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## AmiA and AliA peptide ligands, found in *Klebsiella pneumoniae*, are imported into pneumococci and alter the transcriptome

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*Klebsiella pneumoniae* releases the peptides AKTIKITQTR and FNEMQPIVDRQ, which bind the pneumococcal proteins AmiA and AliA respectively, two substrate-binding proteins of the ABC transporter Ami-AliA/AlIB oligopeptide permease. Exposure to these peptides alters pneumococcal phenotypes such as growth. Using a mutant in which a permease domain of the transporter was disrupted, by growth analysis and epifluorescence microscopy, we confirmed peptide uptake via the Ami permease and intracellular location in the pneumococcus. By RNA-sequencing we found that the peptides modulated expression of genes involved in metabolism, as pathways affected were mostly associated with energy or synthesis and transport of amino acids. Both peptides downregulated expression of genes involved in branched-chain amino acid metabolism and the Ami permease; and upregulated fatty acid biosynthesis genes but differed in their regulation of genes involved in purine and pyrimidine biosynthesis. The transcriptomic changes are consistent with growth suppression by peptide treatment. The peptides inhibited growth of pneumococcal isolates of serotypes 3, 8, 9N, 12F and 19A, currently prevalent in Switzerland, and caused no detectable toxic effect to primary human airway epithelial cells. We conclude that pneumococci take up *K. pneumoniae* peptides from the environment via binding and transport through the Ami permease. This changes gene expression resulting in altered phenotypes, particularly reduced growth.

*Streptococcus pneumoniae* (pneumococcus) is a Gram positive bacterium listed amongst the priority pathogens by the World Health Organization in 2017<sup>1</sup>. It is the most common cause of bacterial community-acquired pneumonia globally and causes meningitis and septicaemia with significant morbidity and mortality particularly in young children, the elderly and the immunocompromised<sup>2</sup>. Current vaccines only protect from a fraction of pneumococcal serotypes and not from non-vaccine serotypes or nonencapsulated pneumococci with antibiotic resistance becoming a growing concern<sup>3</sup>.

It has been proposed that *S. pneumoniae* senses the environment via its ABC transporter Ami-AliA/AlIB permease by uptake of oligopeptides, modulating gene expression<sup>4</sup> and nasopharyngeal colonization<sup>5</sup>. By expressing and purifying the transporter's oligopeptide binding proteins AmiA and AliA as recombinant proteins, we identified the peptides AKTIKITQTR and FNEMQPIVDRQ as their respective ligands from nasal wash of children<sup>6</sup>. The peptide sequences matched ribosomal proteins of *Klebsiella pneumoniae* and were found in the secretome of *K. pneumoniae*<sup>7</sup>. The peptides altered pneumococcal phenotypes as they reduced pneumococcal growth, capsule size and transformation rate<sup>8</sup>. Mutation of the genes encoding the oligopeptide binding proteins

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in strain D39 abrogated the peptide effect on pneumococcal growth<sup>7,8</sup>, therefore peptide interaction with the oligopeptide binding proteins of the Ami permease is essential for the effect. This is in line with the identification of pneumococcal strains which have mutations in the oligopeptide-binding proteins of the Ami permease and whose growth is not inhibited by the peptides, such as the previously-identified R6 and ATCC 700903<sup>7</sup>.

Here, we aimed to determine whether uptake of the AmiA and AliA peptides via the Ami-AliA/AlkB permease is necessary for their effect and to find clues about their mode of action by exploring changes at the transcriptional level. By using fluorescently labelled peptides, we show that the peptides are taken up into the pneumococci and this uptake was necessary to suppress bacterial growth. Both peptides altered the pneumococcal transcriptome with similar but distinct effects.

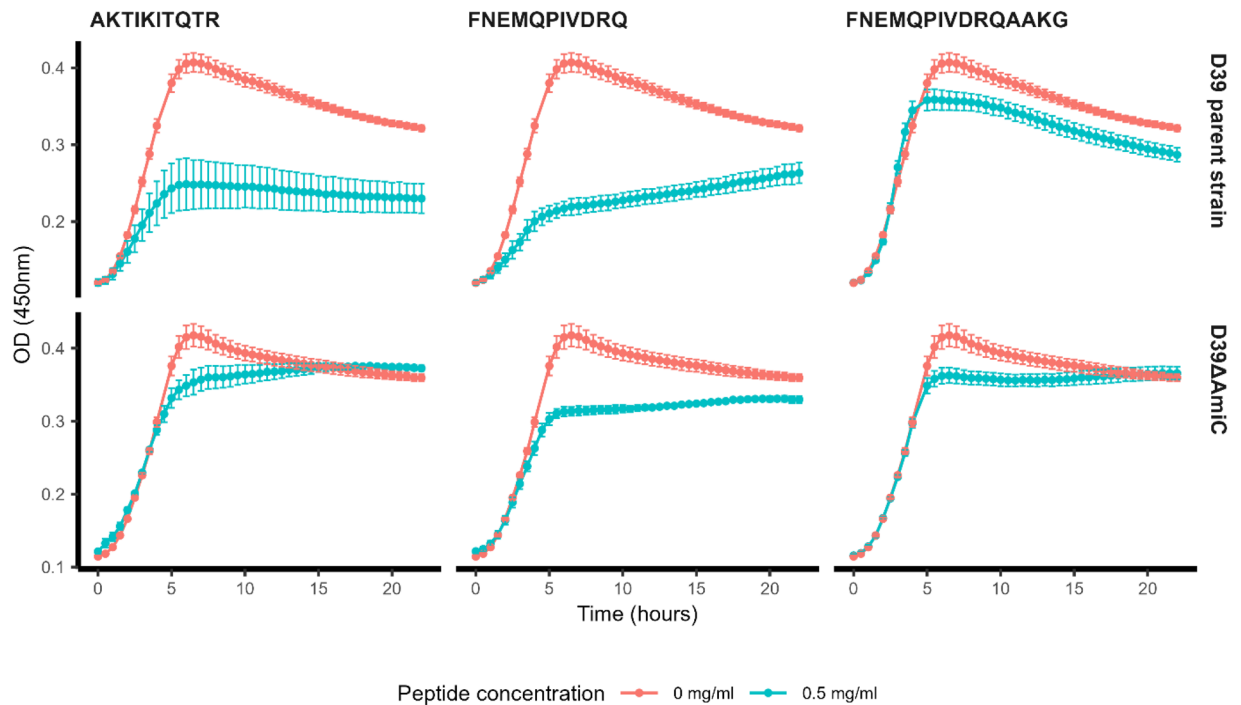
## Results

### Pneumococcal growth inhibition by AmiA and AliA peptide ligands requires uptake via Ami-AliA/AlkB permease

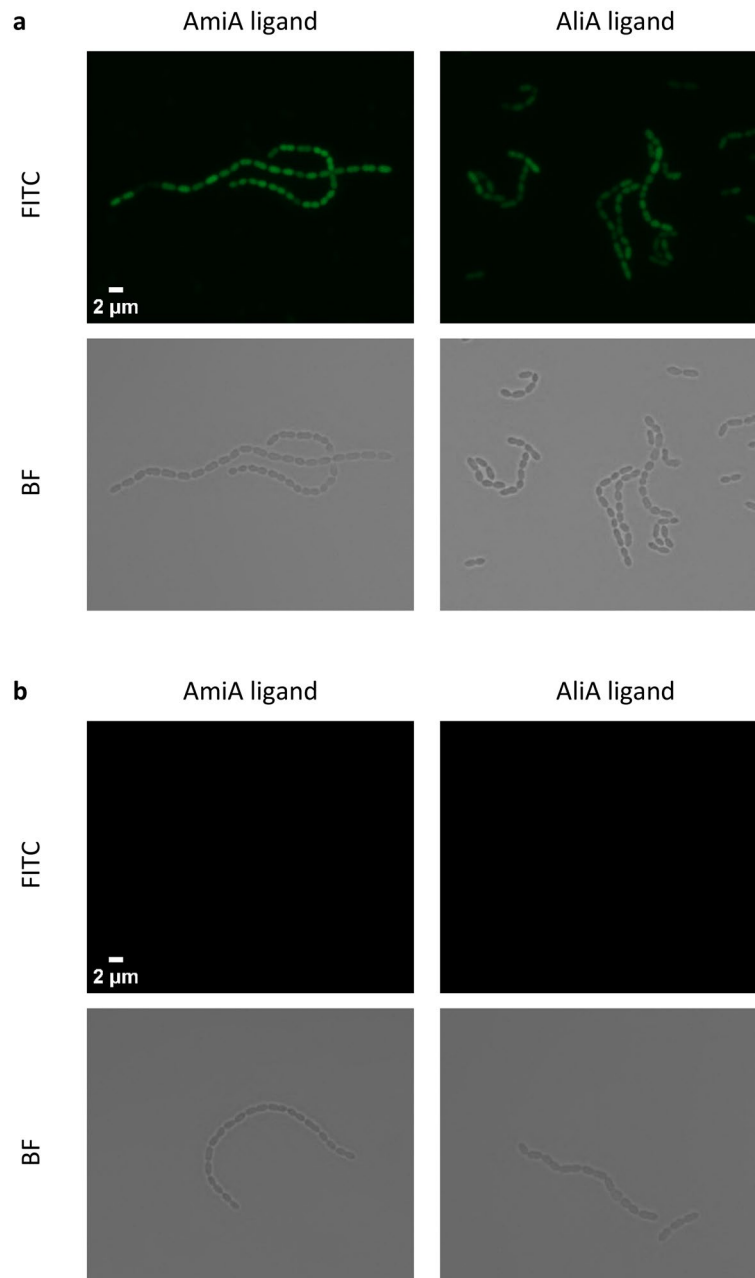
As the role of the Ami permease is to import oligopeptides, we hypothesized that uptake may be necessary for response to the peptides. We confirmed this using a mutant in which one of the two permease subunits, AmiC, was disrupted. The AmiA and AliA peptide ligands inhibited the *S. pneumoniae* D39 parent strain, but growth suppression was lost or greatly reduced in the  $\Delta$ amiC mutant (Fig. 1). A nonspecific ligand control had little effect on growth of either parent strain or its AmiC mutant (Fig. 1). The AmiC mutant had no growth deficiency compared to its parent strain in the absence of peptide (Supplementary Fig. S1). To confirm peptide uptake by *S. pneumoniae* and that this requires a functional Ami-AliA/AlkB oligopeptide permease, we incubated the D39 parent strain and  $\Delta$ amiC mutant for 5 min with FITC-labelled peptides and performed epifluorescence microscopy. In the D39 strain with a functional Ami permease, but not in the  $\Delta$ amiC mutant, we found FITC staining intracellularly (Fig. 2) confirming peptide uptake. In the orthogonal view of a z-stack, the FITC peptide staining was inside the bacterial cell and not on the membrane (Fig. 3). We did not find FITC staining intracellularly in a D39  $\Delta$ amiA mutant strain (Supplementary Fig. S2). Thus, peptide uptake via a functional Ami permease is required for pneumococcal growth suppression by the AmiA and AliA peptide ligands.

### AmiA and AliA peptide ligands affect pneumococcal transcriptome

To identify the underlying mechanisms by which the AmiA and AliA peptide ligands caused phenotypic changes such as growth inhibition and decreased capsule size and transformation rate<sup>8</sup>, we identified differentially expressed genes in *S. pneumoniae* strain D39 treated with AmiA or AliA peptide ligands compared to the untreated control. AmiA ligand caused more dramatic gene expression changes than AliA ligand as shown

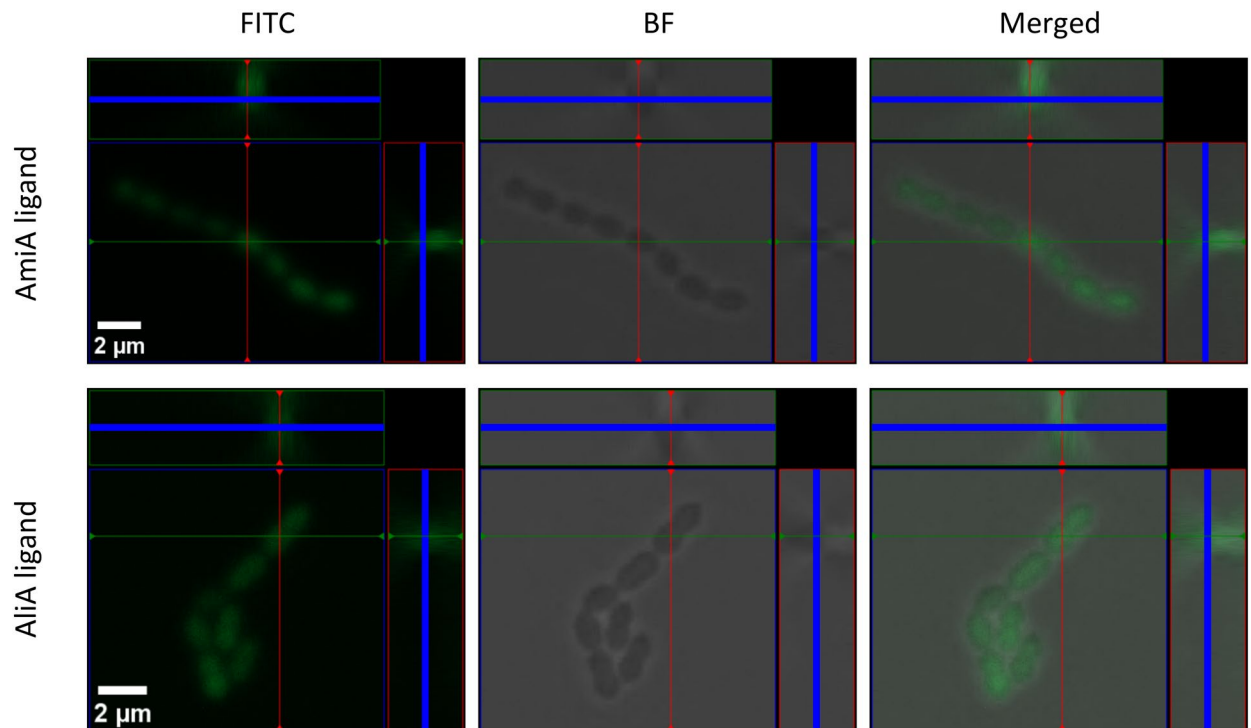


**Figure 1.** Growth suppression requires uptake of AmiA and AliA ligands via the Ami permease. The *S. pneumoniae* D39 parent strain was inhibited by AmiA ligand (AKTIKITQTR) and AliA ligand (FNEMQPIVDRQ), but inhibition was greatly reduced in the  $\Delta$ amiC mutant. This indicates that the peptides are taken up via the Ami permease to act. Nonspecific ligand control, FNEMQPIVDRQAAGK, had little effect on growth of D39 parent strain or the  $\Delta$ amiC mutant. Growth curves were performed in peptide-free chemically defined medium (CDM) by measuring optical density OD (450 nm) over time in absence or presence of peptide (0.5 mg/ml). Results represent 3 independent experiments, error bars indicate SEM.



**Figure 2.** AmiA and AliA peptide ligands are taken up via Ami permease. Peptides located inside of *S. pneumoniae* D39 parent strain (a), but not  $\Delta amiC$  mutant (b). Representative images of pneumococcal cells after incubation with FITC-labelled AmiA and AliA peptide ligands taken at mid-bacterium localization in z position showing FITC and Brightfield (BF) channel. Scale bar indicates 2  $\mu\text{m}$  for all pictures in a and b.

in the heatmap (Fig. 4). There, we clustered the differentially expressed genes and identified biological process pathway enrichment within the clusters. Both peptides strongly downregulated genes in cluster 1, which is enriched for peptide and protein transport, branched-chain amino acid (BCAA) biosynthesis and amino acid biosynthesis. Cluster 2, enriched for aromatic amino acid biosynthesis, tryptophan biosynthesis, glycolysis, amino acid biosynthesis and stress response, was downregulated by both peptides, with stronger  $\log_2\text{FC}$  ( $\log_2$  fold change) values for the AmiA ligand. Cluster 3 was not enriched for any biological process, but for the molecular function ribonucleoprotein, ribosomal protein, rRNA binding, RNA binding and upregulated by the AmiA ligand, but downregulated by the AliA ligand. Cluster 4, enriched for cell wall biogenesis/degradation, cell shape, DNA repair, DNA damage, rRNA processing and protein biosynthesis, contained overall more downregulated genes and did not show the same pattern for both peptide treatments. Cluster 5, enriched for transport, sugar transport and phosphotransferase system, included more upregulated genes for both peptide



**Figure 3.** AmiA and AliA peptide ligands locate inside pneumococcal cells of strain D39. Representative images of *S. pneumoniae* strain D39 parental strain after 5 min incubation with FITC-labelled AmiA and AliA peptide ligands. Scale bar indicates 2  $\mu\text{m}$  for all pictures treated with the same peptide. Orthogonal view from z stack shows colocalization of peptide in FITC channel with cytosol of bacterium in Brightfield (BF) channel.

treatments. Cluster 6, enriched for purine and pyrimidine biosynthesis, was strongly upregulated by the AmiA ligand, but not by the AliA ligand.

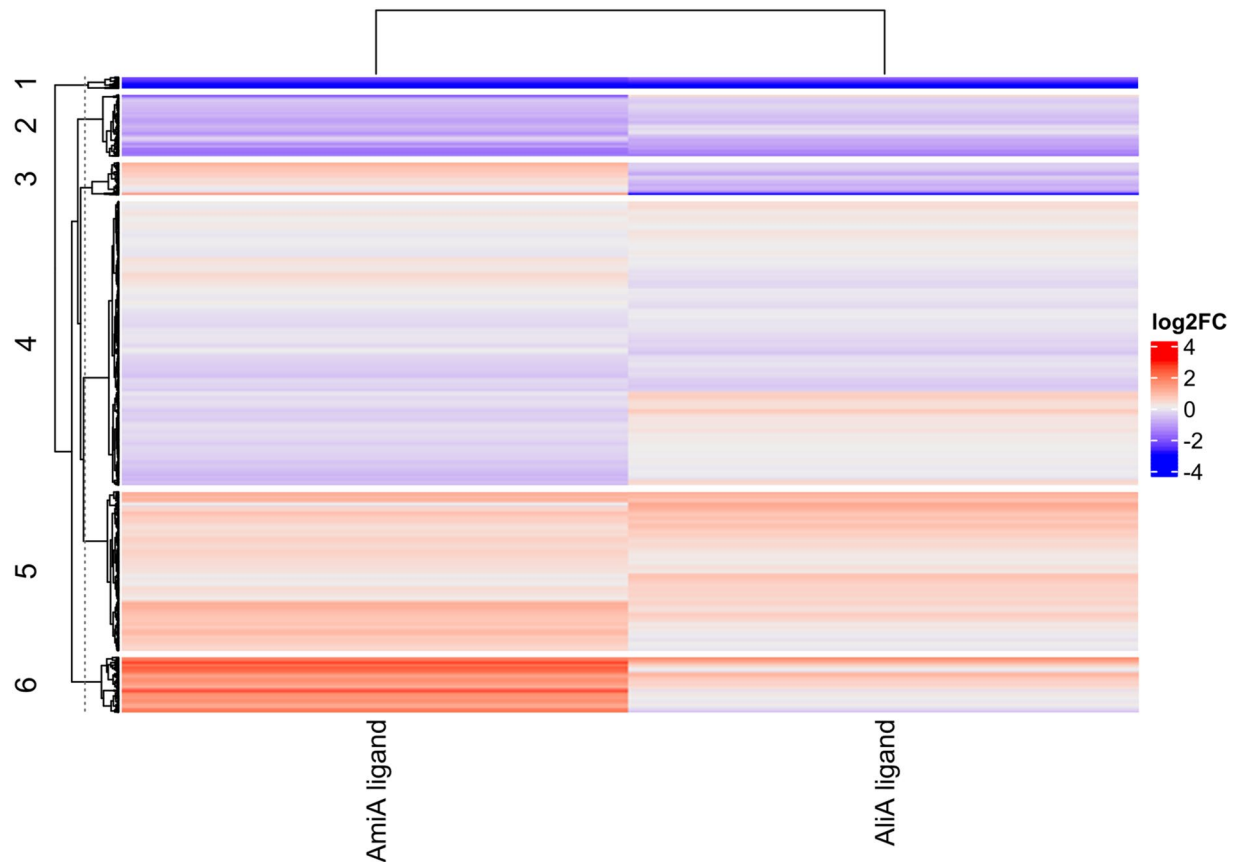
To better understand which biological processes are represented by the top differentially expressed genes by each peptide treatment, we identified enrichment in the datasets of significantly ( $p$  value  $< 0.05$ ) downregulated ( $\log_2\text{FC} \leq -1.25$ ) genes and separately significantly upregulated ( $\log_2\text{FC} \geq 1.25$ ) genes (Supplementary Fig. S3). Among the top upregulated genes by both peptides were those involved in the fatty acid biosynthesis process and malonyl-CoA biosynthesis. Expression of genes was increased in additional processes by AmiA ligand, including inosine monophosphate (IMP), uridine monophosphate (UMP), purine and pyrimidine biosynthesis. The process enrichment of the top downregulated genes reflects findings from the heatmap clusters including amino acid synthesis processes for isoleucine and valine, which are both BCAAs, enriched in the dataset of both peptide treatments. Furthermore, threonine biosynthesis, peptide and protein transport processes were enriched in the top downregulated genes by both peptide treatments.

We made a gene interaction network with the top differentially expressed genes, for AmiA ligand in Fig. 5 ( $-2 \geq \log_2\text{FC} \geq 2$ ) and for AliA ligand in Fig. 6 ( $-1.25 \geq \log_2\text{FC} \geq 1.25$ ). Among genes downregulated by both peptides we see the following clusters: Ami permease (*ami* genes and *alia*), BCAA biosynthesis (*ilv* genes) and BCAA transporter (*liv* genes), by AmiA ligand in addition phenylalanine, tyrosine, tryptophan biosynthesis (*trp* genes) and by AliA ligand riboflavin biosynthesis (*rib* genes). Among genes upregulated by AmiA ligand we see ribonucleoside monophosphate biosynthesis (*pur* and *pyr* genes) and by AliA ligand fatty acid biosynthesis (*fab* genes). We confirmed significant downregulation of *alia*, *ilvB* and *livJ* and significant upregulation of *fabK* by real-time RT-PCR for both peptide treatments (Supplementary Fig. S4).

On the single gene level, the top downregulated gene was SPD\_0161 (NRAMP family Mn<sup>2+</sup>/Fe<sup>2+</sup> transporter) by both peptides with  $< -5 \log_2\text{FC}$ . The top upregulated gene was SPD\_0553 (hypothetical protein) by AmiA ligand (3.76  $\log_2\text{FC}$ ) and SPD\_0684 (bioY, Substrate-specific component BioY of biotin ECF transporter) by AliA ligand (1.88  $\log_2\text{FC}$ ). We did not find any *com* genes differentially expressed, but found several single genes significantly downregulated by both peptides with  $\log_2\text{FC}$  values in Supplementary Table S1. In summary, the AmiA and AliA peptide ligands altered the pneumococcal transcriptome, affecting transcriptional regulators and downregulating genes for amino acid and protein metabolism.

### AmiA and AliA peptide ligands inhibited growth of clinical isolates of currently prevalent serotypes of *S. pneumoniae*

We previously found that AmiA and AliA peptides inhibited growth of genetically diverse pneumococcal strains of serotypes 1, 2, 6B, 7F, 18C, 19A and non-typeable<sup>7,8</sup>. As the AmiA and AliA peptide ligands are of potential interest as therapeutics to treat pneumococcal diseases, we tested their effect on growth of Swiss clinical pneumococcal isolates from 2019 of the most prevalent serotypes causing invasive disease in Switzerland<sup>9</sup>: 3, 8, 9N, 12F and 19A. These serotypes were also prevalent in pneumococcal infections, invasive pneumococcal disease



**Figure 4.** AmiA and AliA peptide ligands cause differential gene expression in *S. pneumoniae* strain D39. AmiA and AliA peptide ligands caused similar changes (upregulation or downregulation) in clusters 1,2,5 and 6, whereas AmiA peptide ligand upregulated and AliA peptide ligand downregulated more genes in cluster 3. Patterns are also different in regions of cluster 4.

or amongst the most virulent serotypes in South Africa and China<sup>10,11</sup>. Both peptides reduced pneumococcal growth of all isolates tested (Fig. 7). In summary, the AmiA and AliA peptide ligands reduced growth of clinical pneumococcal isolates of serotypes 3, 8, 9N, 12F and 19A.

#### No toxic effect of AmiA and AliA peptide ligands to human airway epithelial cells

Well-differentiated airway epithelial cell (AEC) cultures are organotypic cultures with air-liquid interface that mimic several aspects of the respiratory tract, including mucus production, airway cell heterogeneity and ciliary beating activity<sup>12</sup>. We evaluated whether the peptides are cytotoxic to primary human AEC (hAEC) cultures by incubating them with the peptides for 30 h, collecting medium from the apical and basolateral sides and determining the presence of LDH. LDH release was similar and low in media collected from hAEC cultures treated with or without peptides indicating no cytotoxicity (Supplementary Fig. S5a). Immunofluorescence staining showed the same cell morphology with ciliated cells and tight junctions present in all samples (Supplementary Fig. S5b). To summarize, we did not detect cytotoxic effects of the AmiA or AliA peptide ligands *in vitro* to hAEC cultures.

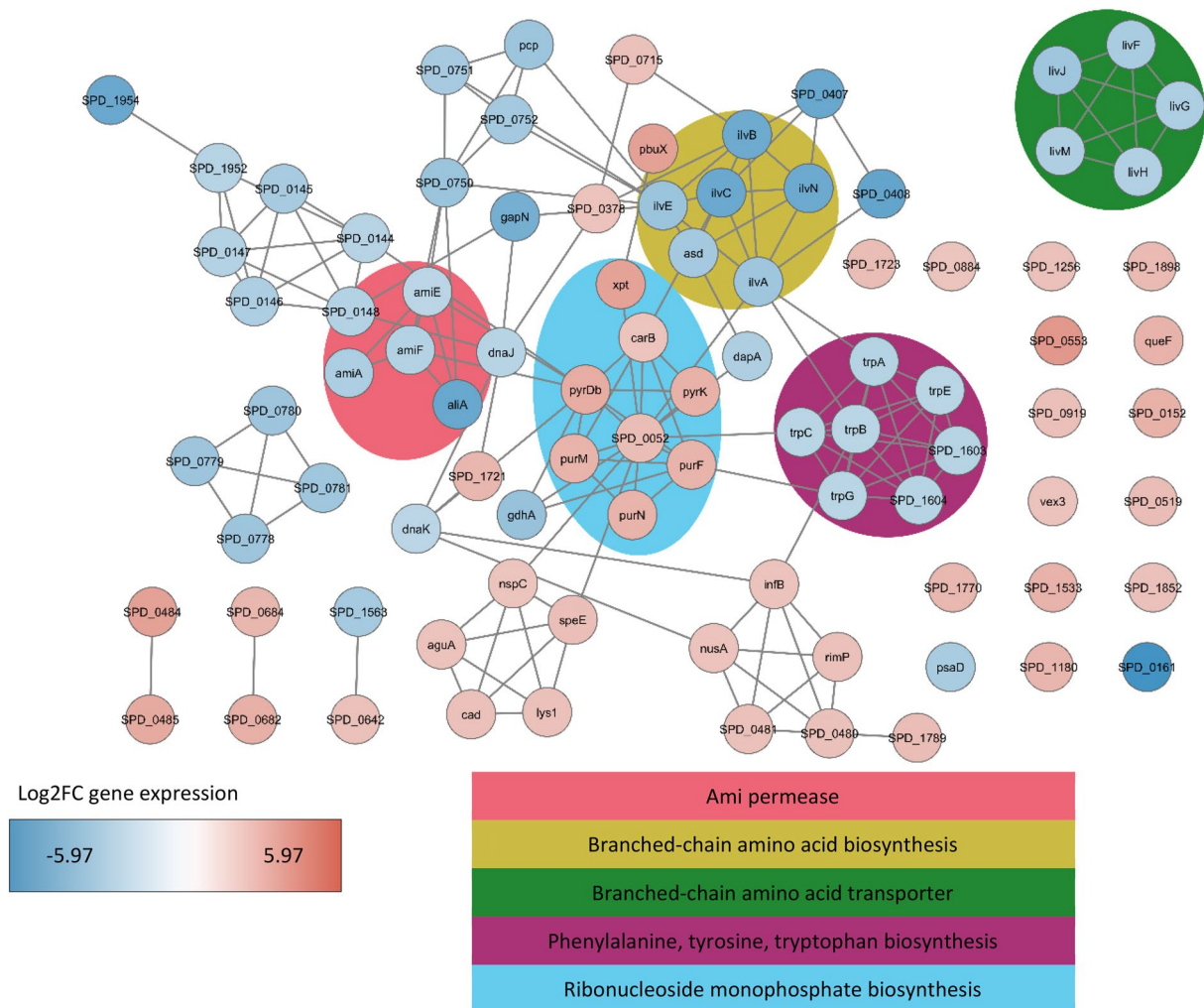
#### Discussion

We found that the suppression of pneumococcal growth by the AmiA and AliA peptide ligands was dependent on uptake via the Ami permease, which altered the pneumococcal transcriptome. The peptides inhibited growth of pneumococcal isolates of currently-circulating serotypes not previously tested and were not cytotoxic to hAEC cultures.

We can associate growth inhibition and other previously observed phenotypic changes with alterations in the pneumococcal transcriptome caused by the AmiA or AliA peptide ligands. The genes most downregulated by both peptides were associated with energy or synthesis and transport of branched-chain amino acids (BCAAs).

BCAAs indicate the general nutritional status of bacterial cells and are an important constituent of the amino acid pool in bacterial proteins<sup>13</sup>. A feature of pneumococcal adaptation to nasopharyngeal colonisation is efficient biosynthesis, liberation or acquisition of BCAAs<sup>14</sup>. The BCAA transporter encoded by *liv* genes is necessary for disease pathogenesis as deletion led to reduced virulence *in vivo*<sup>15</sup>. The *ilv* operon encodes enzymes which condense threonine and pyruvate or two pyruvates into branched-chain keto acids, precursors of BCAAs<sup>16</sup>. Ablation of *ilvC* has been demonstrated to diminish BCAA concentration and impair growth, potentially by depriving the

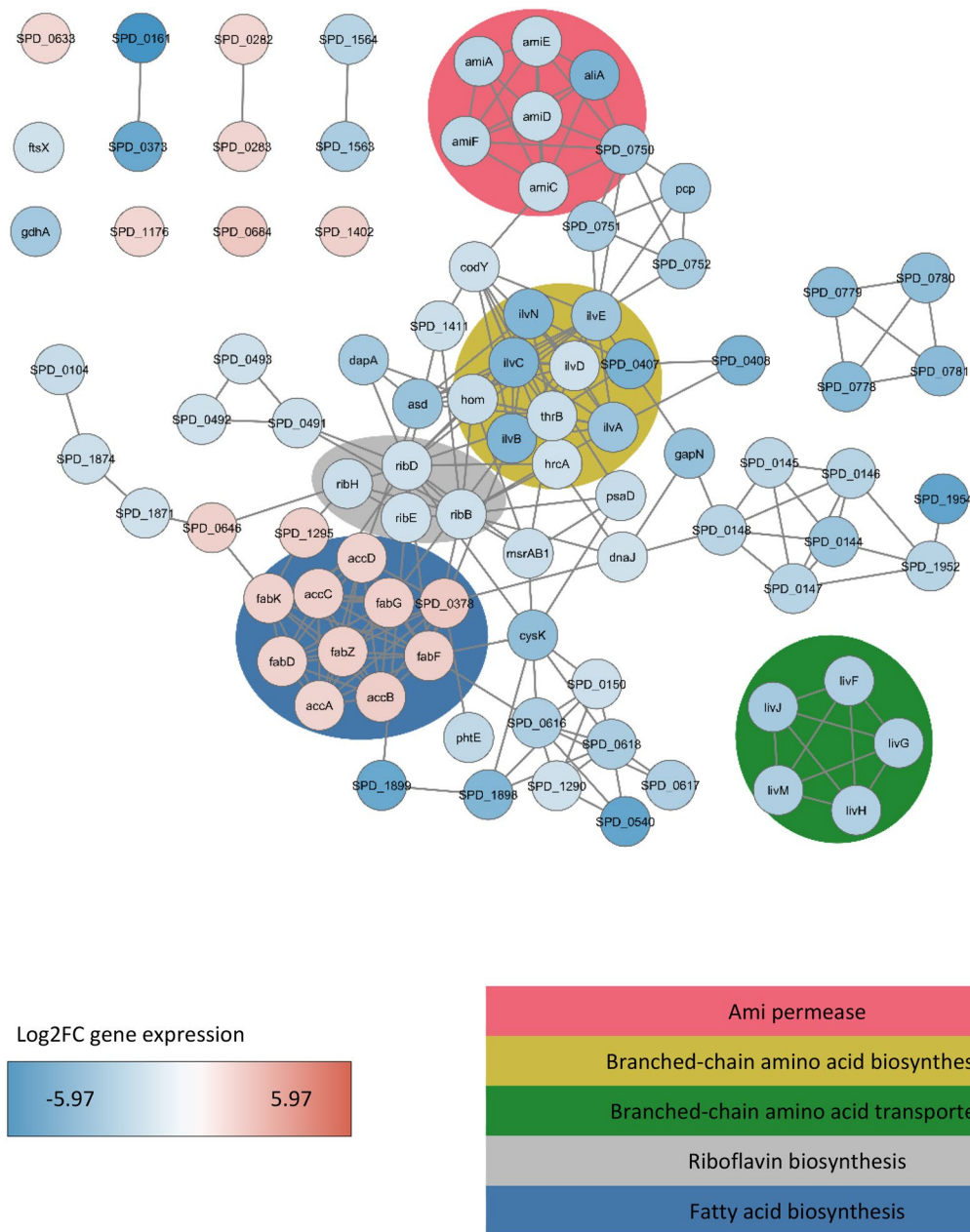




**Figure 5.** Top downregulated and upregulated genes by AmiA peptide ligand. Gene association network of significantly ( $p$  value  $< 0.05$ ) downregulated ( $\log_2FC \leq -2$ ) and upregulated ( $\log_2FC \geq 2$ ) genes in *S. pneumoniae* strain D39 after 15 min of incubation with AmiA peptide ligand. Lines (edges) represent interactions between 2 genes (nodes) and were imported from the STRING database. Nodes are colour coded according to  $\log_2FC$  expression change: blue represents negative  $\log_2FC$  (downregulated) and red positive  $\log_2FC$  (upregulated). Gene Ontologies or keywords of groups of genes are highlighted in the background.

bacteria of nutrients in the stationary phase<sup>13</sup>. Both the AmiA and AliA peptide ligands downregulated *liv* and *ilv* genes. Designed antimicrobial peptide DM3 and penicillin also altered expression of genes in pneumococcal amino acid biosynthesis processes, most strongly affecting branched chain family amino acids<sup>17</sup>. Reduced ability to synthesise or acquire BCAAs might lead to the growth inhibiting effect of the AmiA and AliA peptides.

In *Staphylococcus aureus*, BCAA metabolism controls the phospholipid structure as BCAAs, mainly isoleucine, are converted to precursor molecules used to initiate fatty acid synthesis<sup>18</sup>. Altering the membrane fatty acid composition to modify membrane fluidity can be used in response to different environments. In *S. pneumoniae* fatty acid biosynthesis is regulated by the type II fatty acid biosynthesis cluster at a single genomic location and the transcriptional factor FabT<sup>19</sup>. The proteins FabK, FabG and FabF, which are encoded in the *fabKDGf-accB-fabZ-accCDA* operon, participate in enzymatic reactions of fatty acid biosynthesis by adding malonyl-coenzyme-A molecules to the growing hydrocarbon chain<sup>20</sup>. Alterations leading to changes in membrane fluidity have been associated with resistance mechanisms to intracellular oxidative stress<sup>21</sup>, importance for cell division site selection<sup>22</sup> and FabT with indirect regulation of capsular polysaccharide<sup>23</sup>. It has been hypothesized that *S. pneumoniae* has the ability to alter membrane homeostasis in response to the production of quorum-sensing peptides, such as competence-associated peptide BriC which altered fatty acid biosynthesis<sup>24</sup>. It is interesting to note that penicillin treatment downregulated *fab* and *acc* genes<sup>25</sup>. AmiA and AliA peptide treatment upregulated *fab* and *acc* genes, which might alter fatty acid composition and be an adaptation mechanism of the pneumococcus to the peptide treatment in preparation for a changing environment. The above-mentioned indirect connections to cell division site selection and capsular polysaccharide may be relevant, as the peptides reduced pneumococcal growth and capsule size<sup>8</sup>. FtsE and FtsX are essential cell division proteins which interact with and control the

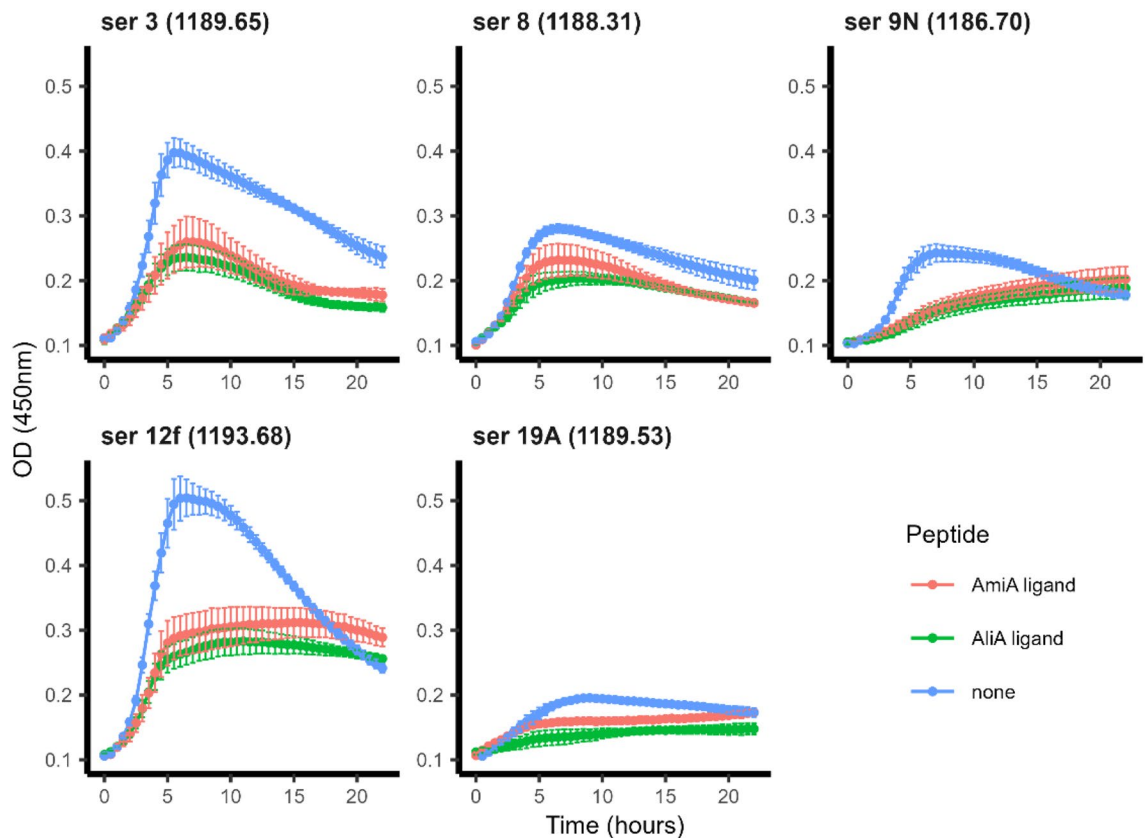


**Figure 6.** Top downregulated and upregulated genes by AliA peptide ligand. Gene association network of significantly ( $p$  value  $< 0.05$ ) downregulated ( $\log_2FC \leq -1.25$ ) and upregulated ( $\log_2FC \geq 1.25$ ) genes in *S. pneumoniae* strain D39 after 15 min of incubation with AliA peptide ligand. Lines (edges) represent interactions between 2 genes (nodes) and were imported from the STRING database. Nodes are colour coded according to  $\log_2FC$  expression change: blue represents negative  $\log_2FC$  (downregulated) and red positive  $\log_2FC$  (upregulated). Gene Ontologies or keywords of groups of genes are highlighted in the background.

peptidoglycan hydrolyse PcsB which separates daughter cells<sup>26,27</sup>. Their downregulation by the peptides might be a consequence of reduced growth which would require less expression of the daughter cell splitting machinery.

*S. pneumoniae* is naturally competent, which contributes to its genomic plasticity and can facilitate antigenic variation, vaccine escape and the acquisition of antibiotic resistance<sup>28</sup>. We cannot explain the reduction of transformation efficiency by AmiA and AliA peptide treatment with differential expression of *com* genes, which are involved in regulation and development of competence for genetic transformation<sup>29</sup>. Nevertheless, we found downregulation of other genes, namely *hrcA*, *grpE*, *dnaK* and *dnaJ*, which have been associated with regulating competence<sup>30,31</sup>. HrcA (heat-inducible transcription repressor), a key regulator of pneumococcal physiology, might repress the induction of competence in intracellular stress conditions and regulates *grpE* encoding a heat shock protein, *dnaK* and *dnaJ*, which both encode chaperone proteins<sup>31</sup>.

The *codY* gene, encoding a master nutritional regulator involved in amino acid metabolism<sup>32</sup>, was downregulated by both peptides. Among the genes regulated by CodY, we found downregulation of genes that encode the



**Figure 7.** Growth curves of Swiss clinical isolates of *S. pneumoniae* in presence and absence of AmiA and AliA peptide ligands. Effect of peptides on growth was tested on isolates of different serotype (ser) with isolate number in brackets: 3 (1189.65), 8 (1188.31), 9N (1186.70), 12F (1193.68), 19A (1189.53). Growth curves were performed in peptide-free chemically defined medium (CDM) by measuring optical density OD (450 nm) over time in absence or presence of AmiA or AliA peptide ligands (0.5 mg/ml). Results represent 3 independent experiments, error bars indicate SEM.

Ami permease, BCAA transporter (*liv* operon), *ilv* operon, *pcp*, *gdhA*, *asd*, *dapA* and *gapN*<sup>32</sup>. CodY has been hypothesized to sense peptide uptake through Ami and to be able to repress competence<sup>33</sup>. In *Streptococcus suis*, a mutation in *codY* decreased capsule thickness due to altered gene expression in sialic acid synthesis, leading to decreased sialic acid content in capsule<sup>34</sup>. Phenotypic changes related to CodY are interesting, as the AmiA and AliA peptide ligands decreased transformation efficiency and capsule size<sup>8</sup>.

In pneumococcus the translation inhibitors erythromycin, chloramphenicol, puromycin and tetracycline caused increased transcription of genes in purine biosynthesis, but antibiotics that target other cellular processes did not<sup>35</sup>. Furthermore, in response to these translation inhibitors, ribosomal proteins (*rpl* and *rps* genes) were upregulated and amino acid biosynthesis downregulated (including *ilvN*, *ilvC*, *ilvD*, *ilvE*, *trpA*, *trpC*, *trpD*, *trpG*). This reflects part of the observations of AmiA peptide ligand treatment, indicating that it might affect translation.

The effects of AmiA and AliA peptide ligands on the pneumococcal transcriptome had many similarities, but the regulation of genes involved in purine and pyrimidine biosynthesis and genes encoding ribosomal proteins was distinct for the AmiA peptide ligand. We hypothesize that the difference occurs intracellularly rather than depending on which substrate binding protein the peptides bind, since in D39 both peptides bind the AmiA protein<sup>7</sup>.

In conclusion, we found that the AmiA and AliA peptide ligands are taken up via the Ami-AliA/AlkB permease into the pneumococcal cells. Together with our previous studies, we hypothesize that the peptides cause pneumococcal growth inhibition and phenotypic changes by modulating expression of genes involved in metabolism, as the processes affected were mostly associated with energy or synthesis and transport of amino acids. The peptides inhibited growth of clinical pneumococcal isolates of currently prevalent serotypes (3, 8, 9N, 12F and 19A) and we did not detect cytotoxic effects on human cells. AmiA and AliA peptide ligands may be useful tools to manipulate the transcriptome and control pneumococcal growth.

## Methods

### Bacterial strains and culture conditions

Bacterial strains, culture conditions and media were as previously<sup>7</sup>. Carlo Casanova kindly provided Swiss clinical *S. pneumoniae* strains. Daniel Straume kindly provided the D39 parent strain (ds865, D39 ( $\Delta comAB::erm$ )) and



its mutant  $\Delta amiC$  (ds1039, D39,  $\Delta amiC::Janus$ ). Peter W. M. Hermans kindly provided D39 parent strain and its D39  $\Delta amiA$  mutant, with construction of mutants described previously<sup>5,36</sup>.

### Peptides

Synthetic peptides (Genscript, Thermo Fisher Scientific) were ordered with  $\geq 95$  % purity. Peptides for fluorescent microscopy were labelled at the C-terminus with Lys (FITC).

### Growth assay

Growth assays were done as described previously<sup>7</sup> and plotted in R, error bars indicate SEM of 3 independent biological replicates.

### Protein sequence alignment

Protein sequence alignment was done as described previously<sup>7</sup>.

### Epifluorescence microscopy

After overnight culture on agar plates, we grew *S. pneumoniae* D39 parent strains, D39 $\Delta amiA$  and D39 $\Delta amiC$  in BHI to  $OD_{600nm} = 0.1$  to  $0.2$ . Next, we centrifuged 1 ml culture for 1 min at 10,000 g and resuspended the pellet in 100  $\mu$ l BHI and 50  $\mu$ l of 1 mg/ml fluorescently labelled peptide. After incubating for 5 min, we washed the bacteria three times with 1 ml PBS and resuspended the pellet in 40  $\mu$ l of PBS. Then, we spotted 1.5  $\mu$ l onto an acrylamide pad inside a gene-frame on a microscope slide, which we prepared as described in the supplementary methods. We performed microscopy using a Zeiss Axio Imager M1 fluorescence microscope with a 100 $\times$  oil immersion objective (EC Plan-Neufluor 100x/1.30 Oil M27), filter with 493 nm excitation and 517 nm emission wavelength for the FITC channel and photographed images with a Zeiss AxioCam 712 mono camera. We edited pictures with the same settings for all samples to maintain comparability between samples and intensity of fluorescence using the Software Zeiss ZEN 3.8.: first, we applied denoising to the brightfield channel picture setting real wavelets to 1.0, then we adjusted the histogram upper white limit in the FITC channel to 5,000.

### Gene expression analysis by RNA-Seq

After overnight culture on agar plates, we grew *S. pneumoniae* strain D39 in BHI + 5 % FCS to  $OD_{600nm} = 0.5$  and sub-cultured 100  $\mu$ l of overnight culture in 10 ml of fresh CDM until  $OD_{600nm} = 0.2$ . We split the culture into two 5 ml cultures and added 0.5 mg/ml peptide to one sample. After 15 min incubation at 37 °C, we stopped transcription with RNprotect bacteria reagent (Qiagen) and isolated total RNA with the RNeasy kit (Qiagen) following the manufacturer's instructions. We assessed RNA concentration and integrity with Nanodrop (Thermo Scientific) and Agilent Biol analyzer (Agilent) and submitted 3 biological replicates of each sample to the Next Generation Sequencing Platform (University of Bern). There, quality control assessment, rRNA depletion, generation of libraries and sequencing were carried out as described in the Supplementary Methods.

Lexogen (Vienna, Austria) performed quality control, read processing and differential gene expression analysis up to the log fold change table as follows: quality control (fastqc), UMI extraction (umi\_tools extract), trimming (cutadapt), STAR alignment to the reference genome (*S. pneumoniae* D39), umi deduplication (umi\_tools dedup) and differential gene expression analysis with DESeq2.

For the heatmap in Fig. 4, we clustered differentially expressed genes with the k-means clustering algorithm using ComplexHeatmap<sup>37</sup> in R. We submitted each generated cluster to DAVID 2021 (version of December 2021)<sup>38</sup> to identify functional enrichment.

With the most differentially expressed genes based on their log<sub>2</sub>FC value, we retrieved a STRING network for *S. pneumoniae* D39 in Cytoscape 3.8.0 to create a gene association network.

### Gene expression analysis by RT-qPCR

We synthesised cDNA using SuperScript IV Reverse transcriptase (Invitrogen), including RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), according to the manufacturer's instructions. We performed RT-qPCR according to the manufacturer's instructions using TaqMan Fast Advanced Master Mix (Applied Biosystems) and Custom TaqMan Gene Expression Assays (Applied Biosystems) with sequences in Supplementary Table S2.

### Human airway epithelial cell (hAEC) culture generation

We cultured hAEC cultures in 6.5 mm Transwells<sup>®</sup> on a 0.4  $\mu$ m pore polyester membrane insert in flat bottom 24-well plates (Corning, 3470) as described previously<sup>12</sup>. We seeded  $1 \times 10^5$  cells per insert and performed experiments once a pseudostratified layer of differentiated airway epithelial cells was formed, including physiological mucus production and cilia movement.

### Cytotoxicity assay in hAEC cultures

We determined cytotoxicity of 30 h peptide incubation on hAEC cultures by measuring release of lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis, and visualizing the ultrastructure of the epithelium as done previously<sup>12</sup>. To the apical side of hAEC cultures we added 40  $\mu$ l of TEER solution (NaCl 0.9 %, CaCl<sub>2</sub> 1.25 mmol/l and HEPES 10 mmol/l dissolved in distilled water) as negative control, 40  $\mu$ l of peptide resuspended in TEER solution (0.25 mg/ml, 0.5 mg/ml) and 40  $\mu$ l of 1x LDH+ control solution from the CytoTox<sup>®</sup> Non-Radioactive Cytotoxicity Assay (Promega). After incubation for 30 h at 37 °C and 5 % CO<sub>2</sub>, we added 160  $\mu$ l TEER to the apical side and collected the medium from the apical and basolateral side of each culture to determine cytotoxicity. We measured release of LDH on hAEC cultures using the CytoTox<sup>®</sup> Non-Radioactive

Cytotoxicity Assay (Promega) according to the manufacturer's instructions and calculated %LDH release using the formula of Rayamajhi et al.<sup>39</sup>.

## Ethics

All research was performed in accordance with the relevant guidelines and regulations. For the primary human airway epithelial cell culture model the anonymized post-mortem tissue material was obtained through the Tissue Bank Bern (TBB) in accordance with ethical approval (KEK-BE 1571/2019).

## Data availability

The transcriptomic data for this study has been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB74191.

Received: 14 December 2023; Accepted: 27 May 2024

Published online: 30 May 2024

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## Acknowledgements

We thank Carlo Casanova and Marianne Küffer (Institute for Infectious Diseases, University of Bern) for providing strains and serotypes of Swiss clinical pneumococcal isolates. We would like to thank the Next Generation Sequencing Platform, University of Bern, for performing the high-throughput sequencing experiments. We are grateful to Sabina Berezowska and Irene Ramos-Centeno (Institute of Pathology, University of Bern) for providing the tissues for hAEC culture establishment via the Tissue Bank Bern. We are grateful to Zhian Salehian (Norwegian University of Life Sciences) for their technical support. This work was funded by grant number 192067 from the Swiss National Science Foundation to L.J.H.

## Author contributions

L.J.H. conceived the study, J.L. wrote the first draft of the manuscript. J.L. and L.J.H. participated in the design of the study. J.L., L.S.G, P.C.F. did bacterial growth assays. D.S. provided D39 parental strain and D39 $\Delta$ amiC. P.W.M.H. provided D39 parental strain and D39 $\Delta$ amiA. J.L. and P.C.F. did epifluorescence microscopy after preliminary experiments and establishment of a protocol with C.G. and J.W.V. J.L. analyzed RNA-Seq. N.J.C. provided support for analysis of RNA-Seq data in R. J.L., L.L., M.F.L and R.D. performed or advised on hAEC culture assays. All authors gave final approval for publication.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-63217-2>.

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