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Selective SLAM/CD150 Receptor-Detargeting of Canine Distemper Virus

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Running title: Selective SLAM-detargeting of A75-CDV

Key words: Wild-type CDV, cell entry, SLAM receptor, attachment protein H, selective SLAM-blind H.

Highlights

- Single mutations (S546Y, Y547N or T548R) in A75/17-H do not disturb SLAM-dependent fusion-triggering
- The triple A75/17-H mutant (546-SYT/RNR-548) interferes with SLAM-dependent fusion-triggering
- A75/17-H (546-SYT/RNR-548) exhibits impaired interaction with SLAM
- Viruses harboring the H-mutant selectively lost SLAM-dependent cell entry

Abstract

The envelope attachment (H)-protein of canine distemper virus (CDV) mediates receptor engagement and fusion-triggering; two key functions in viral cell entry and spread. Signaling lymphocyte activation molecule (SLAM) and Nectin-4 (N4) act as morbilliviral entry receptors in immune and epithelial cells, respectively, which defines very similar pathogeneses. High incidence of brain disorders is however unique to CDV. The wild-type CDV-A75/17 strain (A75) preferentially infects glial cells and spreads from astrocyte-to-astrocyte without inducing massive fusion events, despite the fact that SLAM and N4 expressions remained below detection levels. To investigate whether an A75 H-microdomain required to interact with SLAM may additionally contribute to promote viral spread between astrocytes, we initially engineered a novel A75 H-protein variant (546-SYT/RNR-548) that lost SLAM-binding property and, consequently, lacked fusion-triggering activity specifically in SLAM-expressing cells. Collectively, this approach provides the molecular tool to decipher the role of the selected H-microdomain in supporting A75-spread in glial cells.

Belonging to the genus *Morbillivirus*, canine distemper virus (CDV) is highly contagious and can infect unvaccinated domestic dogs, a wide range of wild animals of the order Carnivora as well as some members of other orders [1]. It is closely related to important pathogens from the same genus such as measles virus (MeV), peste-des-petit-ruminants virus (PPRV) and already eradicated rinderpest virus (RPV). Known for its potential to spillover to different species, CDV outbreaks sporadically in wildlife with devastating mortality [2]. One of the unique feature of CDV is high incidence of neurovirulence in infected animal hosts [3]. Nonetheless, different CDV strains display diverse levels of neurovirulence, affecting different regions of the brain and causing neurologic symptoms at distinct time points. For example, while some strains manifests as acute encephalitis, A75/17-CDV (A75) results in a long-term progressive and focal demyelinating disease [4,5].

In vivo studies using MeV indicated that two receptors are required for virus pathogenesis and transmission [6–9]. While CD150/SLAM is responsible for rapid spread in immune cells [10–14] causing viremia and immunosuppression, PVRL4 (also known as nectin-4 (N4)) is expressed in epithelial cells [15–18] and is crucial for the development of clinical signs in addition to functioning as a host "exit" receptor allowing virus shedding in the trachea [8,19]. Furthermore, in dog brains, N4 has been found in ependymal cells and meninges, as well as in epithelia of choroid plexus, neurons, granular cells and Purkinje's cells, but absent in astrocytes [20–22]. Interestingly, astrocytes were reported to be a preferential site of replication for the demyelinating A75 strain [23]. Employing primary dog brain cell cultures (mainly consisting of astrocytes), it was reported that A75 spread via astrocyte-to-astrocyte contacts without inducing substantial cytolysis and progeny virus production [24]. Combined with the fact that viral spread was efficiently inhibited by a small molecule fusion inhibitor [24], the possible existence of a host cellular factor promoting non-cytolytic, astrocyte-to-astrocyte transmission, was therefore suggested [22].

Morbilliviruses employ two surface glycoproteins (H and F) to enter a target cell. While H mediates receptor binding, F merges the viral envelope with the host cell plasma membrane, thereby resulting in the formation of a fusion pore and injection of the ribonucleocapsid into the host cell [25–27]. CDV H is a type II transmembrane tetrameric protein, where each protomer contains 604 to 607 amino acids (aa), depending on the viral strain [28]. H consists of three major domains: N-terminal cytoplasmic tail (aa 1 to 35), transmembrane domain (aa 36 to 58) and a large ectodomain (aa 59 to 604 or 607) [29,30]. Ectodomain can be further segmented into three parts: stalk (aa 59 to 154), connecting region (aa 155 to 187) and receptor binding C-terminal head domain (aa 188 to 604 or 607) [30,31].

High-resolution structures of MeV H in complex with SLAM and N4 indicated that H-head domains assume a β-propeller conformation that interacts with SLAM and N4 through a similar side of the β-propeller, although their precise mode of interaction exhibit variations [32,33].

The importance of SLAM in contributing to MeV pathogenicity was elegantly demonstrated by the generation of so-called SLAM "blind" viruses by substituting an asparagine residue into an alanine at position 533 of H (MeV H-R533A) (homologous residue in CDV-H: R529) [34]. Such viruses were selectively disabled in triggering membrane fusion through SLAM. In contrast, with regard to the H-protein of the ferret-adapted 5804P-CDV [35], a combination of six amino acids, locating in two nearby clusters, required alanine/serine substitutions (526-9 + 547/8) in order to ablate SLAM-dependent membrane fusion-triggering [10]. Interestingly, when those six mutations were transferred to the H-protein of A75 (H-SB), receptor-detargeting was indeed achieved, although membrane fusion-triggering was impaired in presence of both SLAM and N4 [29]. A similar phenotype was observed in case of a single point CDV H-variant (H-R529A) [29], which indicated possible strain-specific variations in the mechanisms underlying receptor-dependent fusion-triggering.

Despite of recent advancement in deciphering CDV pathogenesis, the exact role of cellular receptors in different stages of neuro-infection as well as the role of microdomains in H and F remains largely unexplored. Although expression of SLAM and N4 remained below detection levels in canine astrocytes [22,24,29], key residues of the A75 H-protein implicated in SLAM binding may nevertheless contribute to the promotion of non-cytolytic viral cell-to-cell spread (perhaps by contributing to the interaction with another cellular factor). Since our previous studies indicated that A75 H-SB and H-R529A were defective in promoting membrane fusion in presence of both SLAM and N4, we here aimed at engineering truly selective SLAM "blind" A75 H-proteins.

Based on a previously designed CDV H/SLAM 3D structural model [36] as well as the MeV H/N4 co-crystal structure [33] (note that no major differences are expected to occur in how CDV H may bind to canine N4), residues at positions 546, 547 and 548 (546-SYT-548) were selected for mutagenesis, since those three amino acids may be specifically involved in productive interaction with SLAM (**Fig. 1A and B**). To potentially destroy the H/SLAM interaction, we performed non-conservative substitutions at these positions, thereby generating three single point variants (H-S546R, H-Y547N, H-T548R). Furthermore, a mutant carrying all three mutations was also generated (H-RNR). The fusion promotion ability of those protein variants was then assessed by employing previously described qualitative and quantitative transient cell-cell fusion assays [30,37]. Note that, in contrast to the experiments conducted in Vero-cSLAM cells, where F-wt has been coexpressed with H to induce membrane fusion, we employed a hyperfusogenic F-variants (F-V447T) [30,38] to facilitate the monitoring of cell-cell fusion events in Vero-cN4 cells.

Clearly, the single amino acid mutants H-S546R, H-Y547N, H-T548R promoted similar fusion efficiency to H-wt in both Vero cells expressing canine SLAM or N4, whereas H-R529A exhibited deficiency in both cell types. In sharp contrast, the triple mutant H-RNR was

defective in promoting fusion in a SLAM-dependent manner but retained full functionality in presence of N4 (**Fig. 1C and D**).

We next determined whether the lack of fusion-triggering recorded with selected H-variants correlated with impaired interaction with SLAM, thereby unambiguously highlighting the mode of action. To this aim, we established an enzyme-linked immunosorbent assay (ELISA), which relied on the coating of recombinant soluble H-proteins (wt or mutants) and adding a recombinant soluble SLAM molecule. Both proteins were expressed as covalent dimers: while V-domain (aa 27-140) of SLAM was fused to a human Fc domain (cSLAM.V-Fc), H (aa 154-607) was fused N-terminally to a dimeric GCN motif. In this set of experiments, the single point mutant H-Y539A was also added, since previously reported to exhibit N4-dependent fusion-promotion defects [22,39]. Reduced binding affinity between N4 and a MeV H-protein variant harboring the Y543A substitution (homologous position of the CDV H-Y539A mutation) was reported [40]. Strikingly, in presence of cSLAM.V-Fc, efficient interaction was recorded for H-wt and H-Y539A, but not for H-R529A and H-RNR (**Fig. 2**). Collectively, these data demonstrated that H-RNR specifically lost interaction with SLAM, thereby explaining the SLAM-dependent fusion-triggering deficiency.

We next wished to confirm these results in the background of recombinant viruses. We selected the H-R529A and H-RNR constructs and transferred the genes into a cDNA clone of A75 that additionally expressed the monomeric GFP variant "mNeonGreen" (neon) [37,41]. Because A75 did not efficiently grow both in Vero and Vero-cN4 cells, and the recombinant viruses generated in this study (A75^{neon}-H-R529A and A75^{neon}-H-RNR) were designed to exhibit growth-defects in Vero-cSLAM cells, recombinant viruses were rescued in presence of the transient expression of the VSV G-glycoprotein. Such a system was recently successfully employed to rescue matrix protein-defective recombinant viruses [42,43]. This approach aimed at generating particles entering target cells via the support of VSV G, whereas the ensuing replication, spread and growth phenotypes relied on A75 components, including the various H-protein variants under investigation. VSV G-pseudotyped A75 particles were thus inoculated into Vero, Vero-cN4 and Vero-cSLAM cells and pictures of infected cells (monitored by fluorescence microscopy) were taken 7 days post infection (**Fig. 3**).

As expected, while the wt recombinant A75 virus did grow very efficiently in Vero-cSLAM cells, it replicated in Vero-cN4 cells in a very limited manner (producing only few and small syncytia). As previously documented, infection of Vero cells was strongly defective. Corroborating the data obtained in our transient cell-cell fusion assays, recombinant A75 viruses carrying H-RNR were completely defective in syncytia formation in Vero-cSLAM cells, whereas indications of limited infections (but without large syncytia formation) was observed in case of A75^{neon}-H-R529A. Infectivity in Vero-cN4 was also clearly impaired by A75^{neon}-H-R529A, while A75^{neon}-H-RNR preserved a phenotype very similar to the wt recombinant virus.

In this study, based on a 3D structural model of the CDV H/SLAM interaction, we identified three residues in the H-protein of the A75-CDV strain (residues 546-SYT-548) that, once mutated in a combinatorial manner, generated an H-protein variant (H-RNR) exhibiting a significant defect in interacting with SLAM as well as in SLAM-dependent fusion-triggering. Conversely, the fusion-promotion efficiency triggered by the triple A75 H-variant in Vero-cN4 cells remained unaltered. Collectively, this led us to successfully engineer a selective SLAM-detargeted recombinant A75 virus, since we recorded wt-like spread of A75^{neon}-H-RNR in Vero-cN4 cells but growth impairments in Vero-cSLAM cells. Furthermore, our data confirmed that A75 H-protein harboring the R529A mutation generated a receptor-binding protein variant that was unable to bind to both receptors, thereby translating into a recombinant virus displaying growth-defects in both Vero-cSLAM and Vero-cN4 cells. These results are different from a previous study where six mutations locating in two nearby clusters of H had to be performed (526-9 + 547/8) in order to obtain SLAM "blind" CDV (5804P)-viruses. Interestingly, the strategy employed to "blind" MeV to the human SLAM receptor relied on a single amino acid substitution: R533A (corresponding to the R529A mutation in CDV H). Collectively, those findings indicated potential morbilliviral- and even CDV strain-specific subtle variations of their mode of interaction with the SLAM receptor. Alternatively, mutations at those positions in various morbilliviral H-proteins may have led to differences in protein folding. In the case of A75 H-protein, mutations within the first amino acid cluster (526-529) may prevent the proper folding of a productive "pre-receptor-bound" conformation. Further structural studies are warranted to fully understand such discrepancies.

Noteworthy, the recombinant A75 virus carrying H-R529A nevertheless displayed some residual signs of spread in Vero-cSLAM cells, which indicated some restored functionality in the context of a viral infection. The presence of the fusion protein and/or the matrix protein may have supported a better folding of the H-R529A variant. In turn, this may have translated into the recovery of a limited population of properly folded "pre-receptor-bound" H-protein states. In any cases, in the context of A75, our data clearly demonstrated that a combination of three mutations in the second amino acid cluster of H (546-548) were required to successfully engineer SLAM "blind" recombinant A75 viruses.

The recombinant viruses generated in this study were trans-complemented with the VSV G-glycoprotein. This strategy was employed since (i) A75 did not grow efficiently in Vero-cN4 cells, and (ii) we aimed at the rationale design of SLAM "blind" viruses, thereby precluding the amplification in Vero-cSLAM cells. Although not highly efficient, recombinant viruses could be successfully produced and used to unambiguously demonstrate the selective SLAM-detargeting phenotype of A75^{neon}-H-RNR. Furthermore, this strategy may provide an attractive option to better study A75-spread in glial cells, since, although cell-to-cell transmission is efficient, the initial infection step of canine astrocytes with A75 remained very limited [5,22].

VSV-G-pseudotyped A75 particles may exhibit enhanced initial entry in canine astrocytes, thereby substantially facilitating studies of viral transmission.

In summary, we present in this study the identification of three consecutive residues in the receptor-binding H-protein of the A75-CDV strain that, once mutated in a combined manner, generated selective SLAM "blind" viruses. Recombinant A75 viruses, combined with our VSV G trans-complementation system, provide excellent tools to further investigate the impact of selected H-microdomains in supporting astrocyte-to-astrocyte transmission; a phenotype that may be implicated in CDV-induced brain diseases.

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Figure legend

Figure 1. Residues 546-SYT-548 of A75 H are important SLAM-dependent fusion-triggering. (**A**) Structural model of CDV H-head domain (grey) bound to SLAM (surface representation in red with 50% transparency). (**B**) Co-crystal structure of MeV H-head domain (grey) bound to N4 (surface representation in red with 50% transparency) [33]. Potential residues on the H protein affecting SLAM-dependent fusion-triggering (CDV H R529, S546, Y547, T548; MeV H R533, S550, Y551, F552) are depicted in green, cyan, light blue and dark blue, respectively. Residue on the H protein affecting N4-dependent fusion-triggering (CDV H Y539 and MeV H Y543) is color-coded in red. (**C**) Qualitative assessment of membrane fusion. Fusion was obtained by co-expressing A75 H (wt or mutants) with A75 F. GFP was included to facilitate recording of cell-cell fusion. Pictures were taken 24 hours post-transfection and captured using an inverted fluorescence microscope (EVOS M5000). (**D and E**) Quantitative cell-to-cell fusion assay based on the split nanoluciferase (nLuc) reporter protein system (Promega). Fusion was triggered upon the mixing of two HEK-293T cell populations (effector and target). While effector cells were transfected with plasmids encoding H (wt or derivative mutants), F and one part of nLuc, target cells were transfected with plasmids encoding a receptor (cSLAM or cN4) and the second part of nLuc. Upon addition of the nLuc substrate, light emission was recorded 24 hours post-transfection using a multiplate reader (Cytation 5 device, BioTek). Note that for the both fusion assays performed in presence of cN4, a hyperfusogenic F-variant was employed [30,38]. The values show the means ± SD from three independent experiments performed in duplicates. Dunnett's multiple-comparison test was applied

after one-way analysis of variance (one-way ANOVA) (* $p < 0.05$; *** $p < 0.001$; ns, non-significant).

Figure 2. **The triple mutants A75 H-RNR lost interaction with SLAM.** Serial dilutions of soluble cSLAM.V-Fc were added on plastic wells coated with recombinant soluble H proteins (wt or derivative mutants). To reveal the SLAM binding activity, goat anti-human IgG antibody coupled with HRP (AP113P, Merck) was added after washing, followed by adding Ultra TMB-ELISA (Thermo Fisher Scientific) and measuring the optical density (OD) at 450nm (Cytation 5 microplate reader).

Figure 3. **Generation of a selective SLAM "blind" A75 recombinant virus.** Infection profiles mediated by G-complemented recombinant A75 viruses in Vero cells expressing (or not) cSLAM or cN4. Seven days post-infection, multiple images of (fluorescent) infected cells per well were captured by inverted fluorescence microscopy (Cytation 5 microplate reader) and stitched together (using Gen5.10 software).

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Figure 1

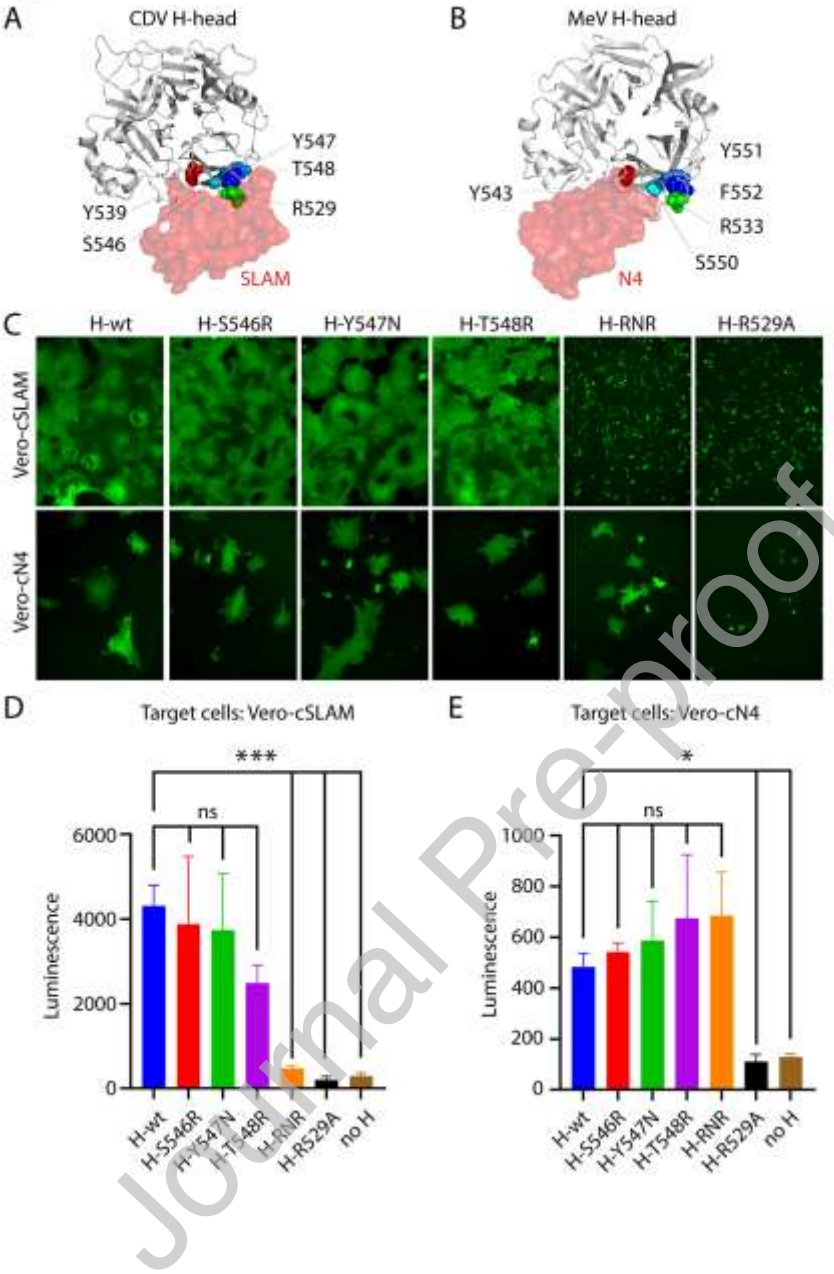


Figure 2

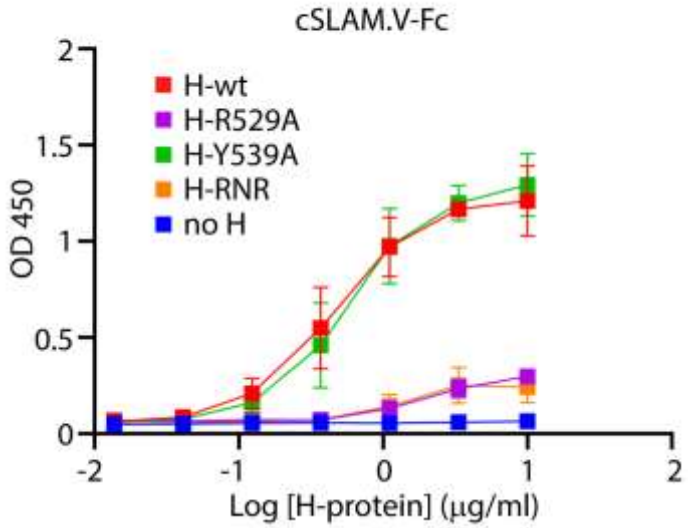
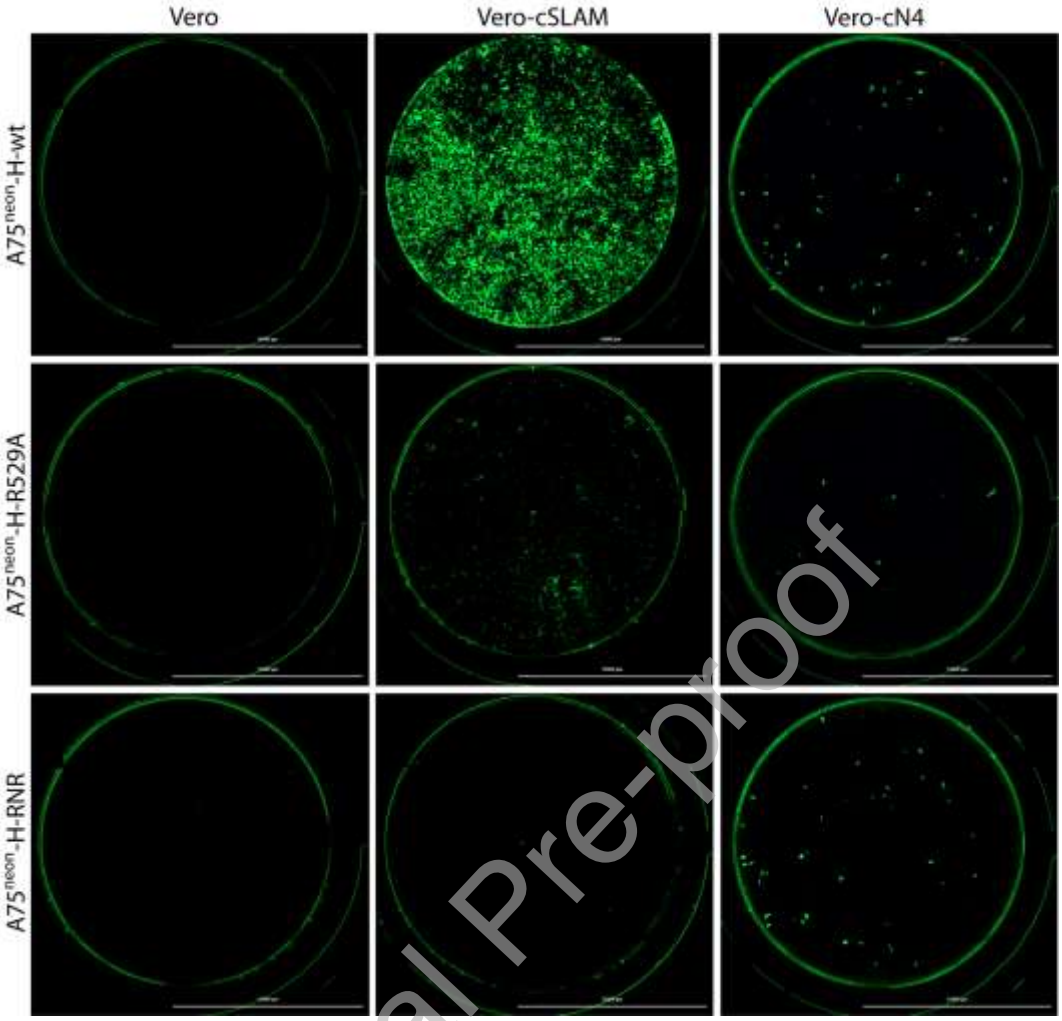


Figure 3



Author contributions

Vaiva Gradauskaite: Conceptualization, Methodology, Data curation, Writing—review & editing, **Mojtaba Khosravi:** Investigation, Methodology, Writing—review & editing, **Philippe Plattet:** Conceptualization; Supervision; Funding acquisition; Investigation; Visualization; Writing—original draft; Project administration; Writing—review & editing

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: