



Characterization of extracellular vesicles from primary skin epithelial cells and cell lines from Atlantic salmon

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ABSTRACT

Extracellular vesicle (EV) research in fish, particularly in aquaculture-relevant species, is gaining momentum, with interest in biomarker development, while the availability of *in vitro* tools to study EV-mediated cellular communication in fish is limited, particularly with respect to primary cell lines. As salmon represents one of the most valuable fish species in global aquaculture, identifying robust biomarkers for fish health monitoring is a high priority. This study introduces a novel *in vitro* model for EV research in salmon, utilising primary and immune relevant cell lines to closely mimic *in vivo* conditions. EVs were profiled from primary scale derived keratocyte cells (SKC), in addition to EVs from previously established salmon cell lines (ASK and SSP-9 from salmon head kidney). EV size profiles were identified overall in the 50–450 nm range, with some differences between the cell lines. EV protein cargoes were analyzed by LC-MS/MS alongside associated pathway enrichment, providing insights for roles of EVs in cell-type-specific communication, including immune responses. Notably, the SKC-derived EVs revealed proteins and pathways associated with wound healing, keratinization, and immune defense, highlighting their potential as therapeutic targets. Additionally, shared pathways identified in EVs from all cell lines, included the non-canonical NF- κ B pathway and ubiquitination, offering new perspectives into EV-mediated signaling in fish. Our *in-vitro* model system may provide a translational platform to develop EVs as robust biomarkers of fish health, with potential applications in aquaculture.

1. Introduction

Extracellular vesicles (EVs) are 30–1000 nm lipid bilayer structures secreted by cells into the extracellular environment and taken up by neighboring cells (Couch et al., 2021). EVs carry a variety of biomolecules, such as proteins, lipids, RNA species, and DNA, and play a crucial role in cell-to-cell communication (Wessler et al., 2025); a mechanism which is conserved across phylogeny (Lange, 2023; Di Naro et al., 2025). While the majority of EV research to date has been in relation to human pathologies, due to their usability as biomarker tools, EV research in other species has grown in recent years, including in relation to the fish aquaculture sector (Magnadóttir et al., 2019a, 2020, 2021). Atlantic salmon (*Salmo salar* L.) is a fish species of major economic importance in global aquaculture (Barasa et al., 2022). The health and productivity of salmon are vital, and EVs may offer biomarker tools for improving aquaculture practices, both relating to disease

management and vaccination strategies. However, to date studies on EVs in salmonids remain limited, particularly with respect to primary cell lines. EVs have been assessed in the serum of virus infected salmon (Lagos et al., 2017), in response to bacterial stimulation in salmon head kidney leukocytes (Iliev et al., 2018) and in relation to stress responses in rainbow trout (Faught et al., 2017). In this study, we characterized EVs at baseline (without any immune stimulus), derived from two different head kidney of salmon that are often used to study immune responses (ASK and SSP-9), and a primary cells derived from fish scales; keratocytes (SKC). The aim was to establish an experimental framework and tools for future *in-vitro* modelling of roles for EVs in response to stressors, including infection and environmental changes. By identifying EV protein cargo contents, associated pathway enrichment analysis can provide insights into their biological functions, including immune associated ones. Identification of robust biomarkers is critical for salmon farming, where various environmental challenges due to the open cage

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system allow for opportunistic infection, with high mortality rates caused by various factors including jellyfish blooms (Mitchell et al., 2021), parasitic infections (Pincinato et al., 2025) and temperature fluctuations (Bui et al., 2022). Therefore, investigations into EV mediated communication from salmon immune competent organs and tissue, and particularly skin, are of considerable interest. Our previous findings have shown that primary keratocytes from salmon scales can be used as *in-vitro* cellular models for various mechanisms of cellular communication, including the formation of tunneling nanotubes (TNTs), as well as internalization of microparticles (Thiyagarajan et al., 2024) and pathogens (Mikkelsen et al., 2025; Åsbakk, 2001). The current report characterizes EVs from these cells, including their proteomic cargo profiles, providing insights into these EVs' biological roles to establish a platform for further *in vitro* EV research models for salmon immunobiology.

2. Materials and methods

2.1. Ethical statement

Atlantic salmon post-smolts (0.5–2 kg) were obtained from the Research Station in Tromsø, Norway. Fish were reared on commercial diets in flow-through tanks following EU and Norwegian animal welfare protocols. Euthanasia was performed with a sharp blow to the head, adhering to EU Directive 2010/63/EU (<https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063>). Fish were transported on ice to the University of Tromsø, and scales were processed within 1 h.

2.2. Cell line models

Three salmon cell lines were used for comparative analysis of EV profiles and EVs' proteomic cargo content: Atlantic salmon head kidney (ASK) cells (ATCC, CRL-2747TM), *Salmo salar* pronephros 9 (SSP-9) cells (Rodriguez Saint-Jean et al., 2014), and primary cells from scales (SKC), as previously described (Thiyagarajan et al., 2024), were grown in HBSS-mix media, consisting of 0.22 μ m-filtered HBSS (VWR, 21-023-CM) with 100 μ g/ml streptomycin, 100 IU/ml penicillin (Sigma-P0781), and 1 μ g/ml of Amphotericin B solution (Sigma-A2942), with 10 % FBS. ASK and SSP-9 cells were cultured according to recommended methods as monolayers at 20 °C in Leibovitz (L-15) medium, with ASK medium supplemented with 10 % FBS and P/S, and SSP-9 medium with 8 % FBS (Peñaranda et al., 2019). Approximately 2×10^6 cells were seeded per T-75 flask and grown to confluence. For primary SKC cells, 4 scales (1–5 mm in size) per T-25 flask were explanted and cultured in HBSS supplemented with antibiotics and 10 % FBS. Keratocyte migration occurred within 3–5 days at 4 °C, as described previously (Thiyagarajan et al., 2024) until reaching 70–80 % confluence. Before isolation of EVs from all three cell types, the cells were washed, thoroughly, three times with sterile DPBS, and for the isolation of EVs released from the cells, 5 ml of fresh FBS-free medium was added per flask for 3 h (to avoid any contamination with FBS-derived EVs), whereafter the EV containing cell medium was collected for EV isolation. EV preparation was performed in triplicates for each cell line.

2.3. EV isolation and characterization

EVs were isolated from the cell culture media of the three cell lines, according to previously established protocols (Lange et al., 2024) using differential centrifugation, and characterized adhering to the recommendations of MISEV 2023 (Welsh et al., 2024), by nanoparticle tracking analysis (NTA) for quantification and size profile distribution, for two surface markers by western blotting and for morphology by transmission electron microscopy (TEM). In brief, for EV isolation, the EV containing media was centrifuged at 4000 g for 20 min at 4 °C to remove aggregates and apoptotic bodies, the supernatant was then ultracentrifuged at 100,000 g for 1 h at 4 °C, collecting the EV-enriched

pellets which were washed with 1 ml sterile DPBS, and then centrifuged again at 100,000 g for 1 h, followed by reconstitution in 100 μ L DPBS. For size profiling by NTA, 10 μ L of the EV solution was diluted in 990 μ L DPBS and analyzed using the NTA NS300 system equipped with a sCMOS camera and a 488 nm diode laser (Malvern Panalytical, Ltd, Malvern, UK). Five 60-s videos were recorded, and the replicate histograms were averaged using the NTA 3.4 software histograms assessing EV size profiling and quantification, also categorizing EVs into small (≤ 100 nm), medium (101–200 nm), and large (> 200 nm) subgroups.

The EVs were assessed for two EV surface markers, CD63 (ab216130, Abcam, UK) and flotillin-1 (Flot-1, ab41927, Abcam), using dot blot and Western blotting. Samples were prepared in $2 \times$ Laemmli buffer, boiled at 100 °C for 5 min, and separated by SDS-PAGE on 4–20 % TGX gels. Proteins were transferred onto 0.45 μ m nitrocellulose membranes via semi-dry transfer at 15 V for 1 h. Membranes were blocked with 5 % BSA in TBS-T for 1 h, followed by overnight incubation at 4 °C with primary antibodies (1:1000 in TBS-T). After washing, membranes were incubated with HRP-conjugated secondary antibodies in TBS-T (1:3000; BioRad) for 1 h, washed, and visualized using ECL and a UVP BioDoc-ITTM System.

For TEM imaging, EV pellets were resuspended in 100 mM sodium cacodylate buffer (pH 7.4) and 3–5 μ L was applied to a glow-discharged carbon-coated TEM grid. After air-drying for 10 min, grids were fixed with 2.5 % glutaraldehyde for 1 min, washed with distilled water, and stained with 2 % uranyl acetate for 1 min. Excess stain was removed, and grids were air-dried. Imaging was performed using a JEOL JEM 1400 microscope at 80 kV with 30,000 $\times$ to 60,000 $\times$ magnification, and digital images were captured with a GATAN Rio 16 digital camera (Ametek GB Limited, Leicester, UK).

2.4. LC MS/MS and PPI network analysis

Protein cargoes from the three different salmon cell line derived EVs were analyzed by LC-MS/MS after in-gel digestion (performed by Cambridge Proteomics, University of Cambridge, UK). For a baseline profile, EV samples were pooled from three replicate EV isolates from each cell line. EV cargo proteins were identified using the Mascot search algorithm against the *Salmo salar* database (86,699 sequences), with ion scores > 34 indicating identity or homology ($p < 0.05$). Fragment and peptide mass tolerances were set at 0.1 Da and 20 ppm, respectively. Protein-protein interaction networks were generated using the STRING (<https://string-db.org/>) database at medium confidence settings, choosing *Salmo salar* as a species and analyzed for Gene Ontology (GO), KEGG, and Reactome pathways. Results were exported as gif images and Excel tables.

2.5. Statistical analysis

For comparison and for graphical representation of datasets from the three different cell lines, GraphPad Prism version 10 was used. STRING analysis was carried out with medium confidence. NTA histograms are presented with mean and standard error of mean.

3. Results

3.1. EV characterization

EVs from the ASK, SSP-9 and SKC were characterized following isolation by ultracentrifugation for size distribution and particle quantification using NTA (Fig. 1A), Western and dot blotting for the EV-specific markers CD63 and Flot-1 (Fig. 1B and C) and visualized by TEM for morphology (Fig. 1D). NTA revealed a poly-dispersed population with the majority of EVs falling in the size range of approximately 50–450 nm with slight variations between cell lines, with main peaks at, 58, 111, and 186 nm for ASK-EVs; at 73, 101–125 and 179 nm for SSP-9 EVs; and at 96, 159 and 245 nm for SKC EVs (Fig. 1A). In addition, very

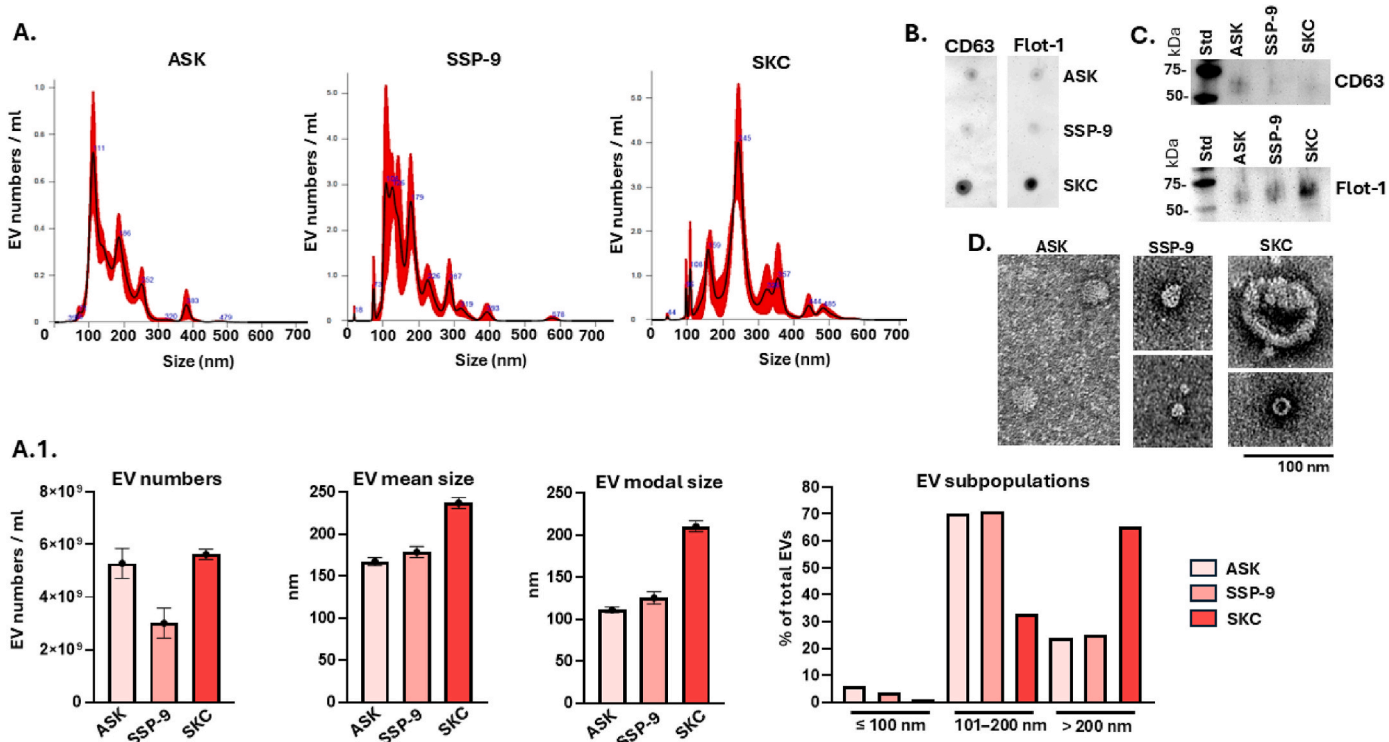


Fig. 1. EV analysis from ASK, SSP-9 and SKC: (A) Representative NTA histograms for EV profiles (the black line represents the mean, and the red line represents the standard error of the mean, SEM); (A.1) Represents EV profiles relating to EV numbers, size (mean and modal), and sub-populations as a % of total EVs across the three salmon cell lines; (B)–(C) Dot blot and western blotting analysis of EVs from the three cell lines for the EV-specific markers CD63 and flotillin-1, size standard indicated in kDa. (D) TEM imaging of EVs, showing representative images of EVs from all three cell lines; scale bar represents 100 nm.

minor peaks at 18 nm and 14 nm were observed for SSP-9 and SKC EVs respectively, which may be indicative of exomeres - but these peaks were considered negligible. Overall, modal EV sizes observed by NTA were 111 nm for ASK EVs, 125 nm for SSP-9 EVs, and 210 nm for SKC

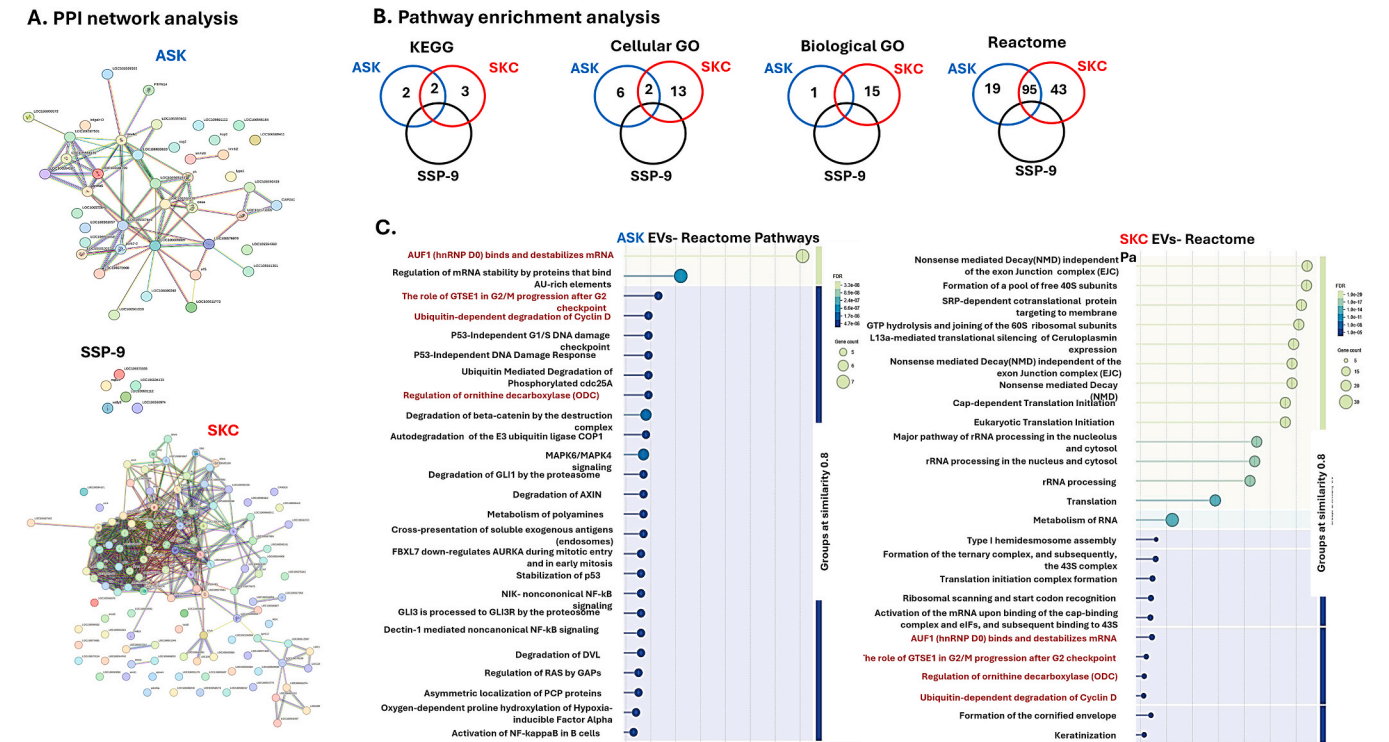


Fig. 2. Protein-Protein Interaction (PPI) analysis of the EV proteomes of the three cell types: (A) PPI network analysis; (B) Venn diagrams illustrating unique and shared pathways between the EV proteomes of the three cell types; (C). Top 25 reactome pathways for ASK EV and SKC EV proteomes. The graphs display signal on the x-axis and False Discovery Rate (FDR) on the y-axis.

EVs, respectively (Fig. 1A1). EV concentrations were $\sim 5 \times 10^9$ particles/ml for ASK and SKC cells, and $\sim 3 \times 10^9$ particles/ml for SSP-9 cells. The ASK and SSP-9 cells were seeded in T75 flasks, while the SKC cells were seeded in T25 flasks and overall, our results support that the SKC primary cells can yield similar amounts of EVs compared to the immortalized cell lines. When assessing distribution of EV subpopulations, the majority of the EVs were at 101–200 nm for ASK and SSP-9 cells, while SKC-EVs were predominantly at 101–200 nm and >200 nm.

3.2. Salmon EV proteome cargoes and associated protein-protein interaction networks and pathway enrichment analyses

The protein cargoes of the EVs from the three salmon cell types were analyzed using LC-MS/MS against the Atlantic salmon UniProt database, considering only salmon-specific hits. Based on this analysis, SKC-EVs contained 230 protein hits, ASK-EVs had 83 hits, while SSP-9-EVs had only 10 hits (Supplementary Tables 1–3). This variation may reflect some differences between the primary cells (SKC) and the immortalized cell lines (ASK and SSP-9), and number of passages of cell lines may need consideration in future studies as the SSP-9 cells used here were at a passage 178. Differences were observed in protein-protein interaction (PPI) networks and associated pathway enrichment (GO, KEGG, Reactome) following STRING analysis (Fig. 2). The ASK and SKC EV proteomes showed numerous shared, but also unique enriched pathways (Fig. 2B), while no pathway enrichment was found for the small proteome identified in the SSP-9 EVs, and this will need further investigation as this may possibly have been influenced by the high passage number of this cell line as the processing of EV isolation from all three cell lines was carried out following the same procedure. The comparative pathway enrichment, showing unique and shared KEGG, Cellular GO, Biological GO and Reactome pathways, is illustrated in the Venn diagrams in Fig. 2B. The top 25 Reactome pathways for ASK and SKC EVs, based on signal and false discovery rate (FDR) are furthermore presented in Fig. 2C.

4. Discussion

Extracellular vesicle (EV) research in fish and aquaculture research is gaining momentum, but knowledge of EV mediated communication in cellular processes is still scarce. Recent studies have characterized EVs in various commercially valuable/important fish species, including cod (Magnadóttir et al., 2019a; Lange et al., 2019), and halibut (Magnadóttir et al., 2019b). In salmon, some studies have been carried out on EVs during infection (Lagos et al., 2017; Smith et al., 2020), while this report fills a knowledge gap on EVs from primary salmon scale cells. As salmon is amongst the most valuable commercial fish species in the aquaculture sector globally, it is of high priority to identify robust biomarkers to monitor fish health. Thus, there is also an urgent need for developing reliable *in-vitro* models that can be used to assess the roles for EVs in cellular communication, also in response to infection, pollution and other external stimuli (Dias et al., 2024). Furthermore, there is considerable value in using primary cell such as the SKC cells, combined with immortalized cell lines. Our study reports a novel platform for *in-vitro* modelling of EVs in salmon, importantly including a primary skin keratocytes which may be critical for modelling fish skin responses (Krasnov et al., 2025). Importantly, explant-derived SKCs may be of considerable value as primary cell-model which may mimic the *in-vivo* environment. Salmon cell-specific EV profiles identified in the current study indicate that EVs derived from both primary (SKC) and widely used salmon cell lines (e.g., ASK, SSP-9) exhibit distinct proteomic and pathway enrichment profiles. This may stem from their different tissue origins and passage numbers. For instance, GO enrichment analysis indicated that intracellular non membrane bound and intracellular molecules in SKC EVs may aid studies in cell-type-specific communication (Gordaliza-Alaguero et al., 2019). As substantial amount of

transcriptomic data is already available for salmon (Sveen et al., 2023), it may be of interest to compare this with the proteomic data from cell-released EVs for further insights into potential therapeutic targets for fish diseases. Notably, our reported data also includes several proteins relevant to wound healing, keratinization and immune responses (Supplementary Table 4), which was also reflected in the associated pathway enrichment analysis. For example, proteins involved in keratinization and wound healing in SKC EVs show promise as future therapeutic targets (Eslamloo et al., 2022). The identification of pathways associated with wound healing, cell-cell communication, and immune responses in SKC-derived EVs underscores the potential of this model for studying tissue repair and host defense mechanisms in salmon (Krasnov et al., 2025). These findings are particularly relevant also for aquaculture, where understanding these processes can aid in improving fish health and resilience (Barasa et al., 2022; Hvas et al., 2021). In addition, the discovery of unique pathways, such as the non-canonical NF- κ B pathway and ubiquitination, shared between ASK and SKC cells, provides novel insights into the molecular mechanisms mediated by EVs in relation to inflammatory responses (Das et al., 2023; Kumar et al., 2024; Huang et al., 2025). These findings open new avenues for exploring EV-mediated signaling in fish (Tacchi et al., 2012). For methodological consideration, it must be noted that we used stepwise ultracentrifugation for EV isolation, obtaining total EV preparations in an approximate 50–450 nm size range, while some co-purification with non-vesicular extracellular nanoparticles (NVPs) and/or exomeres cannot be fully excluded, given minor (while almost negligible) small EV peaks observed by NTA at 14 and 18 nm for two of the cell lines. Additional steps, including high-purity isolation techniques such as density gradient centrifugation and size exclusion chromatography can therefore be tested and applied in further studies. The proteomic EV data obtained here showed absence of “contaminants” associated with exomeres or NVPs (e.g. GALNS, MAN2B1, AGO2, albumin, cystatin C) while the EV characterization included two EV surface markers (CD63 and flot-1) - in addition to NTA and TEM. This meets the MISEV guidelines. In conclusion, the current study highlights the use of primary cell-derived EVs, as well as EVs from cell lines, as a platform for identifying fish health biomarkers such as EV-signatures linked to stress or disease in salmon. The reported EV-proteome SKC data, furthermore, provides valuable insights into potential EV functions in skin and pave the way for using EVs in aquaculture. This study also provides a platform for future testing of various agents (e.g., immunostimulants, mucosal vaccines, xenobiotics) on EV formation and EV-mediated communication in skin.

CRediT authorship contribution statement

Dhivya Borra Thiyagarajan: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marie K. Mikkelsen:** Writing – review & editing, Methodology. **Igor Kraev:** Writing – review & editing, Visualization, Methodology. **Roy Ambli Dalmo:** Writing – review & editing, Project administration, Funding acquisition. **Sigrun Lange:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Competing interest statement

The authors declare that there are not any non-financial or financial

competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.dci.2026.105555>.

Data availability

No data was used for the research described in the article.

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