

Supplementary Information

Intranasal vaccination with pneumococcal protein antigens presented on *Salmonella* outer membrane vesicles confers broad, serotype-independent protection

Authors

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Affiliations

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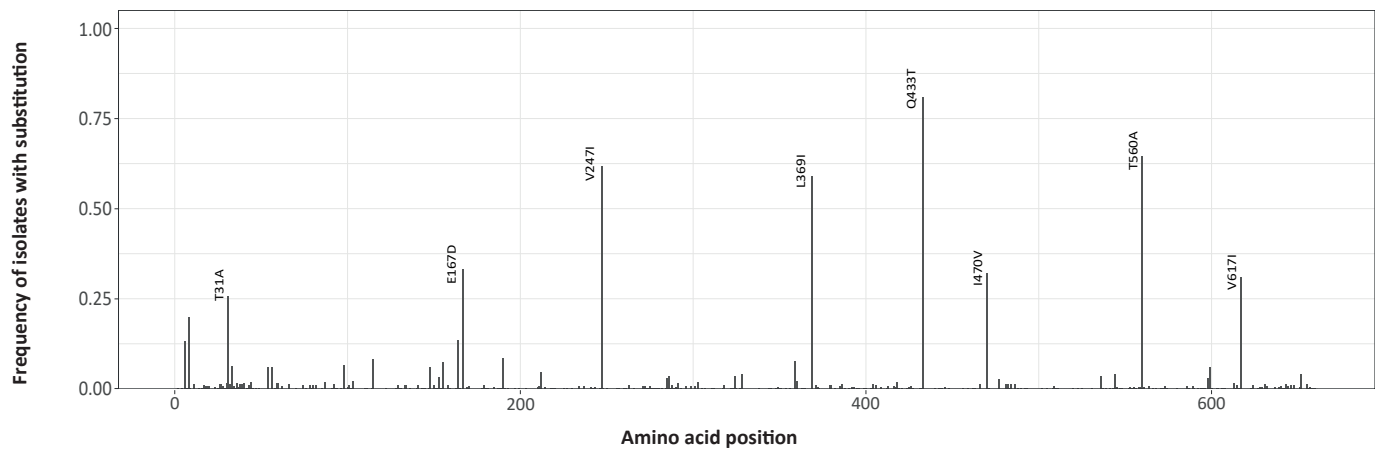
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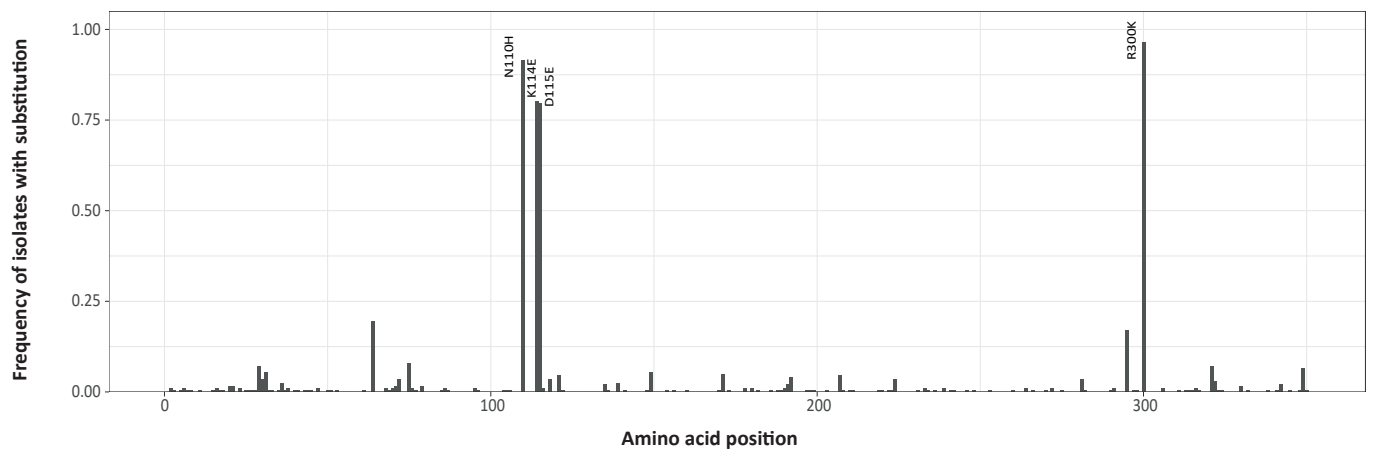
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AliA substitutions (SP_0366 reference)

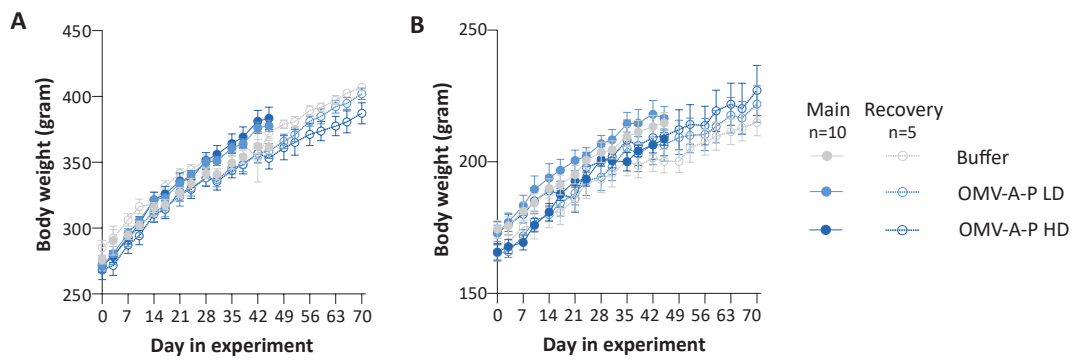


PnrA substitutions (SP_0845 reference)



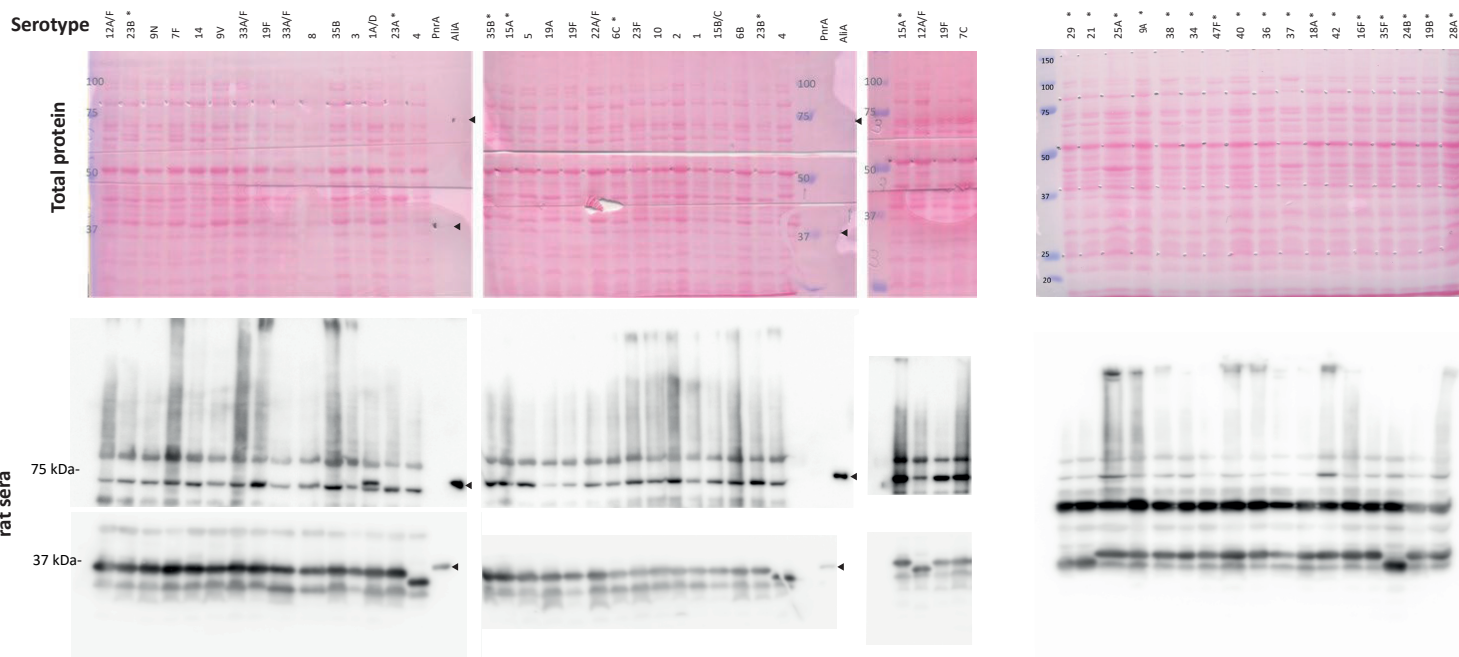
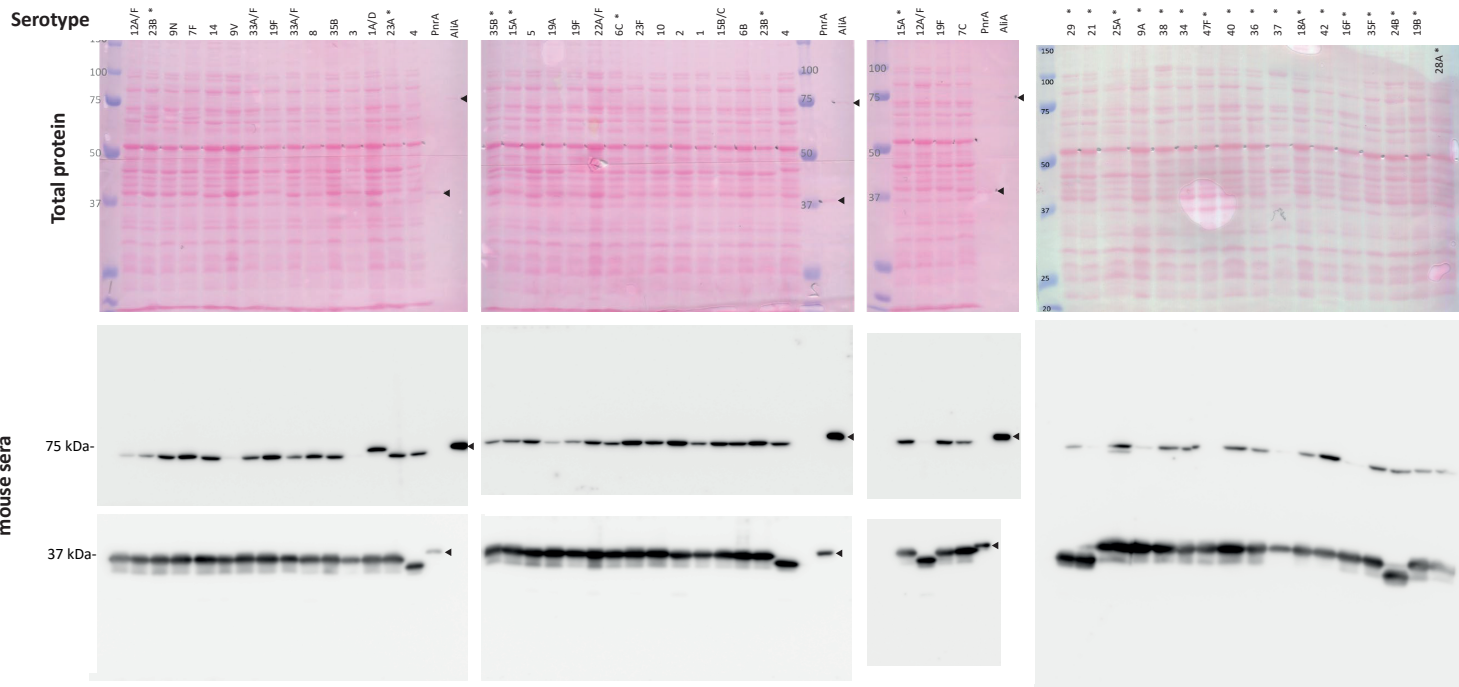
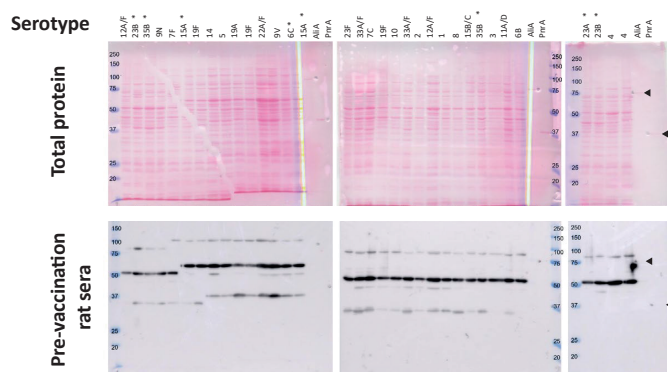
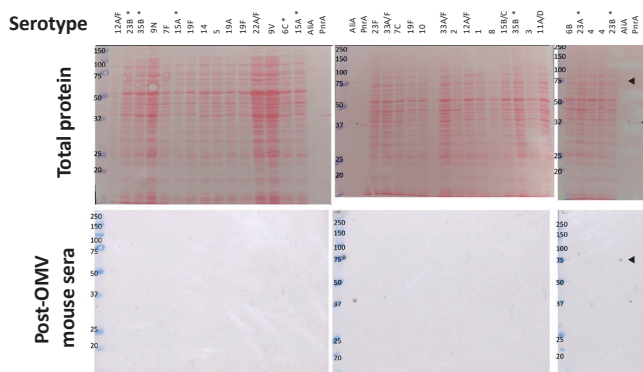
Supplementary Figure 1. Amino acid homology analysis of pneumococcal antigens AliA and PnrA.

Amino acids sequences of AliA and PnrA of 21,155 strains were compared to the reference sequence (TIGR4). The frequency of isolates with substitutions per amino acid position are shown. Most abundant amino acid substitutions are highlighted.

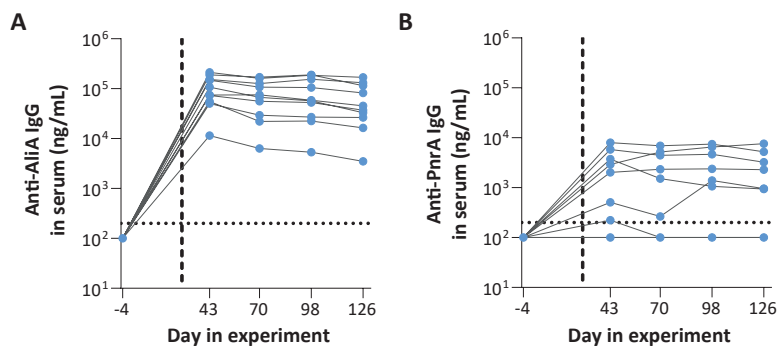


Supplementary Figure 2. Body weights of rats are not affected by intranasal vaccination with OMV-A-P.

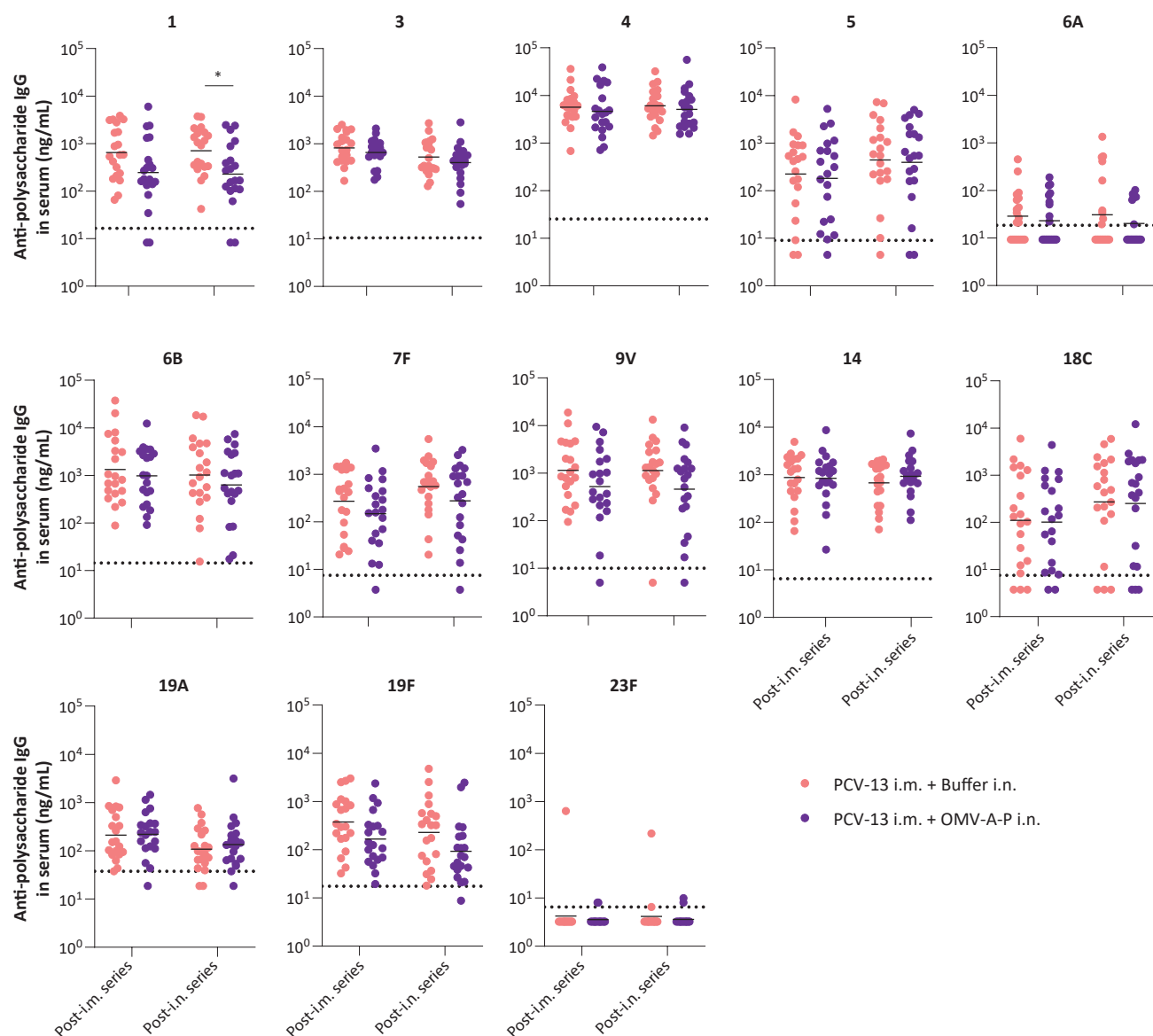
Rats (male n=15, female n=15) received OMV-A-P (LD: 8.7 μ g; HD: 87 μ g) or buffer intranasally on day 0, 14, 28 and 42. Body weights were measured twice a week. Mean body weight $\pm$ standard error of mean is depicted. **A)** Body weight of male rats. **B)** Body weight of female rats.

A**B****C****D**

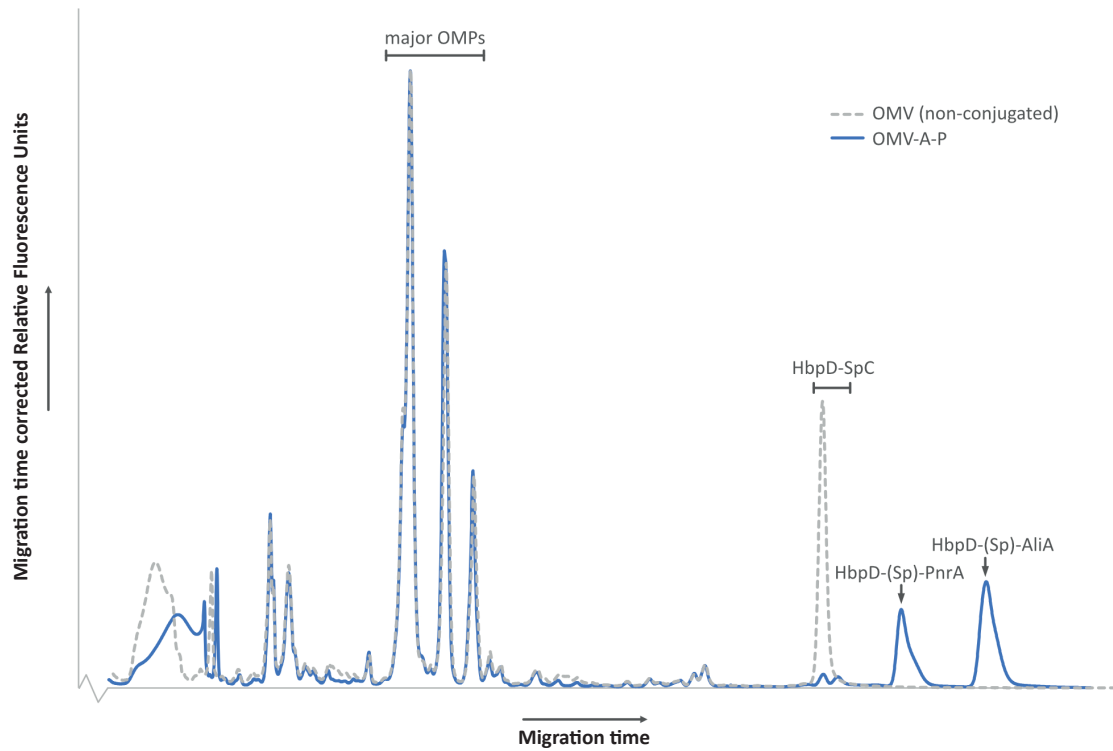
Supplementary Figure 3. Sera of OMV-A-P vaccinated rats and mice react specifically with AliA and PnrA in lysates of 51 pneumococcal isolates. Rats (male n=15, female n=15) and mice (n=10) received OMV-A-P intranasally. Pooled post-third vaccination sera were used for Western blot analysis of 51 pneumococcal isolates to identify antibody binding to AliA and PnrA in the lysate. Ponceau S stainings were performed to confirm equal loading of samples. Besides pneumococcal lysates, purified AliA and PnrA were loaded for reference (◄). Specificity of binding was checked using pre-vaccination sera for rats, and OMV-vaccinated sera for mice. **A)** Ponceau S staining and full blots corresponding to Western blots using rat sera shown in Figure 2C. **B)** Ponceau S staining and full blots corresponding to Western blots using mouse sera shown in Figure 2C. **C)** Ponceau S staining and Western blot on a subset of strains using pre-vaccination rat sera. **D)** Ponceau S staining and Western blot on a subset of strains using OMV-vaccinated mouse sera.



Supplementary Figure 4. Serum IgG responses against AliA and PnrA remain stable for more than 3 months following intranasal vaccination with OMV-A-P. Mice (n=10) were vaccinated intranasally three times with OMV-A-P. Serum samples were collected on day 43, 60, 98 and 126 and AliA- and PnrA-specific IgG concentrations were determined. **A)** AliA-specific serum IgG over time. **B)** PnrA-specific serum IgG over time. Horizontal dashed lines indicate the detection limits. Data points from the same animals are connected.



Supplementary Figure 5. Vaccination with OMV-A-P does not alter antibody responses to PCV-polysaccharides. Mice (n=20) were intramuscularly (i.m.) vaccinated three times with PCV-13, followed by three intranasal (i.n.) administrations with OMV-A-P or buffer. Serum was collected after completing the first (post-i.m.) and second (post-i.n.) vaccination series to assess IgG concentrations against all thirteen polysaccharides. Horizontal dashed lines indicate the detection limits. Horizontal solid lines indicate the group geometric mean. Statistical analysis was performed using unpaired *t*-tests, comparing animals assigned to different groups at the same moment in the experiment. * $p < 0.05$.



Supplementary Figure 6. Overlay of CE-SDS analysis of GLP-produced OMV and OMV-A-P formulations. Peaks for major outer membrane proteins (OMPs), HbpD-SpyCather (HbpD-SpC), conjugated PnrA to HbpD (HbpD-(Sp)-PnrA) and conjugated AliA to HbpD (HbpD-(Sp)-AliA) are indicated.

21 **Supplementary Table 1. Metadata Global Pneumococcal Sequencing Database and**
22 **presence/absence of aliA / AmiA / AliB / PnrA and amino acid variation AliA / PnrA**
23 See separate .xls file.

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26 **Supplementary Table 2. GLP toxicology data of OMV-A-P vaccinated rats**

27 See separate .xls file.

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Supplementary Table 3. Pneumococcal strains used for Western blot analysis

STRAIN ID	SEROTYPE	GPSC	ORIGIN (DISEASE/ COLONISATION)	COUNTRY OF ORIGIN	REFE REN CE
BACONSPR A0015	19F	1	Invasive disease	the Netherlands	2
PBCN0356	11A/D	3	Invasive disease	the Netherlands	1
PBCN0394	19F	4	Invasive disease	the Netherlands	1
PBCN0651	23B	5	Invasive disease	the Netherlands	1
PBCN0048	19A	10	Invasive disease	the Netherlands	1
PMEN1	23F	16	commercially available; patient; Streptococcus pneumoniae (Klein) Chester - 700669 ATCC	Spain	
TIGR4	4	27	commercially available; blood; https://www.atcc.org/products/baa-334	Norway	
PBCN0357	7F	32	Invasive disease	the Netherlands	1
PBCN0433	14	18	Invasive disease	the Netherlands	1
PBCN0085	33A/F	3	Invasive disease	the Netherlands	1
PBCN0120	9V	6	Invasive disease	the Netherlands	1
PBCN0133	6B	24	Invasive disease	the Netherlands	1
PBCN0392	6C	89	Invasive disease	the Netherlands	1
PBCN0150	1	31	Invasive disease	the Netherlands	1
PBCN0431	35B	36	Invasive disease	the Netherlands	1
PBCN0378	35B	36	Invasive disease	the Netherlands	1
PBCN0490	9N	16	Invasive disease	the Netherlands	1
BACONSPC W0203	7C	16	Invasive disease	the Netherlands	2
PBCN0477	12A/F	26	Invasive disease	the Netherlands	1
PBCN0457	23A	7	Invasive disease	the Netherlands	1
PBCN0364	8	3	Invasive disease	the Netherlands	1
PBCN0173	12A/F	32	Invasive disease	the Netherlands	1
PBCN0230	10	35	Invasive disease	the Netherlands	1
PBCN0272	22A/F	19	Invasive disease	the Netherlands	1
PBCN0315	33A/F	3	Invasive disease	the Netherlands	1
PBCN0488	23B	7	Invasive disease	the Netherlands	1
PBCN0240	3	12	Invasive disease	the Netherlands	1
D39	2	Unknown	commercially available		
PMEN19	5	8	Commercially available; Streptococcus pneumoniae (Klein) Chester - BAA-341 ATCC	Colombia	

PMEN25	15A	9	commercially available; colonisation; https://www.atcc.org/products/baa-661	Portugal	
PBCN0618	15A	13	Invasive disease	the Netherlands	1
G54	19F	9	commercially available		
PBCN0289	15B/C	4	Invasive disease	the Netherlands	1
AMC	9A	Unknown	Invasive disease	the Netherlands	5
2020010					
JN P020	16F	Unknown	Colonisation	Tanzania	3
JN S041	18A	Unknown	Colonisation	Tanzania	3
AHOY 1166	19B	Unknown	Colonisation	the Netherlands	4
JN P032	21	Unknown	Colonisation	Tanzania	3
JN P165	24B	Unknown	Colonisation	Tanzania	3
PBCN0257	25A	Unknown	Invasive disease	the Netherlands	1
AMC	28A	Unknown	Invasive disease	the Netherlands	5
2020186					
JN P044	29	Unknown	Colonisation	Tanzania	3
JN P160	34	Unknown	Colonisation	Tanzania	3
JN P043	35F	Unknown	Colonisation	Tanzania	3
AHOY901	36	Unknown	Colonisation	the Netherlands	4
JN S111	37	Unknown	Colonisation	Tanzania	3
AMC	38	Unknown	Invasive disease	the Netherlands	5
2021414					
AMC20208	40	Unknown	Invasive disease	the Netherlands	5
65					
AMC	42	Unknown	Invasive disease	the Netherlands	5
2021231					
AHOY1904	47F	Unknown	Colonisation	the Netherlands	4

31 GPSC: Global Pneumococcal Sequencing Cluster

- 1 Cremers, A.J.H., et al., *The post-vaccine microevolution of invasive Streptococcus pneumoniae*. Scientific Reports, 2015. **5**(1): p. 14952.
- 2 Sanches Ferreira, A.D., et al., *Investigating two decades of Streptococcus pneumoniae bacteraemia in the Gelderland area, the Netherlands, using whole-genome sequencing*. Microb Genom, 2025. **11**(3).
- 3 Ngocho, J.S., et al., *Emerging of non-vaccine Streptococcus pneumoniae serotypes colonizing the nasopharynx of children under the age of five years in the 13-valent pneumococcal conjugate vaccine era in Moshi district, Tanzania. A short communication*. Vaccine, 2025. **48**: p. 126724.
- 4 Bogaert, D., et al., *Pneumococcal Carriage in Children in The Netherlands: a Molecular Epidemiological Study*. Journal of Clinical Microbiology, 2001. **39**(9): p. 3316-3320.
- 5 the Netherlands Reference Laboratory for Bacterial Meningitis; Amsterdam; Kindly provided by prof. Dr. N. van Sorge

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34 **Supplementary Table 4. Amino acid sequences of antigens displayed on OMVs**

Target	Amino acid sequence
SpT-PnrA	MG AHIVMVDAYKPTK GSGGTGGNRSSRNAASSSDVKTAAIVTDT GGVDDKSFNQSAWEGLQAWGKEHNLSKDNGFTYFQSTSEADYAN NLQQAAGSYNLIFGVGFALNNAVKDAAKEHTDLNYVLIDDVIKDQK NVASVTFADNESGYLAGVAAAKTTKTKQVGFVGGIESEVISRFEAGF KAGVASVDPSIKVQVDYAGSFGDAAKGKTIAAAQYAAGADIVYQVA GGTGAGVFAEAKSLNESRPENEKVWVIGVDRDQEAEGKYTSKDGKE SNFVLVSTLKQVGTTVKDISNKAERGEFPGGQVIVYSLKDKGVDLAVT NLSEEGKKAVEDAKAKILDGSVKVPEK
SpT-AliA	MG AHIVMVDAYKPTK GSGGTSGSGSSTKGEKTFSYIYETDPDNLNY LTTAKAATANITSNVVDGLENDRYGNFVPSMAEDWSVSKDGLTYTY TIRKDAKWYTSEGEEYAAVKAQDFVTGLKYAADKKSDALYLVQESIKG LDAYVKEIKDFSQVGIKALDEQTVQYTLNKPESFWNSKTTMGVLAP VNEEFLNSKGDDFAKATDPSSLLYNGPYLLKSIVTKSSVEFAKNPNYW DKDNVHVDKVLSFWDGQDTSKPAENFKDGSLTAARLYPTSASF LEKSMKDNIVYTQQDSITYLVGTNIDRQSYKYTSKTSDEQKASTKKA LLNKDFRQAIAFGFDRTAYASQLNGQTGASKILRNLFVPPTFVQADG KNFGDMVKEKLVTYGDEWKDVNLADSQDGLYNPEKAKAEFAKAK SALQAEGVQFPIHLDMPVDQTATTKVQRVQSMKQSLEATLGADN VIIDIQQLQKDEVNNITYFAENAAGEDWDLSDNVGWGPDFADPST YLDIIKPSVGESTKTYLGFDSGEDNVAKKVGLYDYEKLVTEAGDETT DVAKRYDKYAAAQAWLTDSALIPTTSRTGRPILSKMVPFTIPFALSG NKGTSPEVLYKYLELQDKAVTVDEYQKAQEKWMKEEESNKKAAQE DLAKHVK

35 *Spy tag is indicated in red. PnrA antigen sequence is indicated in blue. AliA antigen sequence is indicated in*
 36 *green.*

37 **Supplementary Table 5. Overview of preclinical experiments included in this study**

Study	Species	Design	Outcome measures	Vaccine batch ID	GLP
1. <i>Long-term protection by OMV-A-P against pneumococcal colonisation in mice</i>	Mouse C57BL/6J F n=10 animals/group n=6 animals/group for cytokines and nasal lavage (sentinel animals) n=72 in total*	Groups: • OMV (12.0 µg) • OMV-A-P (12.0 µg) • Buffer Vaccination: 3x IN; 2-week interval Pneumococcal challenge: 3 or 15 weeks post-vaccination; ST4.	Efficacy (primary): Reduction in bacterial load (Fig 5) Immunogenicity: IgG in serum (Fig 2; 5; Fig S3, S4) IgA/IgG in nasal lavage (Fig 3) IgA in nasal tissue (Fig 5) Cytokines in nasal, lung, spleen tissue (Fig 4)	FS1 batch DoM 2022-09-09	No
2. <i>Protection by OMV-A-P in combination with PCV-13 against a PCV- and non-PCV serotype in mice</i>	Mouse C57BL/6J F n=10 animals/group n=80 in total*	Groups: • Buffer + Buffer • Buffer + OMV-A-P (9.2 µg) • PCV-13 (50 µL) + Buffer • PCV-13 (50 µL) + OMV-A-P (9.2 µg) Vaccination: 3x IM + 3x IN; 2-week interval Pneumococcal challenge: 3 weeks post-vaccination; ST4 or ST19B	Efficacy (primary): Reduction in bacterial load (Fig 6) Immunogenicity: IgG in serum (Fig 6; Fig S5) IgA in nasal tissue (Fig 6) Cytokines in spleen (Fig 6)	FS1 batch DoM 2022-09-09	No
3. <i>Pilot study of OMV-A-P in rats</i>	Rat Wistar Han M/F n=6 animals/group n=18 in total*	Groups: • Buffer • OMV-A-P (17.4 µg) • OMV-A-P (87.0 µg) Vaccination: 4x IN; 2-week interval	Immunogenicity: IgA/IgG in nasal lavage (Fig 3)	Tox batch DoM 2022-12-19	No
4. <i>GLP toxicology study of OMV-A-P in rats</i>	Rat Wistar Han M/F n=30 animals/group of which 10 recovery animals n=90 in total*	Groups: • Buffer • OMV-A-P (8.7 µg) • OMV-A-P (87.0 µg) Vaccination: 4x IN; 2-week interval	Toxicology (primary): Clinical/ respiratory observations, body weight, blood parameters, urinalysis, full tissue macro and micro pathology (Fig S2) Immunogenicity: IgG in serum (Fig 2, Fig S3)	Tox batch DoM 2022-12-19	Yes

38 DoM: date of manufacturing; GLP: good laboratory practice; M: male; F: female; IN: intranasal; IM:
39 intramuscular; ST: serotype of pneumococcal challenge strain. * Data of n=152 mice and n=108 rats were
40 used in this study.

41 **Supplementary Table 6. Data overview immunogenicity and efficacy of OMV-A-P in mice**
42 **and rats**
43 See separate .xls file.



The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item	Recommendation	Section/line number, or reason for not reporting
Study design	1 For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	Supplementary Table 5; Figure legends of corresponding figures. Figure legends of corresponding figures.
Sample size	2 a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any <i>a priori</i> sample size calculation, if done.	Supplementary Table 5 Methods- Statistics section
Inclusion and exclusion criteria	3 a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established <i>a priori</i> . If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of <i>n</i> in each experimental group.	Methods - statistics section Figure legends of corresponding figures. Figure legends of corresponding figures; Supplementary Table 5
Randomisation	4 a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Methods - Ethics statement section Methods - Ethics statement section
Blinding	5 Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Methods - Ethics statement section
Outcome measures	6 a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	Supplementary Table 5 Supplementary Table 5; Methods- Statistics section
Statistical methods	7 a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	Supplementary Table 5; Figure legends of corresponding figures Not assessed
Experimental animals	8 a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Supplementary Table 5; Methods- Ethics statement section All relevant information is in Methods- ethics statement section
Experimental procedures	9 For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	Methods- lines 476-516; Methods- lines 476-516; Methods- Ethics statement section Methods- lines 476-516;
Results	10 For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	Geometric means and statistics are indicated in corresponding figures; raw data in supplementary Table 2 and 6. effect sizes are indicated in text of results section where applicable