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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Highlights
- Isolation of exosomes from a *Drosophila* cell line under nutrient deprivation
- Purification of exosomes using gel exclusion chromatography from conditioned media
- Immunoblotting to characterize the proteins enriched in exosomes
Protocol

Purification of exosome-enriched proteins produced in a Drosophila cell line by size exclusion chromatography

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SUMMARY

Exosomes are a class of extracellular vesicles that play a role in intercellular signaling under diverse contexts. Here, we describe a protocol that has been optimized for the isolation and characterization of exosomes from a Drosophila melanogaster cell line using size exclusion chromatography (SEC). The specific focus of this protocol was to examine the starvation-induced exosome loading of a protein. For complete details on the use and execution of this protocol, please refer to Pandey et al. (2021).¹

BEFORE YOU BEGIN

This protocol describes the isolation of exosomes from Drosophila melanogaster Kc167 cells grown in complete medium and under starvation to look for nutrient regulated proteins (a FLAG-tagged protein is used here as an example). Sterile cell culture practices for growing cell lines should be followed. The cells should be actively dividing and free of any contamination and the medium should be filter sterilized prior to use.

Institutional permissions

This protocol requires institutional biosafety committee (IBSC) permissions for handling the invertebrate Drosophila melanogaster cell lines. For this protocol, handling of Drosophila cell lines has been approved by the IBSC at the Regional Centre for Biotechnology as per guidelines of the Department of Biotechnology, Government of India.

Preparation of medium and buffers

@ Timing: 1–3 h, reagents/solutions should be prepared prior to the experiment

1. Filter-sterilize 250 mL of Complete medium (CCM3) and starvation medium (2mg/mL Glucose) with a 0.2 μm Polyethersulfone (PES) filter and store at 4°C (Complete medium).
2. Prepare 10× PBS and filter using 0.22 μm filter.

Note: The volume of these media can be scaled-up according to the downstream application.

Preparation of cells

@ Timing: 45 min for plating of cells; 72 h culturing prior to starvation

3. Plate cells in four T75 flasks (CCM3 medium) till they reach a density of around 1.5 × 10⁶ cells/mL (Figure 1).
Note: The cells should be maintained in a minimum media volume of 10–15 mL and cell density should be accurately estimated before starting the experiment. The incubator should be set at 25°C.

△ CRITICAL: The cells should be maintained in a minimum media volume of 10 mL and cell density should be accurately estimated before starting the experiment.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Flag (mouse) antibody | Sigma-Aldrich | RRID: AB_262044 |
| Working dilution: 1: 2500 |        |            |
| Anti-dCsp1(mouse) antibody | DSHB | RRID: AB_2307340 |
| Working dilution: 1:1000 |        |            |
| Anti-Syntaxin 1A (mouse) antibody | DSHB | RRID: AB_528484 |
| Working dilution: 1:500 |        |            |
| Anti-mouse HRP antibody | Jackson ImmunoResearch Laboratories, Inc | RRID: AB_10015289 |
| Working dilution: 1:2000 |        |            |
| Chemicals, peptides, and recombinant proteins |        |            |
| HyClone CCM3 cell culture media | Cytiva | SH30061.01 |
| D-(+)-Glucose | Sigma-Aldrich | G8270 |
| Lipofermectin 3000 Transfection Reagent | Thermo Fisher Scientific | L3000015 |
| Phospho Safe Extraction reagent | Sigma-Aldrich | 71296 |
| Benzonase | Sigma-Aldrich | E1014-5KU |
| Sodium dodecyl sulfate | Sigma-Aldrich | L4390 |
| Trizma® base | Sigma-Aldrich | T6066 |
| Glycine | Sigma-Aldrich | G8898 |
| Methanol | Sigma-Aldrich | 494437 |
| Clarity Western ECL Substrate | Bio-Rad | 170-5061 |
| Skimmed milk powder | MP Bio | 902887 |
| Tween® 20 (Polyisorbate) | VWR Life Science | M147-1L |
| Critical commercial assays |        |            |
| HiSpeed Midi Kit | QIAGEN | 12643 |
| Experimental models: Cell lines |        |            |
| Kc167 cell line | DGRC | RRID:CVCL_Z834 |
| Recombinant DNA |        |            |
| Flag-Chinmo pUASTattB | Geetanjali Chawla’s lab | N/A |
| Software |        |            |
| GraphPad Prism | GraphPad Software | RRID:SCR_002798 |
| Other |        |            |
| Amicon Ultra 15 Centrifugal Filter Unit, Ultracel, 3 KDa, 15 mL | Millipore | UFC900308 |
| qEV single/35 nm Column | Ison Science | qEV single/35nm Column |
| qEV Rack | Ison Science | qEV Rack |
| Corning® bottle-top vacuum filter system | Corning | CLS431097 |
| PVDF membrane | Millipore | IPVH00010 |

MATERIALS AND EQUIPMENT

The centrifugation steps described in this protocol were performed in Eppendorf centrifuge 5810R and Eppendorf centrifuge 5427R. The Western blots were developed using the ImageQuant software and ImageQuant LAS 4000 system from GE Healthcare. The exosome size was measured by the Nano Zetasizer ZS 90 by Malvern.
### CCM3 medium

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| HyClone CCM3 medium      | NA                  | 28.6 g  |
| NaHCO₃                   | 4.1 mM              | 0.35 g  |
| CaCl₂                    | 4.5 mM              | 0.5 g   |
| Autoclaved water         | NA                  | Make up to 1,000 mL |
| **Total volume**         |                     | 1,000 mL |

Adjust the pH to 6.3 with 10N NaOH and filter sterilize with a 0.22 μM filter. Store the medium at 4°C for one year.

### 10x phosphate buffered saline

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Na₂HPO₄·2H₂O             | 100 mM              | 17.8 g  |
| NaCl                     | 1.37 M              | 80 g    |
| KCl                      | 27 mM               | 2 g     |
| KH₂PO₄                  | 18 mM               | 2 g     |
| Autoclaved water         | NA                  | Make up to 1,000 mL |
| **Total volume**         |                     | 1,000 mL |

Adjust the pH to 7.4 with HCl or NaOH. PBS can be filter sterilized or autoclaved. Store at room temperature for one year. Dilute to 1× prior to use.

### Starvation medium (2 mg/mL glucose)

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Glucose                  | 1.1 mM              | 2 g     |
| Autoclaved water         | NA                  | Make up to 1,000 mL |
| **Total volume**         |                     | 1,000 mL |

The medium should be filter sterilized and stored at 4°C for one year.

### 10x running buffer for PAGE gel

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Tris base                | 0.25 M              | 30 g    |
| Glycine                  | 1.92 M              | 144 g   |
| Sodium dodecyl sulfate   | 1%                  | 10 g    |
| Autoclaved water         | NA                  | Make up to 1,000 mL |
| **Total volume**         |                     | 1,000 mL |

pH of the buffer is around 8.3, adjustment is not required. Store the buffer at room temperature for one year. Dilute to 1× before use.

### 10x transfer buffer for western blot analysis

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Tris                     | 0.25 M              | 30 g    |
| Glycine                  | 1.92 M              | 144 g   |
| Autoclaved water         | NA                  | Make up to 1,000 mL |
| **Total volume**         |                     | 1,000 mL |

pH should be around 8.3, adjustment not required. Store at room temperature. For transfer, dilute the buffer to 1× and add 20% methanol. Store the diluted buffer at 4°C for one year.
Transfection of cells: Day 1

1. Transfect the cells in four T75 flasks with the required DNA construct using Lipofectamine 3000 transfection reagent.

   Note: The DNA: Lipofectamine ratio is determined by the cell number and the manufacturer recommended protocol can be followed for transfection. The DNA: P3000: Lipofectamine ratio was 1µg DNA: 2 µL P3000: 1.5 µL Lipofectamine 3000. 35 µg DNA was transfected/T75 flask with 70 µL P3000 and 52.5 µL Lipofectamine 3000.

2. Incubate the cells at 25°C for 72h before initiating starvation.

   Critical: All the steps must be performed in a biosafety cabinet under completely sterile conditions.

Transfer of cells to starvation; day 4

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2. Incubate the cells at 25°C for 72h before initiating starvation.

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b. Centrifuge the cells at 500 $\times$ g for 5 min at room temperature (25°C) in a benchtop centrifuge.

c. Aspirate the Complete medium from both the falcon tubes.

4. Resuspend the cell pellet gently in one falcon tube (labeled as ‘Complete’) in 30 mL of CCM3 and the cell pellet in other falcon tube (labeled as ‘Starvation’) in 30 mL of 2mg/mL glucose.

a. Transfer 15mL from each falcon tube to two new T75 flasks. Keep the flasks for 8 h under this condition.

Note: It is important to spin down cells that are to be kept under complete medium (for 8 h) and resuspend them into fresh medium along with the cells subjected to starvation. This medium is to be collected for exosome isolation and hence the time given for exosome enrichment in both cases should be equal.

Collection and concentration of conditioned medium and lysis of cells; day 4

① Timing: 1 h

5. After 8 h of starvation, transfer the cells in CCM3 (complete medium) and 2 mg/mL glucose (starvation medium) to two 50 mL falcon tubes. Each falcon tube will have 30 mL of cells resuspended in the medium.

a. Centrifuge the cells at 500 $\times$ g for 10 min at room temperature (25°C) in a benchtop centrifuge.

b. Transfer the medium (30 mL) from both the conditions to new 50 mL falcon tubes and label appropriately. Keep the tubes on ice.

c. Resuspend the cell pellet in 5mL ice-cold PBS and centrifuge the cells at 500 $\times$ g for 5 min at 4°C.

d. Lyse the cells using 100–200μL of the cold lysis buffer (Phospho Safe Extraction reagent with Benzonase at a concentration of 250U per mL of lysis buffer) by pipetting up and down several times. Sonication is not required for lysis. Incubate the samples on ice for 20 min.

e. Store the whole cell lysate at −80°C until immunoblotting or centrifuge at 16,000 $\times$ g for 10 min at 4°C.

f. Transfer the clarified lysate to a new 1.5 mL microcentrifuge tube and stored on ice for later use.

Note: Alternative Lysis buffers with required proteinase inhibitors can also be utilized for this step. We utilized Phospho Safe Extraction reagent for cell lysis as this buffer can be efficiently used both for cell lysis and maintaining the proteins in their phosphorylated state. It has a cocktail of four phosphatase inhibitors- sodium fluoride, sodium vanadate, β-glycerophosphate, and sodium pyrophosphate along with a proprietary lysis buffer.

6. The conditioned medium (from step 5b) in 50mL falcon tube is centrifuged at 1,000 $\times$ g for 5 min at 4°C to remove any remaining cells and debris.

7. Gently transfer the supernatant avoiding any pelleted debris or residual cells using a 10 mL serological pipet to a new 50mL falcon tube.

8. Transfer 15mL of the exosome containing medium into the reservoir of a 15mL 3KDa Amicon centrifugal filter.

9. Centrifuge the sample at 1,950 $\times$ g until 150 μL sample remains.

10. Repeat steps 8 and 9 for the remaining 15 mL supernatant.

11. Discard the flow through and transfer the concentrated medium from ‘Complete’ and ‘Starvation’ centrifugal filter reservoir to prelabeled microcentrifuge tubes.

△ CRITICAL: All the centrifugation steps must be performed at 4°C to avoid degradation of RNA and protein.
Validation of exosomes in the concentrated media by dynamic light scattering analysis; day 4

© Timing: 3–5 min per sample

The size of the exosomes in the conditioned medium or purified exosomal fractions can be determined by several methods including Nanoparticle tracking analysis (NTA), Electron microscopy (EM), Flow cytometry, Atomic Force microscopy (AFM), Resistive pulse sensing, and Dynamic light Scattering (DLS).

In this protocol we utilized a Zetasizer Nano to validate the presence of exosomes in the concentrated medium. The Zetasizer system determines the particle size by measuring the Brownian motion of the particle in a sample by Dynamic Light Scattering (DLS) followed by interpretation of the size. The collected fractions can be concentrated for particle analysis using an Amicon ultrafiltration device.

Note: It is advisable to make 3–4 dilutions of the conditioned medium (complete and starvation) before starting the DLS analysis. Highly concentrated or highly diluted samples will likely cause errors in the estimation and the result will not be as desired. According to the Zetasizer nano manual, for particle sizes between 100 nm to 1 μm the concentration of a sample can be as low as 0.01 mg/mL. The maximum concentration largely depends upon whether the particles tend to aggregate at a high concentration and cause multiple scattering. For exosomes a range of 0.1mg/mL to 1 mg/mL can be tested and the best scattering profile can be chosen. For this protocol, the best profile was obtained at 0.5 mg/mL out of three concentrations (0.5 mg/mL, 1 mg/mL, and 1.5 mg/mL). Additionally, the samples should be kept on ice throughout the experiment.

12. The protocol here refers to the use of Zetasizer Nano (ZS90) instrument for DLS analysis. Different instruments can have variations in the operating procedure and thus respective manuals are to be referred. First, turn on the instrument and open the Zetasizer Nano ZS 90 software.
13. Place around 100 μL of the sample to be analyzed in a quartz cuvette compatible with the instrument and place it in the instrument holder.
14. On the computer start the software, choose the option particle size analysis.
   a. Set-up the required parameters like refractive index and absorption of the material to be analyzed and which solution it is dispersed into.
      Note: In this protocol the solution was dispersed in 1× PBS, and PBS is a preset option in the machine with a refractive index of 1.338. Based on the manufacturer’s website, the recommended refractive index (RI) and absorption (k) values suggested for exosomes are 1.37–1.39 and 0.01, respectively (https://www.materials-talks.com/faq-how-important-are-refractive-index-absorption-for-nanoparticles/). Set up the number of measurements required (anywhere between 3–5).
15. Start the analysis. The software will take some time to adjust the temperature and other conditions.
   a. Obtain a graph between the size of particles (in nm) and intensity (representing how abundant they are in the sample).
      Note: The particles that are the most abundant in the sample will have the sharpest and strongest peak. For exosome this peak is anywhere between 100–200 nm of size.
16. After one measurement rinse the cuvette with milliQ water and repeat for other samples.
17. When the run is complete, click on an individual run and go to the intensity option on the software. This will show the graph for that run.
Go to the Edit option and select ‘copy graph’ or ‘copy raw values’.

Note: The graph obtained can be directly used or the raw values of size and intensity can be used to plot our own graphs using a software like Microsoft excel or Graph Pad Prism (Figure 2).

**Isolation of exosomes; day 4**

**Timing:** 1 day

This step involves isolation of exosomes from concentrated cell media by size exclusion chromatography (SEC) using columns available from Izon (Figure 3). Other alternatives that can be used for purification of exosomes by SEC include Sephacryl S-500 columns, Sepharose CL-4B/2B columns, mini-PURE-EV columns, Exo-spin mini-columns and Sephacryl S-1000 columns. The exosomes that are larger in size are eluted first compared to proteins which are eluted in later fractions.

18. Equilibrate two pre-labeled Izon qEV/35nm single-use columns (optimum for the isolation of RNA) with 150μL 1× PBS (at a temperature of 18°C–25°C) and load the concentrated ‘Complete’ and ‘Starvation’ medium onto the column (Figure 4).

19. Collect the Buffer volume (1mL) and discard.

20. Collect 100 μL fractions (10–15) in pre-labeled microcentrifuge tubes. The exosomes elute in the 2nd and 3rd fractions.

21. Measure the absorbance of the collected fractions at 600 nm (for exosomes) and 280 nm (for proteins).

Note: When absorbance values are plotted as a graph two separate peaks will be obtained—one for extracellular vesicles and one for proteins (Figure 5). The elute that shows a peak at 600 nm is likely to be a pure exosomal fraction.
2. The exosome fractions can be used for downstream applications.

⚠️ CRITICAL: Incubate the columns at room temperature before starting the purification. Air bubbles can be introduced in the columns if columns removed from 4°C and applying PBS at room temperature. These introduced air bubbles can result in poor separation.

Validation of exosomes by immunoblot analysis; day 5 and day 6

⏱ Timing: 2 days

In this step, the whole cell lysates and the exosome fractions isolated from Complete and Starvation medium are analyzed by Western Blot Analysis. Different exosomal markers are analyzed in the fractions purified from the two nutrient conditions.

- Remove the top cap of an IZON column and attach it in an upright position in the rack provided
- Remove the lower cap and let the buffer (PBS) in the column run through it
- After all the buffer has entered the loading frit, load the concentrated sample onto the frit
- Let the sample enter through the frit completely into the column and discard the buffer volume dispaced (around 1mL)
- Top up the column with more buffer (1 mL PBS) and start collecting 100 μL fractions
- Measure absorbance of the fractions and choose exosomal fractions for immunoblotting

Figure 2. Particle size analysis of the conditioned concentrated CCM3 and starvation medium collected from Kc167 cells using a Zetasizer nano system

Dynamic light scattering analysis was performed on conditioned medium collected from Kc167 cells in complete and starvation medium.

Figure 3. A flowchart depicting the steps involved in exosome purification using size exclusion chromatography
23. Estimate the protein amount in the whole cell lysates and purified exosomes using the Bradford assay.
   a. Add 6× sample buffer after normalizing the concentrations for all the samples.
   b. Denature the samples by heating at 85°C for 5 min.
   c. Load equal amount of protein per lane onto an SDS-PAGE gel.

   Note: The percentage of the gel will vary depending on the size of the protein under study.

24. Resolve the protein samples on the SDS-PAGE gel.
   a. Transfer the proteins onto a 0.45 μm pore sized PVDF membrane (charged with methanol) in a wet transfer system using the Tris Glycine transfer buffer at 4°C.
   b. Standardize the voltage and timing for transfer depending on the proteins of interest.

Figure 4. A gel-filtration column for the isolation of exosomes from concentrated conditioned media

Figure 5. Elution profile of vesicles with 100 μL of concentrated conditioned medium loaded
OD600nm (blue line) and OD280nm (red line) was measured for each of the 100 μL fractions. The peak corresponding to the vesicles occurred at 200μL after void volume (1,000μL). The protein fraction eluted in later fractions (Fraction 30–34).
Note: The transfer can be done for a few hours at a higher voltage or overnight at a lower voltage depending on feasibility.

25. Block the membrane in 5% skimmed milk solution prepared in 0.1% PBST (1× PBS + 0.1% Tween-20) at room temperature on a rocker for 1 h at 25°C.

26. After the blocking is complete wash the membrane with 0.1% PBST 3 times (for 5 min each).

27. Dilute primary antibodies in 5% skimmed milk solution (according to manufacturer’s instructions) and add to the membrane. This protocol uses a FLAG-tagged protein as an example so the Anti-Flag (mouse) antibody is used at a dilution of 1:2,500 for detection of the protein of interest.

Note: It is essential to also perform blotting for a few proteins that are known to be associated with exosomes like cysteine string protein (Csp) and Syntaxin 1A (Syx1A). These will serve as a positive control to indicate that the eluted fractions are exosomes.

28. These antibodies can be used at the following dilution - cysteine string protein (Csp) 1:1,000 and Syntaxin 1A (Syx1A) 1:500.

29. Gently rock the membrane incubated with primary antibodies overnight (12–16 h) at 4°C.

30. Next day, wash the membrane with 0.1% PBST 3–4 times (5 min each) to remove excess of the primary antibody.

31. Dilute appropriate HRP-conjugated secondary antibodies (depending on primary antibodies used) in 5% skimmed milk and add to the membrane.
   a. Rock at room temperature for 1–2 h to allow efficient binding.

32. Wash the membrane with 0.1% PBST 4–5 times (5 min each) and proceed to develop the blot on a ChemiDoc using an appropriate ECL detection system (Figure 6).

Note: It is also a good idea to not only load the fraction with the sharpest peak at 600 nm but a few fractions before and after the peak. This will provide a good idea about the enrichment of exosomes in the fractions and hence the best one can be chosen for further experiments. Along with the eluted fractions, equal amount of protein should also be loaded onto the SDS-PAGE for the cell lysates obtained from both the conditions (complete medium and starvation). This is to make sure the transfection is uniform in both the conditions and the protein of interest is expressed in both. For the Western blot analysis of lysates, conditioned medium and exosome fractions for this protein, please refer to.¹ In addition to proteins, mRNAs and microRNAs are also loaded into exosomes. For purification and characterization of miRNAs loaded into exosomes please refer to.²–⁴

EXPECTED OUTCOMES

This is a detailed protocol for the isolation and validation of exosomes from a Drosophila cell line that is subjected to starvation or nutrient deprivation and to characterize proteins that are selectively enriched onto exosomes under these conditions.
LIMITATIONS
This protocol has been optimized for isolation of exosomes from Kc167 Drosophila cell line. Kc167 can easily grow in CCM3 without the requirement of fetal bovine serum (FBS). However, there are many cell lines that will not grow in a medium devoid of FBS. FBS itself is a source of many extracellular vesicles and hence the protocol will have to be further optimized/modified to use it for cells grown in a medium supplemented with FBS. If the cells are cultured in a medium with FBS and exosomes are to be isolated only from starved cells, then before resuspending the cells in 2 mg/mL glucose (starvation medium) cells can be washed 2–3 times with 1× PBS.

TROUBLESHOOTING
Problem 1
Depending on the cell line used, there might be high or low cell death after the cells are subjected to starvation. If there is too much cell death this might release cellular debris that can contaminate the exosomal fractions (Figure 1).

Potential solution
Monitor the cells under starvation and if required estimate the cell density for both the conditions at the end of 8 h of starvation. If there is a large difference in cell density the starvation time can be reduced and can be optimized accordingly to cause less cell death. In case of Kc167 cells, significant cell death is not observed at around 8 h. However, if the cell density is low, there is higher cell death observed and this requires correct seeding of cells when starting the experiment.

Problem 2
Poor separation profile of exosomes.

Potential solution
The sample being loaded onto the column should be completely free from any debris or particulate matter. Centrifuge the samples appropriately to remove any such impurities. The temperature of the column and of the sample should be in the range 18°C–24°C and there should not be difference in their temperature. Such a difference might result in bubble formation thus leading to poor separation.

Problem 3
High variability in the particle size distribution peaks during dynamic light scattering (DLS).

Potential solution
Make appropriate dilutions of the samples prior to performing DLS with the samples. Low concentrations might result in an undetectable number of particles for analysis and high concentrations might cause multiple scatterings thus causing variability in the results. So, this needs to be standardized according to the experiment undertaken.

Problem 4
Low transfection efficiency (in case a tagged protein is being studied for exosome enrichment under nutrient deprivation).

Potential solution
High quality DNA (free from endotoxins) should only be used for transfection. All media and transfection reagents should be free from any contamination. Cell lines have varying sensitivity towards amount of DNA and transfection reagents (resulting in cytotoxicity) and this needs to be optimized by trying different ratios of these components.

Problem 5
Problems with immunoblotting.
Potential solution
In many cases the objective of an experiment could also be to look at modifications (phosphorylation, acetylation etc.) of an exosome enriched protein along with its presence in the exosomes. These modifications are highly dynamic so lysis buffer being used should be supplemented with appropriate inhibitors (like deacetylase or phosphatase inhibitors), thus preserving these modifications. General cell lysis procedures will not be sufficient to detect such modified proteins.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Geetanjali Chawla (gchawla@rcb.res.in).

Materials availability
This study did not generate new unique materials or reagents.

Data and code availability
This study did not generate new databases or code.

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AUTHOR CONTRIBUTIONS
M.P. wrote the original draft of the manuscript, generated the figures, and performed the experiments. G.C. supervised, wrote and edited the manuscript, and acquired funding. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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