicate the average mRNA expression of three donors (one parallel per donor). (a) indicates significant ($p \leq 0.05$) difference compared to alginate group.
Analysis of mRNA expression

Relative quantification of the mRNA expression in hAD-MSCs showed no significant differences between groups at any time points (Figure 2). For hOB-seeded scaffolds, COL1A1 and TNFRSF11B expression was significantly up-regulated in EMD group after 14 days of culture compared to alginate group (p = 0.011 and p = 0.013, respectively). Also, the expression of BGLAP was significantly higher in the EMD group after 14 (p = 0.004) and 21 (p = 0.006) days of culture when compared to the alginate group. No significant differences were observed in the expression of SPP1 mRNA at any of the time points when comparing the experimental groups (Figure 3).

Quantification of secreted proteins

Few and inconsistent differences were observed between the EMD and alginate groups in the hAD-MSC cultures when comparing the average content of OPG, OPN, and OC to culture medium from hAD-MSCs and hOBs cultured on EMD scaffolds at days 2, 14, and 21. The result is presented in percentage of control, alginate group, and shown for each individual hAD-MSC and hOB donor, respectively (D1, D2, D3). (a) indicates significant (p ≤ 0.05) difference compared to alginate group.
OC in the culture medium from the three donors (Figure 4). In donor 1, OC was significantly higher in the EMD group at day 2 as compared to the alginate group (0 = 0.009), but at day 14 the opposite was found (p = 0.02). No differences were observed for OPN, and for OPG, a difference was only found in donor 3 at day 21 where the concentration was lower in the EMD group (p = 0.01).

In the hOBS, the average content of OPN in the culture medium of the EMD group was significantly increased at days 2 (p = 0.01, p = 0.002) and 14 (p = 0.007, p = 0.02) for both donors 1 and 3, respectively. For donor 2, however, the alginate group demonstrated a higher level of OPN in the cell medium at day 2 only (p = 0.03). No further significant differences were observed between EMD and alginate groups when comparing the average content of OPG and OC in the culture medium from the hOB cultures. The content of OC in the culture medium from the hOB cultures was not detected at a high enough level to allow for quantification. The low level of detectable OC in the medium was most likely caused by repeated freeze thaw cycles during storage.

Variations were seen in the content of proteins in the culture medium when comparing the effect of EMD on the various donors (Figure 4). Significant donor differences were observed for hAD-MSCs in the secretion of OPG between donor 1 and donor 3 at day 21 (p = 0.01) and in the secretion of OC between donor 1 and donor 3 at days 2 (p = 0.03) and 14 (p = 0.04). However, donor differences were also observed for the hOBS in the secretion of OPG at day 14 between donor 2 and donor 3 (p = 0.04) and in the secretion of OPN between donor 2 and 3 at days 2 (p = 0.03) and 14 (p = 0.04).

**Discussion**

The alginate hydrogel coating of the cell-seeded TiO2 scaffolds did not induce a cytotoxic response. The expression of the osteoblast markers COL1A1, TNFRSF11B, and BGLAP and the secretion of OPN were significantly higher in hOBS when cultured on scaffolds with EMD-enriched alginate, whereas the hAD-MSCs seemed unaffected by the EMD.

With an average pore size of 400 µm and an interconnectivity of more than 90% through 200 µm connections,9,21 the TiO2 scaffold may have sufficient space for coating applications. A subtle coating without compromising porosity is desired to allow capillary ingrowth, sufficient cell nutrients, and oxygen supply. Insufficient oxygen delivery to the center of three-dimensional scaffolds may inhibit cell survival,22 and hypoxia has furthermore demonstrated to reduce osteogenic differentiation of adipose-derived mesenchymal cells.23 Except for the first time points, negligible differences were observed across groups with respect to the LDH activity in the medium. This indicates that the open-pore scaffold structure was sufficiently preserved by the alginate hydrogel and the EMD. Larsen et al.24 recently demonstrated the impact of the distance between cells immobilized in alginate to the cell medium interface in terms of oxygen diffusion. The findings from this study suggested that the distance between the immobilized cells to the cell medium interface was small enough to maintain viability. This is in agreement with a previous study reporting high cell viability in alginate hydrogel for 21 days.25

An initial decline of LDH activity was observed in the medium from the hOBS, but not from the hAD-MSCs. The centrifugation steps in conjunction with the coating procedure may have contributed to the removal of loosely adhered cells. The authors cannot soundly explain why this seemed to affect only one cell type. However, no differences were observed between the groups for the hOBS at day 6 onward.

Neither gene expression nor protein analysis indicated that EMD influenced the osteogenic differentiation of hAD-MSCs. This is in agreement with a previous study that evaluated mRNA expression of human umbilical cord MSCs at early time points.13 In that study, EMD was shown to up-regulate osteogenic markers only when osteogenic supplements were present in the culture media. Recently, the impact of donor age on the differentiation potential of hAD-MSCs was demonstrated.26 The effect of donor age was described to mainly influence chondrogenic differentiation. Differences in donor age could explain some of the large donor variations observed in this study, especially when it comes to the chondrogenic differentiation. The lack of osteogenic response in the hAD-MSCs, when cultured on scaffolds with EMD, may also in part be attributed to the relatively high donor age. However, there was no trend of age-related changes in expression and secretion in either cell type.

In contrast to the hAD-MSCs, the hOBS cultured on scaffolds with EMD did significantly up-regulate the expression of the early osteoblast markers (COL1A1, TNFRSF11B) after 14 days of culture and the late osteoblast marker BGLAP after both 14 and 21 days. EMD stimulated the secretion of OPN to the culture medium at early time points for donors 1 and 3, whereas an increase was observed for donor 2 at a much later time point. The stimulatory effects of EMD on osteoblast differentiation are in agreement with previous reports.12,27

The finding that EMD incorporated in the alginate hydrogel could enhance the osteogenic expression suggests a potential for the TiO2 scaffold and the alginate hydrogel coating in BTE. As previously demonstrated for simvastatin,6,7 the results of this study indicate that also substantially larger proteins such as EMD may exert cellular effects when included in an alginate hydrogel. Based on our data, care must be taken when interpreting results from studies based on cells from only one donor and with a narrow window of observations. The huge variation in
The clinical potential of the method described, in vitro cell delivery of factors involved in cell differentiation. Further in vitro studies need to be conducted in order to find the most appropriate factor for MSC differentiation in conjunction with TiO2 scaffolds and alginate hydrogel. Future studies should include more donors and samples in order to more fully elucidate the impact of donor variation and differentiation.

In conclusion, low cytotoxicity and significant up-regulation of the osteoblast markers COL1A1, TNFRSF11B, and BGLAP and secretion of OPN in hOBs demonstrated that the post-seeding alginate hydrogel coating was tolerated by the cells and functioned as a vehicle for in situ delivery of proteins such as EMD. Hence, the alginate coating procedure may have a potential for local delivery of factors to enhance osteogenic differentiation of cells in a porous scaffold.

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Helen Pullisaar and Anders Verket Contributed equally.

Declaration of conflicting interests
The authors declare that there is no conflict of interest.

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