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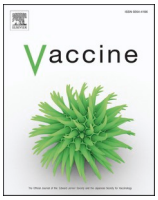

Streptococcus pneumoniae carriage, serotypes, genotypes, and antimicrobial resistance trends among children in Portugal, after introduction of PCV13 in National Immunization Program: A cross-sectional study

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A B S T R A C T

Streptococcus pneumoniae carriage studies are crucial to monitor changes induced by use of pneumococcal conjugate vaccines and inform vaccination policies. In this cross-sectional study, we examined changes within the pneumococcal population following introduction of PCV13 in 2015 in the National Immunization Program (NIP), in Portugal. In 2018–2020 (NIP-PCV13), we obtained 1450 nasopharyngeal samples from children ≤6 years attending day-care. We assessed serotypes, antimicrobial resistance, and genotypes (MLST and GPSC) and compared findings with earlier periods: 2009–2010 (pre-PCV13), 2011–2012 (early-PCV13), and 2015–2016 (late-PCV13). Pneumococcal carriage prevalence remained stable at 60.2 %. Carriage of PCV13 serotypes was 10.7 %, markedly reduced compared to pre-PCV13 period (47.6 %). The most prevalent PCV13 serotypes were 19F, 3, and 19A all showing a significant decreasing trend compared to the pre-PCV13 period (from 7.1 % to 4.7 %, 10.1 % to 1.8 %, and 14.1 % to 1.8 %, respectively), a notable observation given the described limited effectiveness of PCV13 against serotype 3. Non-vaccinated children and children aged 4–6 years were more likely to carry PCV13 serotypes (2.5-fold, 95 %CI [1.1–5.6], and 2.9-fold, 95 %CI [1.3–6.8], respectively). The most prevalent non-PCV13 serotypes were 15B/C, 11A, 23B, 23A, and NT, collectively accounting for 51.9 % of all isolates. In total, 30.5 % of all pneumococci were potentially covered by PCV20. Resistance to penicillin (low-level) and macrolides increased significantly, from 9.3 % and 13.4 %, respectively, in the late-PCV13 period, to approximately 20 % each, mostly due to lineages expressing non-PCV13 serotypes, nearing pre-PCV13 levels. An expansion of lineages traditionally associated with PCV13 serotypes, like CC156-GPSC6 (serotype 14) and CC193-GPSC11 (serotype 19F), but now predominantly expressing non-PCV13 serotypes (11A, 15B/C, and 24F for GPSC6; and 15A and 21 for GPSC11) was noted. These findings indicate that the pneumococcal population is adapting to the pressures conferred by PCV13 and antimicrobial use and indicate the need to maintain close surveillance.

1. Introduction

Streptococcus pneumoniae, commonly known as pneumococcus, is a bacterium that asymptomatically colonizes the upper respiratory tract of humans. It is frequently found in the nasopharynx of young children, with median prevalence rates ranging between 30 and 50 % depending on the income classification of the country [1]. Young children are at increased risk of pneumococcal infection such as otitis media,

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pneumonia, and meningitis, which can lead to severe outcomes, including death [2,3]. The introduction of multivalent pneumococcal conjugate vaccines (PCVs) has significantly decreased the burden of pneumococcal disease [3] and carriage of pneumococcal serotypes targeted by PCVs [4].

PCVs target a limited number of the over 100 pneumococcal capsular types (or serotypes) described to date [5]. PCV13, available since 2010, targets 13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F). More recently, a 15-valent vaccine (PCV15, targeting PCV13 serotypes plus serotypes 22F and 33F) and a 20-valent vaccine (PCV20, targeting PCV13 serotypes plus serotypes 8, 10A, 11A, 12F, 15B/C, 22F, and 33F) have also been approved [6].

In Portugal, PCV7 was commercially available between 2001 and 2010 with increasing uptake over time [7]. In 2010, PCV13 became commercially available. In mid-2015 PCV13 became the first PCV to be introduced into the National Immunization Program (NIP) being offered to all children born since January 1, 2015, with a 2 + 1 doses schedule at 2 and 4 months and a booster at 12 months [8]. There is limited information regarding the uptake of PCVs before their introduction into the Portuguese NIP. However, estimates based on the vaccine administration records of the General Health Directorate suggest that PCV13 uptake was approximately 70 % between 2010 and 2014 [9]. Following its introduction into the NIP, this increased to 99.1 % by 2020, according to the centralized digital records of all vaccines administered in the country [10].

Since 1996, we have been studying pneumococcal colonization in Portugal and the impact of PCVs on nasopharyngeal carriage of young children [4,7,11–13]. The direct effect of private use of PCV7 and PCV13 was notable [4,7,11–13]. Despite this, vaccine serotypes continued to be carried, although at a significantly lower prevalence, especially among non-vaccinated children [4]. Similarly, data from pediatric invasive pneumococcal disease (PIPD) in Portugal indicated a marked effect of private use of PCV7 and PCV13. The proportion of PIPD caused by PCV7 serotypes decreased from 55.9 % in 1999–2002 to 17.0 % in 2006–2008 [14]; the proportion of PIPD caused by PCV13 serotypes decreased from 57.8 % in 2012–2015 to 30.9 % in 2015–2018, although PCV13 serotypes 19F, 3, and 19A were still responsible for the majority of cases [15,16].

As documented worldwide, we observed extensive serotype replacement in carriage upon private use of PCV7 and PCV13, with overall carriage prevalence remaining unchanged due to a significant increase in some (but not all) non-vaccine serotypes [4,7,12]. This increase was often associated with the expansion of specific lineages [12]. Notably, some emerging non-vaccine serotypes, such as 8, 10A, 15B/C, and 24F, were also more frequently detected among pediatric disease isolates [15,16], underscoring the well-documented link between carriage and disease [17].

Following the introduction of PCVs, a decline in resistance to first-line antibiotics occurred in several countries [18]. In Portugal, during the decade of private use of PCV7, changes in antimicrobial resistance prevalence among pneumococci associated with carriage and disease were observed. This was particularly notable for high-level resistance to penicillin, which was primarily linked to serotypes targeted by PCV7 and decreased with the use of PCV7 [7,14,19]. The subsequent private use of PCV13 led to yet another decline in pneumococcal antimicrobial resistance, including low-level resistance to penicillin and macrolides, which was observed among carried pneumococci isolated in 2015–2016 and in disease isolates in 2015–2018 [4,16]. Still, a 2016–2017 report indicated that antibiotic use in Portugal remained high, particularly in primary healthcare settings [20]. More recently, an increase in pneumococcal resistance to macrolides has been documented particularly among non-vaccine serotypes [18]. For instance, in 2020, 15.5 % and 16.8 % of the *S. pneumoniae* disease isolates reported by European countries, including Portugal, were not susceptible to penicillin and macrolides, respectively [21]. The 2024 revised WHO Bacterial Priority Pathogens list classified macrolide-resistant *S. pneumoniae* as a medium-priority category, emphasizing the urgent need to address its public health impact [22].

The observation of ongoing dynamic shifts in the pneumococcal population regarding serotype distribution, prevalent lineages, and antimicrobial resistance trends underscores the importance of regular and long-term surveillance of pneumococcal carriage and disease trends. The Portuguese studies on pneumococcal carriage and disease have collectively emphasized the need for universal vaccination, to maximize the impact of PCV use, which is only attainable with its introduction in the NIP. They also highlighted the crucial importance of regular surveillance for informed public health decisions, evaluation of the impact of such decisions, and guidance in vaccine design. The development of novel multivalent pneumococcal conjugate vaccines with expanded valency, which are likely to replace PCV13 in the near future, further emphasizes this need. These new vaccines are expected to cause shifts in serotype distribution by targeting serotypes not included in current vaccines, leading to adaptations in the pneumococcal population. These changes could impact antimicrobial resistance prevalence, alter prevalent lineages, and affect pneumococcal carriage and disease patterns.

Having all the above issues in mind, in this study, we aimed to assess changes within the pneumococcal population following the introduction of PCV13 in the NIP and to establish a baseline essential to monitor the impact of novel expanded vaccines, likely to replace PCV13 in the near future. Specifically, our objectives were to evaluate changes in pneumococcal serotype distribution, antimicrobial resistance and genetic lineages in circulation in 2018–2020 (NIP-PCV13 period) in Portugal and to compare these findings with data from three prior epidemiological periods: 2009–2010 (pre-PCV13), 2011–2012 (early-PCV13), and 2015–2016 (late-PCV13).

2. Methods

2.1. Ethics statement

The study was registered and approved by the Ethics Research Committee of the NOVA Medical School/Faculdade de Ciências Médicas – Universidade Nova de Lisboa (CEFCM) (30/2017/CEFCM, approved February 9, 2018). Approval was also obtained from the directors of all participating day-care centers. Investigators, along with the pediatrician associated with this study, met with the directors of each day-care center to explain the project in detail. Written letters and questionnaires were distributed to all legal guardians of children attending the day-care centers. These letters included a detailed explanation of the project and the sampling process, along with two copies of the informed consent (one for the parents or guardians to keep and another for the investigators). The informed consent included contact information (email and phone) for the principal investigator and the Ethics Research Committee, indicating both were available to address any questions regarding the project. Written consent was obtained from the parents or guardians of all participating children. All signed consents were stored securely at ITQB NOVA. Sampling was conducted by a trained pediatric nurse who first explained the process to each child individually (except for those too young to understand). Sampling was done in the presence of the child's teacher with the child's agreement to participate. All samples and questionnaires were numerically coded at the time of sampling and processed anonymously thereafter. Data was stored in a dedicated secure database and was fully and irreversibly anonymized upon completion of data analysis.

2.2. Study design

Three yearly cross-sectional studies were carried out between 2018 and 2020, during the months of January to April, in the Municipality of Oeiras, an urban area situated in the metropolitan area of the Capital, Lisbon, Portugal.

The target population of this study, as in similar ones previously

conducted by the group [4], was a convenience sample of children up to 6 years old attending day-care centers. The latter included fully private (fully paid by families), semi-private (paid by the family depending on income and subsidized by the state) and public (free of charge) day-care centers, reflecting the spectrum available in the community. This population was considered representative of the broader population of children in Portugal since, according to data from Statistics General Directorate for Education and Science, approximately 95 % of children attended day-care centers in Portugal between 2018 and 2020.

For the analysis, data from 2018, 2019, and 2020 were grouped and defined as one single period where PCV13 was already integrated in the National Immunization Program (named NIP-PCV13 period). We followed the same strategy as previous studies from our group and others [4,23] grouping sampling years according to PCV13 coverage to allow for comparisons over time. In 2018, 2019, and 2020, PCV13 was already integrated into the National Immunization Program, and coverage among children completing 1 year of age was 98.7 %, 98.8 %, and 99.1 %, respectively [10]. Data from previous studies encompassing 2009 to 2016 (with the exception of 2013 and 2014) were included in the present study for comparison [4] and were grouped in three periods, also according to PCV13 coverage, as described before: pre-PCV13 (corresponding to 2009–2010, before PCV13 introduction in Portugal), early-PCV13 (corresponding to 2011 and 2012, soon after PCV13 introduction in the private market, with a coverage of 69 % among children aged 0–<2 years), and late-PCV13 (corresponding to 2015 and 2016, years after PCV13 introduction in the private market, with a coverage of 85.4 % among children aged 0–<2 years) [4].

To estimate potential coverage of PCVs, serotype 6C was grouped with PCV13 serotypes since it has been shown that the 6A antigen confers cross-protection to 6C [24]. Serotypes 22F and 33F were collectively named PCV15 additional serotypes; serotypes 8, 10A, 11A, 12F and 15B/C were collectively named PCV20 additional serotypes (compared to PCV15).

2.3. Questionnaires

A questionnaire, in Portuguese, regarding demographic information and antibiotic consumption was filled in on paper by the children's parents or guardians. The questionnaire used has been employed consistently since 1996, when formal pre-testing was done, undergoing minor updates overtime to reflect changes such as vaccination schemes. Questions are straightforward and free of technical jargon, making it accessible to respondents. To ensure accurate and consistent data collection, demographic data were verified on sampling day by the team visiting the day-care center, antimicrobial use was reported by the guardians with indication of the commercial name, and information regarding PCV13 immunization schedule was transcribed directly from the child's immunization bulletin.

2.4. Sample collection

Nasopharyngeal samples (one per child) were collected by a trained nurse, accordingly to WHO recommendations for pneumococcal carriage studies [25]. Briefly, a flexible swab (Neonatal FLOQSwabs™, Copan Flock Technologies, Italy) was inserted through one nostril until reaching the nasopharynx, turned, and removed. The swabs were immediately placed into tubes containing 1 mL of skim milk-tryptone-glucose-glycerol (STGG) medium and placed in a tray within a cooler filled with wet ice. To ensure that sample integrity and quality were maintained, samples were kept on the cooler and transported to the laboratory within four hours of collection. Upon arrival, samples were immediately vortexed for 20 s, frozen in liquid nitrogen, and stored at −80 °C.

2.5. Isolation and identification of pneumococci

Isolation of pneumococci was performed as previously described [25]. Briefly, sample tubes were fully thawed on ice, vortexed for 20 s and 50 µL were plated onto blood agar supplemented with 5 µg/mL of gentamicin and were incubated overnight in anaerobic jars at 37 °C. On the following day, suspected pneumococcal colonies were isolated and identified following routine procedures based on colony morphology, occurrence of α-hemolysis and optochin susceptibility. Bile solubility test was performed in optochin resistant isolates. To ensure the accuracy of pneumococcal identification, positive and negative controls for the tests being made were regularly included. Pure pneumococcal cultures were frozen at −80 °C in STGG medium.

2.6. Antimicrobial susceptibility testing

Susceptibility to erythromycin was tested by the disc diffusion method (Oxoid Hampshire, England) following Clinical & Laboratory Standards Institute (CLSI) recommendations and interpretative criteria [26]. Minimum inhibitory concentrations (MIC) to penicillin were determined by Etest (Biomérieux, Marcy-l'Etoile, France) and interpreted according to the CLSI recommended breakpoints for oral penicillin V: isolates with MIC to penicillin of 0.12–1 µg/mL were considered intermediately resistant and isolates with MIC ≥2 µg/mL were considered resistant [26]. Reference strain ATCC 49619 was used as a control. Susceptibility to chloramphenicol, clindamycin, tetracycline, sulphamethoxazole-trimethoprim (SXT), amoxicillin, and ceftriaxone, and respective acquired resistance genes (*tetM*), or genes with chromosomal mutations conferring resistance (*folA/folP*) were inferred through Pathogenwatch (http://pathogen.watch). Strains with an intermediately resistant or resistant profile were collectively referred as non-susceptible. Multidrug resistance was defined as resistance to three or more classes of antibiotics.

2.7. DNA extraction for whole genome sequencing

Pneumococcal pure cultures were streaked onto TSA agar plates supplemented with catalase (c.a. 240 U/mL) and incubated overnight at 37 °C with 5 % CO_2. On the following day, 500 µL of sterile PBS was added to each plate, cultures were resuspended and collected. 200 µL of each suspension were transferred into a tube containing 200 µL of lysis buffer (MagNA Pure Compact Nucleic Acid Isolation Kit, Roche Diagnostics GmbH) and 17.4 µL of RNase A (100 mg/mL) and were incubated for 20 min at 37 °C. Total DNA was then extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit (Roche Diagnostics GmbH) as recommended by the manufacturer. DNA quality of each sample was assessed using Nanodrop (ThermoFisher Scientific) for A_{260}/A_{280} and A_{260}/A_{230} ratios evaluation. DNA quantity was assessed using the dsDNA High Sensitivity Qubit kit (ThermoFisher Scientific), according to the manufacturer's instructions. An electrophoresis imaging on 1 % agarose was also performed to evaluate DNA integrity.

2.8. Whole genome sequencing, serotype and genotype assignment

Whole Genome sequencing (WGS) was performed at the Genomics Unit at Instituto Gulbenkian de Ciências, Oeiras, Portugal. Genomes of samples were sequenced on Illumina NextSeq 500 with a target coverage of 100×.

Assembly of the paired-end reads and quality control were performed using INNUca v4.2.2 (https://github.com/B-UMMI/INNUca) using default parameters and inputting the bacterial species and expected genome size to be used as inclusion/exclusion criteria. This software was also used to determine the genome size, number of contigs and for genotyping through Multilocus Sequence Typing (MLST). Then, SeroBA v1.0.0 (https://github.com/sanger-pathogens/seroba) was used to determine the capsular serotypes based on WGS reads [27].

The relationship between sequence types (STs) was determined using the PHYLOViZ Online tool (https://online.phyloviz.net) with the goeBURST algorithm [28]. We defined a clonal complex (CC) as the fittest genotype that may diversify into phylogenetically closely related strains, by increasing its frequency in the population. STs that were different from all the others were defined as singletons.

Pneumococcal isolates were clustered to a Global Pneumococcal Sequencing Cluster (GPSC) on the basis of their shared sequences and gene content using core and accessory distances using the global genomic surveillance platform Pathogenwatch (http://pathogen.watch) and PopPUNK 2.5.0 (https://poppunk.net/) [29,30]. For visualization we used the flag Cytoscape when running visualization with PopPUNK and Cytoscape to reconstruct the clusters and the GPSC from the output of PopPUNK (https://cytoscape.org/).

2.9. Statistical analysis

All statistical analyses were carried out in R v4.3.1 [31]. Individuals with missing data in the variables of interest were excluded from the analyses. Differences in pneumococcal carriage and demographic and epidemiological data (gender, antimicrobial use and PCV13 use) between study periods were tested using the chi-square test or the Fisher's exact test, as appropriate. Mean ages of children from different periods were compared using ANOVA and Tuckey multiple pairwise comparison tests were subsequently carried out to identify between which periods the differences were significant. Assumptions for ANOVA, specifically the homogeneity of variances, were checked using Bartlett's test, while QQ plots were used to check for the normality of the distribution of children's ages for each period under study.

For proportions, 95 % confidence intervals were estimated using the Wilson interval. To test the statistical significance for trends in pneumococcus prevalence and prevalence of serotypes across the four different periods, the chi-square test for trends in proportions was used, if a trend was observed.

A generalized linear mixed-effects model (GLMM) with a logit link function was used to study the association between carriage of PCV13 serotypes and both vaccination status and age, with day-care center included as a random intercept to account for clustering. The model was adjusted for children over 12 months of age (old enough to have completed the PCV13 vaccination scheme) using the 'lme4' package in R. Fit of the model was examined for overdispersion and multicolinearity using the R package 'blmeco' and 'car' respectively.

The adjusted Wallace coefficient (AW), and corresponding confidence intervals (CI) were used as quantitative measures of congruence between MLST and GPSC methods [32]. A p-value of <0.05 was considered statistically significant. The Wallace coefficient is a measure used in classification to quantify the agreement between two partitions of a dataset. It provides insight into how well one partition can be used to predict another partition, ranging from 0 (no correspondence) to 1 (perfect correspondence).

We used odds ratio (OR) and corresponding 95 % CI to measure the strength of association between covariates and the outcome. A 95 % CI OR containing 1 was considered non-significant. For Chi-square and Fisher's exact tests, ANOVA and chi-square test for trends in proportions, a p-value of <0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the population

The characteristics of the population sampled between 2018 and 2020 (defined as NIP-PCV13 period) are described in Table 1. In short, 1450 nasopharyngeal samples were obtained, the mean age was 2.7 ± 1.5, 45.1 % of the participants were aged between 2–<4 years old, and 53.6 % were male. Antimicrobial use among participants, at the time of sampling and in the month preceding sampling, was 4.4 % and 16.5 %,

Table 1
Demographic and epidemiological characteristics of the population.

Characteristics	Period							
			Private use of PCVs				PCV13 in NIP	
	Pre-PCV13[a, b] (2009–2010) n = 1088		Early-PCV13[a] (2011−2012) n = 724		Late-PCV13[a] (2015-2016) n = 630		NIP-PCV13 (2018–2020) n = 1450	
	n/N	%	n/N	%	n/N	%	n/N	%
No. of day care centers	9	–	6	–	7	–	16	–
Mean age (years ± SD)	3.3 ± 1.5	–	3.2 ± 1.4	–	2.8 ± 1.5	–	2.7 ± 1.5	–
Age[c]								
0–<2 years	168	15.4	115	15.9	136	21.7	357	24.7
2–<4 years	395	36.3	275	38.0	266	42.4	654	45.1
4–6 years	525	48.3	334	46.1	226	35.9	432	29.8
Males	561	51.6	358	49.4	334	(53.0)	777	53.6
Antibiotic use								
At sampling[d]	36/1061	3.4	48/709	6.8	25/605	4.1	63/1426	4.4
Previous month[e]	152/1030	14.8	140/664	21.1	96/569	16.9	229/1385	16.5
Any PCV use (≥1 dose)								
0–6 years[f]	818/1065	76.8	545/709	76.9	506/615	82.3	1290/1369	94.2
PCV13 use (≥1 dose)								
0–<2 years[g]	–		78/113	69.0	111/130	85.4	341/343	99.4
2–6 years[h]	–		119/596	20.0	393/483	81.4	945/1022	92.5
Pneumococcal carriage	676/1088	62.1	452/724	62.4	388/630	61.6	873/1450	60.2
No. of serotypes observed	30	–	31	–	31	–	32	–

Abbreviations: PCV13, 13-valent pneumococcal conjugate vaccine; PCVs, pneumococcal conjugate vaccines; NIP, National Immunization Program; SD, standard deviation.

[a] Data previously described in Félix et al. [4].
[b] In the pre-PCV13 period, 75.2 % children received PCV7.
[c] Data missing for 2 in the late-PCV13 period and 7 children in the NIP-PCV13 period.
[d] Data missing for 27, 15, 25, and 24 children in pre-, early-, late- and NIP-PCV13 periods, respectively.
[e] Data missing for 58, 60, 61, and 65 children in pre-, early-, late- and NIP-PCV13 periods, respectively.
[f] Data missing for 23, 15, 15, and 81 children in pre-, early-, late- and NIP-PCV13 periods, respectively.
[g] Data missing for 3, 2, 6, and 14 children in pre-, early-, late- and NIP-PCV13 periods, respectively.
[h] Data missing for 20, 13, 9, and 64 children in pre-, early-, late- and NIP-PCV13 periods, respectively.

respectively. PCV13 uptake was very high: 94.2 % when all children 0–6 years old were considered, and 99.4 % among children aged 0–<2 years. The prevalence of pneumococcal carriage was 60.2 %. For comparison, we included data from a previous study encompassing three epidemiological periods: pre-PCV13 (2009–2010), early-PCV13 (2011−2012), and late-PCV13 (2015–2016) (Table 1) [4]. PCV13 use increased significantly overtime ($p < 0.001$ for trend). There were no significant differences in pneumococcal carriage, which remained high and stable over the different periods: 62.1 %, 62.4 %, 61.6 %, and 60.2 %, respectively ($p = 0.694$) (Table 1).

The number of serotypes detected in NIP-PCV13 period was 32 and this was comparable to previous periods: 30 in the pre-PCV13, 31 in the early-PCV13, and 31 in the late-PCV13 period (Table 1).

3.2. Serotype distribution and association with age and vaccination status

In the NIP-PCV13 period, among the 873 pneumococci, 10.7 % were PCV13 serotypes (includes serotype 6C), a prevalence comparable to the one observed in the late-PCV13 period (12.9 %, $p = 0.289$) and significantly lower than the one observed in previous periods (47.6 % in the pre-PCV13 period and 31.9 % in the early-PCV13 period, $p < 0.001$) (Fig. 1). The most prevalent PCV13 serotypes were 19F (4.7 % of all pneumococci), followed by serotypes 3 and 19A (1.8 % each) (Fig. 2 and Table S1). Compared to previous periods, the prevalence of these three serotypes showed a statistically significant decreasing trend which was more pronounced between the pre-PCV13 and late-PCV13 periods (Table S1). The other PCV13 serotypes remained at a very low prevalence or were not even detected (Table S1).

Carriage of PCV13 serotypes (including serotype 6C) was significantly more frequent among non-vaccinated children (12.7 % vs 5.7 %, $p = 0.024$). In particular, serotypes 3 and 19A were more prevalent among non-vaccinated children (3.8 % vs 0.9 %, $p = 0.051$ for serotype 3 and 3.8 % vs 0.8 %, $p = 0.035$ for serotype 19A); this was not noted for serotype 19F (2.5 % vs 2.6 %, $p = 1.0$) (Table 2).

As older children (4–6 years old) were significantly less vaccinated than children <4 years old (86.4 % vs 97.8 %, $p < 0.001$), the association between carriage of PCV13 serotypes, vaccination status and age was investigated. Only children old enough to have a complete vaccination scheme (>1 year old, with booster) were included in the analysis. Day-care center was used as a random variable.

Non-vaccinated children (3.3-fold, 95 % CI 1.4–7.1, $p = 0.003$) and children aged 4–6 years (3.3-fold, 95 % CI 1.5–7.1, $p = 0.003$) were more likely to carry PCV13 serotypes.

After multivariate analysis, both factors remained significant: non-vaccinated children were 2.5 times more likely (95 % CI 1.1–5.6) to carry PCV13 serotypes compared to children with a complete vaccine scheme. In addition, children aged 4–6 years old were 2.9 times more likely (95 % CI 1.3–6.8) to carry PCV13 serotypes compared to children aged 1–2 years (Table 3).

Carriage of non-PCV13 serotypes was 89.3 % in the NIP-PCV13 period. The most frequent non-PCV13 serotypes were 15B/C (15.7 %), 11A (11.2 %), 23B (9.9 %), 23A (7.7 %), and NT (7.4 %) which, together, accounted for 51.9 % of all pneumococci (Fig. 2, Table S1). Notably, except for NT strains, which prevalence has remained stable overtime, the other four most frequent serotypes increased significantly in prevalence since the pre-PCV13 period ($p < 0.001$ for all, Table S1).

Serotypes 22F and 33F included in PCV15 and PCV20 (but not in PCV13) accounted for 3.6 % of the isolates; serotypes 8, 10A, 11A, 12F, and 15B/C included in PCV20 (but not in PCV13 nor in PCV15) accounted for 30.5 % of the isolates (Fig. 1, Fig. 2, Table S1). Over half (55.2 %) of the serotypes detected in the NIP-PCV13 period are not targeted by any of the PCVs that have recently become commercially available (Fig. 2, Table S1).

3.3. Antimicrobial susceptibility

During the NIP-PCV13 period an increase in non-susceptibility to penicillin and in resistance to erythromycin was noted when compared to the late-PCV13 period: intermediate resistance to penicillin increased from 9.3 % to 18.8 % ($p < 0.001$), and resistance to erythromycin increased from 13.4 % to 19.5 % ($p = 0.013$) (Fig. 3). Notably, the majority of isolates that were non-susceptible to penicillin and/or erythromycin were not targeted by currently available conjugate vaccines (Fig. 4). Similar observations were made in other settings, such as, Poland and Belgium, where carriage studies in the pediatric population have been performed following PCV13 use [33,34].

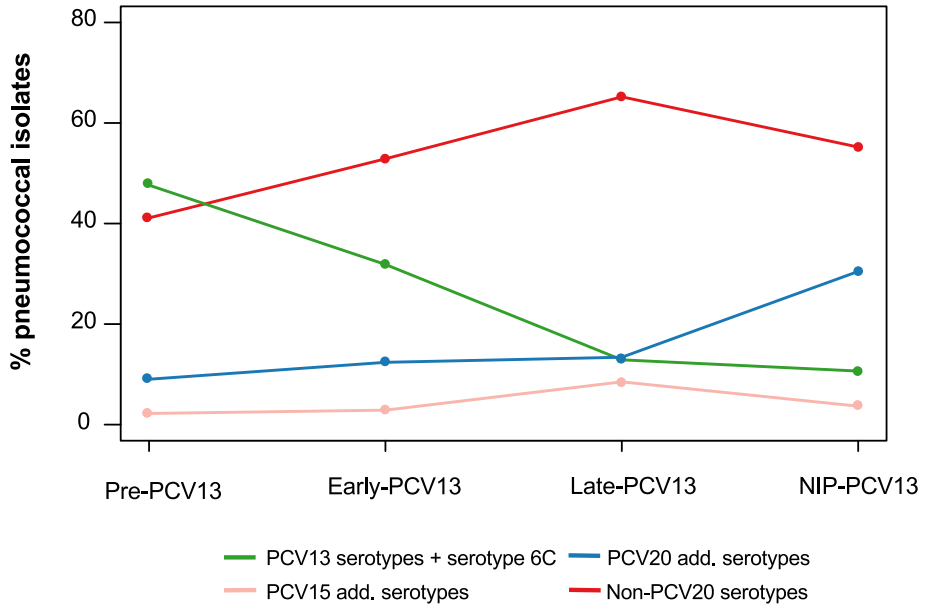

Fig. 1. Prevalence of PCV13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) plus serotype 6C, PCV15 additional serotypes (PCV15 add; 22F and 33F), PCV20 additional serotypes (PCV20 add; 8, 10A, 11A, 12F and 15B/C), and non-PCV20 serotypes (all other serotypes and non-capsulated – NT – isolates) over time. Pre-PCV13 (2009–2010), early-PCV13 (2011–2012), late-PCV13 (2015–2016), and NIP-PCV13 (2018–2020). Data from the first three periods was described before [4] and is shown to allow comparison.

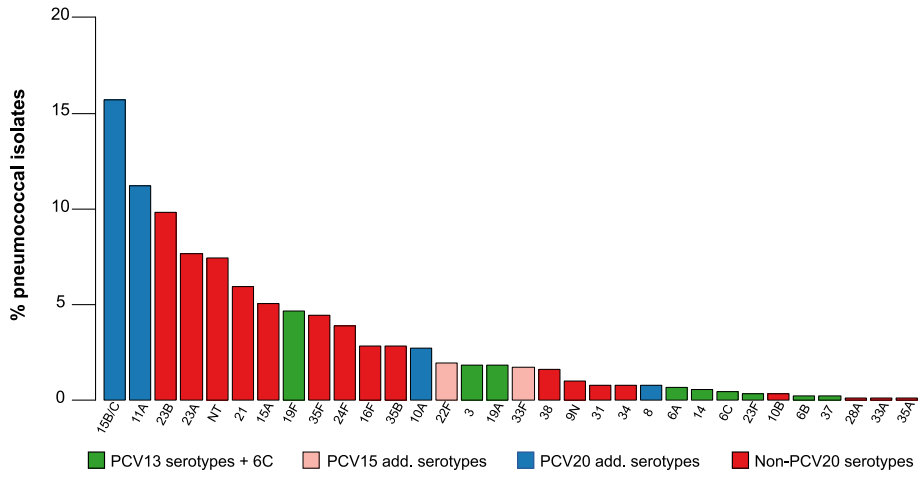

Fig. 2. Serotype distribution of pneumococci in the NIP-PCV13 period (2018–2020).

Table 2
Serotype carriage in PCV13 in NIP period according to vaccination status.

Serotypes	Vaccination status in the NIP-PCV13 period, n (%)[a]				
	Non-vaccinated (n = 79)	Vaccinated with ≥1 dose of PCV13[b] (n = 1290)	p-value (non-vaccinated vs ≥ 1 dose PCV13)	Vaccination with scheme complete[c] (n = 1063)	p-value (non-vaccinated vs 2 + 1 doses PCV13)
PCV13 serotypes plus 6C	10 (12.7)	73 (5.7)	**0.024**	61 (5.7)	**0.025**
3	3 (3.8)	12 (0.9)	**0.051**	11 (1.0)	0.067
6A	0 (0.0)	6 (0.5)	1.000	1 (0.1)	1.000
6B	1 (1.3)	1 (0.1)	0.112	1 (0.1)	0.134
6C	0 (0.0)	4 (0.3)	1.000	4 (0.4)	1.000
14	0 (0.0)	4 (0.3)	1.000	3 (0.3)	1.000
19A	3 (3.8)	10 (0.8)	**0.035**	8 (0.8)	**0.035**
19F	2 (2.5)	34 (2.6)	1.000	32 (3.0)	1.000
23F	1 (1.3)	2 (0.1)	0.122	1 (0.1)	0.134
PCV15 add serotypes	2 (2.5)	30 (2.3)	0.707	23 (2.2)	0.689
PCV20 add serotypes	8 (10.1)	239 (18.5)	0.069	204 (19.2)	**0.050**
Non-PCV20 serotypes	23 (29.1)	433 (33.6)	1.000	359 (33.8)	0.979
All serotypes	43 (54.4)	775 (60.1)	0.345	647 (60.9)	0.284

PCV13 serotypes: 1, 3, 4, 5, 6A, 6B, 7F, 9 V, 14, 18C, 19A, 19F and 23F.
PCV15 add serotypes: 22F and 33F.
PCV20 add serotypes: 8, 10A, 11A, 12F, and 15B/C.
NIP, National Immunization Program.
Statistical differences between groups were assessed with Fisher's exact test.
[a] The vaccination status of 81 children was unknown and these were excluded from the analysis.
[b] A total of 1290 children received at least one dose of PCV13: 1063 completed the vaccination scheme (850 with 2 + 1 doses, 211 with 3 + 1 doses and 2 with 1 + 1 doses), 95 had a vaccination scheme complete for age (e.g. <1 year old) and 132 children had an incomplete vaccination scheme based on age.
[c] The age was unknown for 4 children and these were excluded from the analysis.

High-level resistance to penicillin was mainly due to isolates of serotype 11A (targeted by PCV20, 17 of the 21 high-level resistant isolates); intermediate resistance to penicillin was found in isolates of thirteen distinct serotypes but was mostly due to isolates of serotypes 11A, 23B and NT (76.2 % of 164 intermediately resistant isolates). Resistance to erythromycin was found in isolates of fourteen distinct serotypes but was mostly due to serotypes 15B/C, 19F, 24F, 33F, and NT strains (78.8 % of the 170 isolates resistant to erythromycin). Dual non-susceptibility to penicillin and erythromycin was mostly due to NT isolates (56 % of the 82 isolates with dual non-susceptibility) (Table S2 and Fig. 4).

Regarding other antimicrobial agents, non-susceptibility was inferred through Pathogenwatch for the NIP-PCV13 period isolates. The highest rates of non-susceptibility were observed for SXT (32.9 %), followed by lincosamides (18.1 %), tetracyclines (16.6 %), ceftriaxone (11.4 %), and amoxicillin (7.2 %) (Table S2). None of the isolates was resistant to chloramphenicol. Multidrug resistance, defined as resistance to at least three classes of antimicrobials, occurred in 23.2 % of the 873 isolates which belonged to fourteen distinct serotypes (Table S2).

3.4. Pneumococcal lineages

MLST profiles and GPSC were determined for all 873 pneumococci isolated in the NIP-PCV13 period (Table S2). We observed that the ability of MLST to predict GPSC was very high (adjusted Wallace coefficient ($AW_{MLST \rightarrow GPSC}$) = 0.991 [0.981–1.00]); the reverse, however, was not true ($AW_{GPSC \rightarrow MLST}$ = 0.297 [0.271–0.323]), in agreement with the higher discriminatory power of MLST compared to GPSC.

In total, 114 STs, 73 CC and 46 GPSCs were identified (Table S2). The most common GPSCs were GPSC11 (n = 160, 18.3 %, with isolates of

Table 3
Association of vaccination status and age with carriage of PCV13 serotypes.

Variables	PCV13 serotypes $n = 77$	Non-PCV13 serotypes $n = 684$	OR_{Adj}[a] (95 % CI)	p-value
Vaccination status[b]				
Scheme complete	65	623	Reference	
Non-vaccinated	10	33	**2.5 [1.1–5.6]**	**0.029**
Scheme incomplete	2	28	0.6 [0.1–2.3]	0.109
Age in years				
[1–2[	9	146	Reference	
[2–4[	35	350	1.7 [0.8–3.8]	0.196
[4–6]	33	188	**2.9 [1.3–6.8]**	**0.009**

[a] Results of adjusted linear mixed effects model. The model includes all children with a known vaccination status.
[b] Only children 12 months or older were considered (old enough to have a complete vaccination scheme).

serotypes 15A, 15B/C, 19F and 21), GPSC7 (n = 139, 15.9 %, with isolates of serotypes 23A and 23B), GPSC6 (n = 107, 12.3 %, with isolates of serotypes 11A, 14, 15B/C and 24F), GPSC3 (n = 78, 8.9 %, with isolates of serotypes 8, 11A, 33A and 33F), and GPSC81 (n = 50, 5.7 %, with NT isolates), which, collectively, accounted for 61.1 % of all isolates (Fig. 5).

MLST enabled comparison of pneumococcal lineages in circulation in the NIP-PCV13 period with those identified in previous periods (Fig. 6) [13].

Of the 114 STs, 45 (accounting for 63.1 % of all isolates) were already detected in previous study periods, 37 (accounting for 28.8 % of all isolates) were first detected in the NIP-PCV13 period but were SLVs or DLVs of STs previously detected (suggesting natural evolution within our population), and 32 (8.1 % of all isolates) which were not detected before nor were closely related to STs described in previous periods. Among the latter, 7 STs were associated with PCV13 serotypes: ST232 and ST2049 (serotype 3), ST3111, ST9796, and ST14366 (serotype 19A), and ST423 and ST9972 (associated with serotype 19F) (Fig. 6). The identification of novel STs associated with lineage evolution as well as potentially novel introductions, highlights the importance for continuous genomic surveillance, as new lineages, which may remain undetected if only serotype is determined, can potentially evade the immune system or have different transmission and disease potential [35–37].

We then focused on the population structure of the most frequent serotypes, 15B/C, 11A, 23B, 23A, NT, 21 and 15A (in decreasing order of prevalence). In the NIP-PCV13 period, serotype 15B/C thrived due to the enormous expansion of ST1262 and associated STs (CC8711, GPSC11, also associated with serotypes 15A, 19F and 21 in our collection), which, together, accounted for 72 % of the 137 serotype 15B/C isolates. In this lineage, all isolates but one were susceptible to all antibiotics except SXT. The second most frequent 15B/C lineage, associated with ST162 and ST14368 (DLVs of each other, CC156, GPSC6, also associated with serotypes 14, 15B/C and 24F in our collection) accounted for 13.9 % of 15B/C isolates and included several isolates associated with multidrug resistance (Table S2).

Among serotype 11A, ST62 (GPSC3, also associated with serotype 8), together with ST6521 (CC156, GPSC6) that emerged in the NIP-PCV13 period, were the most prevalent. The latter was responsible for most of the non-susceptibility to penicillin and resistance to erythromycin associated to this serotype (Table S2).

All serotype 23A isolates and all but one serotype 23B isolates were of STs previously identified (or of associated STs), constituting a single lineage (CC439, GPSC7), mostly susceptible to antibiotics.

Most (79.5 %) serotype 15A isolates and approximately half of the serotype 21 isolates belonged to CC193 (GPSC11, described above) identified before in our studies. The remaining serotype 21 isolates belonged to ST432, also detected before.

NT strains were mostly associated with CC344 (GPSC81) and CC448 (GPSC60) previously found in our studies (76.9 % and 10.8 % of all NT, respectively) (Table S2).

3.5. Potential capsular switch events

Capsular switch events naturally occur in the *S. pneumoniae* population due to its inherent competence. Lineages well-suited for colonization that express non-vaccine serotypes, including those resulting from a capsular switch from a vaccine type (VT) to a non-vaccine type (NVT), tend to be selected in the population with the use of PCVs. Thus,

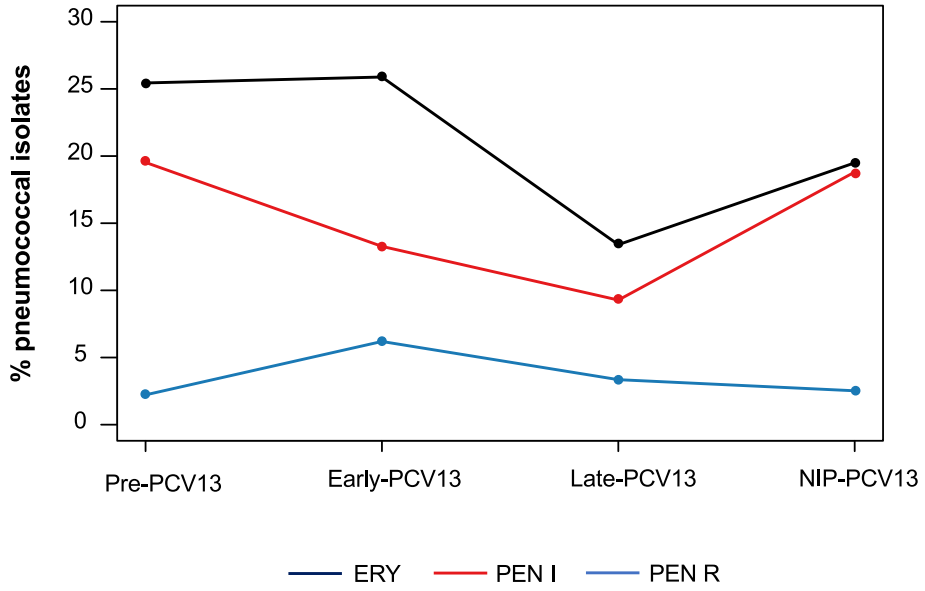

Fig. 3. Levels of penicillin and erythromycin non-susceptibility among pneumococci. Pre-PCV13 (2009–2010), early-PCV13 (2011–2012), late-PCV13 (2015–2016), and NIP-PCV13 (2018–2020); ERY, resistance to erythromycin; PEN I, low-level resistance to penicillin; PEN R, high-level resistance to penicillin. Data from the first three periods was described before [4] and is shown to allow comparison.

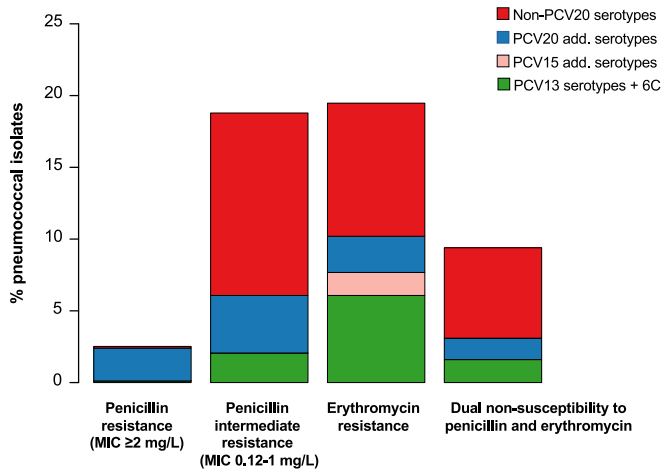

Fig. 4. Proportion of pneumococcal isolates showing intermediate resistance to penicillin and/or resistance to erythromycin in the NIP-PCV13 period (2018–2020). PCV13 serotypes plus serotype 6C: 1, 3, 4, 5, 6A, 6B, 6C, 7F, 9V, 14, 18C, 19A, 19F and 23F; PCV15 add: 22F and 33F; PCV20 add: 8, 10A, 11A, 12F and 15B/C and non-PCV20 serotypes: all other serotypes and non-capsulated (NT) isolates.

the use of PCVs may indirectly select for capsular switch events that result in vaccine-escape lineages, thereby altering population dynamics [13,38].

In the NIP-PCV13 period, an association between ST and serotype continued to be observed for most isolates. There were, however, five cases in which a ST was associated with more than one serotype, suggesting that an event of capsular switch occurred at a given time point. This was the case for ST42–23A/23B (GPSC7), ST162–24F/15(B/C) (GPSC6), ST338–23F/23A (GPSC5), ST673–33F/33A (GPSC3), and ST8959–23B/23A (GPSC7) (Fig. 6). All these associations were described before, either in the MLST or the GPSC databases.

In addition, there were seven cases where isolates which were SLVs of each other expressed different serotypes (Fig. 6). All were detected after the introduction of PCV13 and, in particular, four were only detected in NIP-PCV13 period: ST156–14 and ST166–15B/C (GPSC6), ST156–14 and ST838–11A (GPSC6), ST17325–14 and ST63–15A (GPSC904;9), and ST1228–19F and ST193–21 (GPSC11).

4. Discussion

Continued surveillance of pneumococcal carriage is crucial to monitor changes in vaccine-induced serotype distribution and antimicrobial resistance, as shifts in carriage and disease occur regularly. Our study, along with others conducted worldwide, highlights the importance of this ongoing surveillance [39–41]. This information is essential for making informed decisions about which PCVs to introduce in universal pediatric NIPs, issuing recommendations for vaccinating other at-risk groups based on age or immunocompetence, and informing the design of novel vaccines.

Here we describe pneumococcal serotype distribution, antimicrobial resistance, and genotypes carried by children attending day-care centers in Portugal in 2018–2020 following introduction of PCV13 into the NIP in mid-2015. For comparison, we included data from previous surveillance periods: a pre-PCV13 period (2009–2010), an early-PCV13 period (2011–2012) and a late-PCV13 period (2015–2016). In line with other pediatric vaccines included in the NIP [42], PCV13 uptake was extremely high. In our study, 99.4 % of participants <2 years old had received at least 1 dose of PCV13.

The prevalence of pneumococcal carriage among children up to six years old remained stable at around 60 %. This high prevalence has been consistently observed among day-care center attendees in Portugal and in other countries such as Israel and Iceland [4,40,43]. High transmission rates in day-care centers, contributing to increased prevalence of pneumococcal carriage, have been observed before [44]. While a decrease in pneumococcal carriage prevalence was noted in Canada and Norway after the introduction of PCV13 [39,45], this was not observed in our study nor in studies from other countries, such as France, UK, USA and Israel [40,41,46,47]. The stable prevalence of pneumococcal carriage after introduction of PCV13 is primarily explained by the replacement of PCV13 serotypes with non-PCV13 serotypes well-suited for colonizing young children. In our studies, this was accompanied by an unaltered number of about 30 serotypes in circulation in each period, among a pool of 42 distinct serotypes detected between 2009–2010 and 2018–2020 [4]. Of these, 22 serotypes were common to all periods, and 9 were only detected in a single one. We did not detect significant changes in carriage of PCV13 serotypes (10.7 % in 2018–2020) compared to the previous surveillance period (2015–2016, 12.9 %), as a marked reduction on these serotypes had already been observed by that time [4]. PCV13 serotypes were associated with non-vaccinated children and children over 4 years of age.

In 2018–2020, circulation of PCV13 serotypes was mostly due to serotype 19F (4.7 %) with residual circulation (0–2 %) of other PCV13 serotypes. Circulation of serotype 19F has also been reported in other countries with established PCV13 programs [41,47–49]. In Portugal, most serotype 19F strains belong to the same lineage (PMEN Portugal$^{19F\text{-}177}$, GPSC44) and have been in circulation for over 25 years [13,19]. While in the UK, this lineage, following PCV13 use became associated with non-PCV13 serotypes 7C and serogroup 24, this was not observed in our study [50].

We observed a maintained low prevalence of serotypes 3 and 19A with the few strains that expressed these serotypes being more frequent among non-vaccinated children suggesting some effect of PCV13 on carriage of these serotypes. Understanding the impact of PCVs on carriage of serotype 3 remains challenging and should take into account secular trends in carriage [51], rapidly waning of antibody responses [52], and hypo-responsiveness [53]. In Portugal, when including secular trends, serotype 3 has shown a marked decline in carriage in the past decade, only observed after introduction of PCV13, and in sharp contrast with its increased prevalence during the decade of PCV7 use [51]. Our observations are notable given that a quick decline in the levels of antibodies produced after vaccination leading to reduced long-term protection against serotype 3 have been reported [52], as well as suboptimal immune responses to serotype 3 capsule after vaccination [53].

When focusing on non-PCV13 serotypes, serotypes 11A, 15B/C, 23A, and 23B increased significantly overtime, becoming the most frequent serotypes, and collectively accounting for 44.5 % of all strains. Of these, the first two are targeted by PCV20 and, together, accounted for over one-fourth of all pneumococci. It is therefore anticipated that use of PCV20 could have a significant impact on the pneumococcal population dynamics by decreasing carriage of serotypes 11A and 15B/C.

The hierarchical dominance of serotypes described here has been repeatedly seen in the pneumococcal population following some years upon introduction of conjugate vaccines, suggesting pneumococci have been able to adjust and find a new equilibrium [54]. In addition, this hierarchy appears to have a strong serotype-specific component. In fact, serotypes 11A, 15B/C, 23A and 23B have also become the most frequently carried serotypes among young children in several countries such as UK, Belgium, Hungary, Norway, Sweden, Israel, and USA [23,33,39,47–49,55]. We also noted that serotypes 15A, 21, 24F and 35F, which are becoming increasingly frequent in carriage (collectively accounting for 19.4 % of the isolates); and in particular, 21 and 35(F) were also described among the top 10 serotypes carried by young children in other countries following PCV13 use [39,47–49,55]. As serotype replacement continues to present a challenge for the control of pneumococcal disease, future vaccine development should aim to broaden serotype coverage or target pneumococci universally, regardless of serotype. In addition, tailored multivalent pneumococcal conjugate

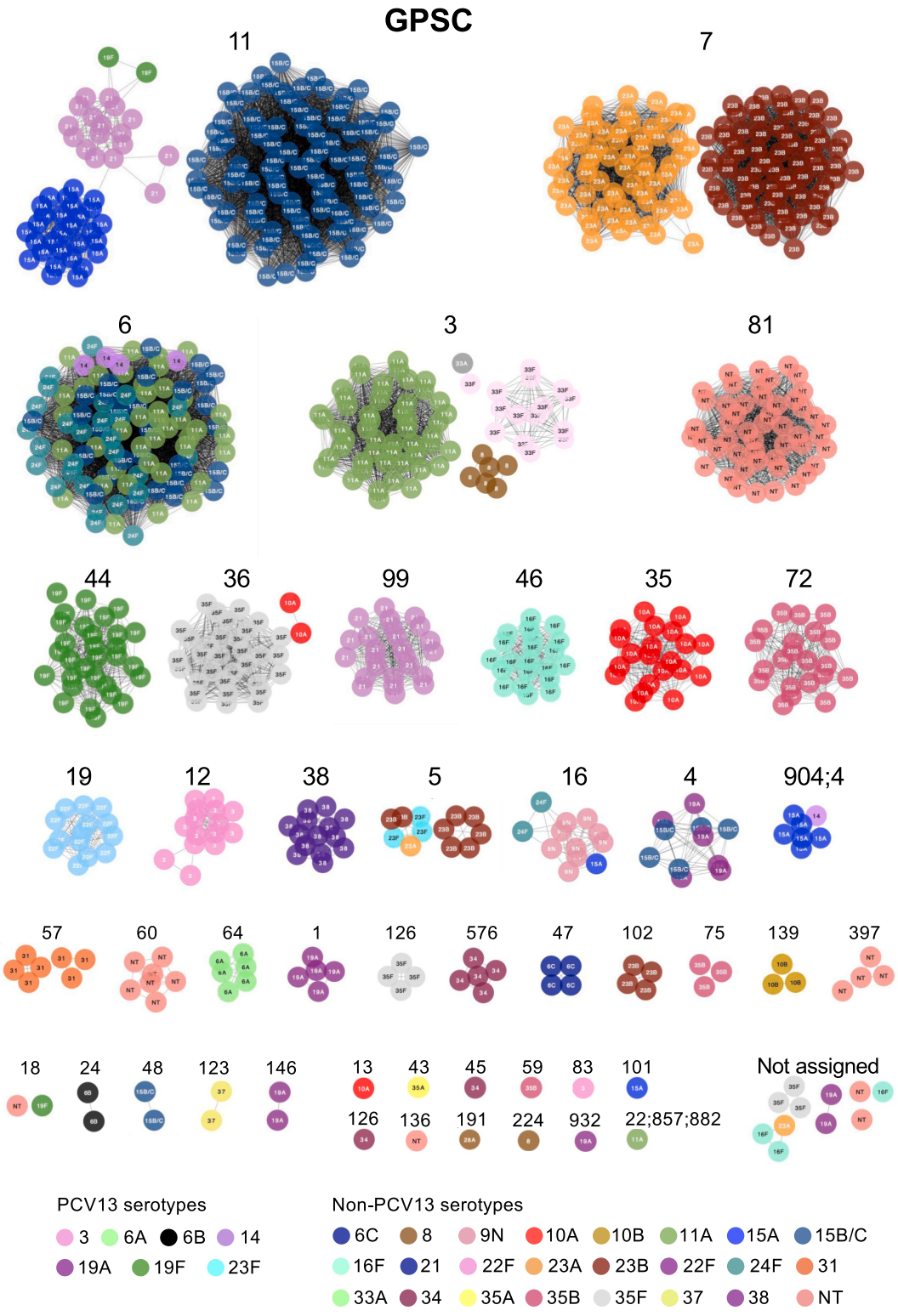

Fig. 5. Global Pneumococcal Sequencing Cluster (GPSC): cytoscape visualization of PopPUNK clusters identified within 873 pneumococci in NIP-PCV13 period. Circles are colored by serotype and labelled by GPSC.

vaccines for specific geographical regions or age groups are also becoming available and may contribute to achieve broader serotype coverage in each setting or population [56,57].

Overall, low-level resistance to penicillin and resistance to macrolides increased in the NIP-PCV13 period compared to the late-PCV13 period being detected in c.a. 20 % of all strains; high-level resistance to penicillin remained infrequent at c.a. 2.4 %. The trends observed in this study align with global patterns of pneumococcal resistance, particularly the rise in low-level penicillin and macrolide resistance [18]. These trends underscore the importance of continuous surveillance to inform treatment protocols and the need for targeted public health initiatives and antibiotic stewardship programs to manage and mitigate resistance effectively [21,22].

To better understand changes in the pneumococcal population, we

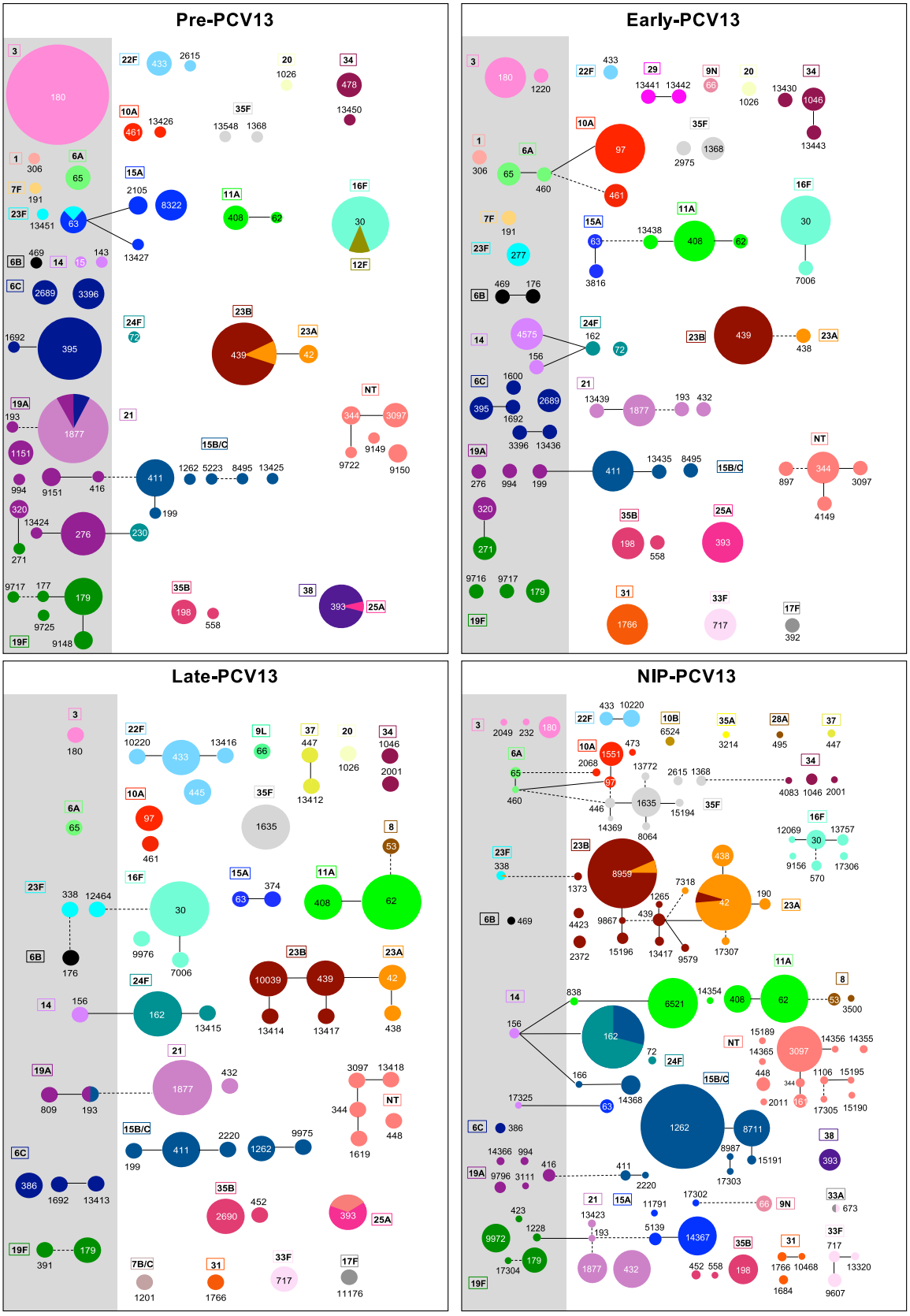

Fig. 6. MLST profiles and serotypes of pneumococci in four periods: Pre-PCV13 period (2009–2010), early-PCV13 period (2011–2012), late-PCV13 period (2015–2016) and NIP-PCV13 period (2018–2020). Each circle represents one ST, which is indicated inside the circle. Each color represents a serotype, which is indicated in the rectangles of the same color. The size of the circle is proportional to the number of isolates. Full lines connect single locus variants (SLVs) and dotted lines connect double-locus variants (DLVs). The grey area highlights serotypes targed by PCV13 + serotype 6C. Data from the first three periods was described before [4] and is shown for comparison.

determined the MLST and GPSC profiles for all strains isolated in the NIP-PCV13 period. MLST profiles enabled direct comparison with our previous study periods, while GPSC facilitated comparison with studies recently conducted worldwide. Most strains (92 %) in circulation in the NIP-PCV13 period belonged to lineages (based on shared MLST profiles, or its SLV or DLV) previously detected in Portugal.

Antimicrobial resistant lineages associated with PCV13-serotypes 6B (CC469), 14 (CC156), 19F (CC179), and 19A (CC411) remained in circulation. Some of these clones have been detected since the 1990's when they were major causes of drug-resistant invasive and non-invasive infections [58,59].

In addition, in the NIP-PCV13 period, we observed expansion of lineages – for example, CC156 and CC193 – mostly linked to non-PCV13 serotypes (11A, 15B/C, 24F; and 15A, 21, respectively) that were traditionally associated with PCV13 serotypes (14, and 19F, respectively). There was also evidence of diversification of lineages linked to non-PCV13 serotypes: for example, serotypes 11A (CC156), 15B/C (CC8711), 23A (CC42), 23B (CC439), and NT (CC344).

We observed that, high level resistance to penicillin remained associated to CC156 isolates now expressing non-PCV13 serotypes 11A and 15B/C. Low-level resistance to penicillin increased to pre-PCV13 period levels mostly due to CC439 (serotype 23B) CC344 (non-encapsulated, NT), and CC156 (11A). Likewise, resistance to macrolides also increased, mostly due to CC156 (15B/C, 24F), CC179 (19F), CC9972 (19F), CC717 (33F), and CC344 (NT). Altogether, these findings show that the pneumococcal population is evolving and adapting to the pressure conferred by PCV13 and antimicrobial use.

Comparison of the GPSC detected in Portugal in the NIP-PCV13 period with those reported abroad revealed several interesting observations. First, GPSC11, which was the most frequent in our study, was associated with serotypes 19F, 15A, 15B/C and 21, and which isolates were mostly susceptible to all antibiotics, has also been reported as one of most prevalent lineages associated with non-PCV13 types causing disease in recent years in other countries such as Brazil and Israel [60,61].

GPSC7, associated with serotypes 23A and 23B, was the second most frequent. While 23A isolates were fully susceptible to antibiotics, several 23B isolates had low-level resistance to penicillin. GPSC7 has also increased in other countries such as UK [50]. Given that it is not targeted by PCV20, and this GPSC has been associated with invasive disease [61], its surveillance deserves special attention.

GPSC6 is particularly noteworthy as it is a global lineage that before PCVs was associated with serotypes 9V/14, frequently expressing penicillin and macrolide resistance and being a major cause of invasive disease worldwide, while also frequent in colonization [7,19,62]. It is now clear that introduction of PCVs did not curtain it, but instead led to its expansion associated with non-PCV13 serotypes such as 11A, 15B/C and 24F detected in our study and in Spain, USA, and Hong Kong, but also 15A, 23A, 23B, and 35B in USA and Peru [63–65]. In Japan, France, Denmark, and UK GPSC6-24F has been linked to an increase of invasive disease cases; in UK an increase in carriage was also described soon after PCV13 introduction [37,65–67]. While the global widespread of serotype 24F has been associated mostly with the multidrug-resistant virulent GPSC10, we have not observed it in our study [37].

GPSC81 was exclusively associated with NT strains not only in Portugal, but also in other countries [68]. Most isolates are multidrug-resistant and typically associated with carriage only [68]. While frequent, we have not seen an increase in these strains.

Our genomic characterization of carried pneumococci has identified specific lineages associated with antimicrobial resistance that are particularly successful in causing colonization and disease in Portugal and other regions. Some of these lineages now express multiple non-vaccine serotypes and continue to disseminate. Monitoring these lineages will be crucial to ensure the adequacy of current treatment guidelines, track the dissemination of antimicrobial resistance, and assess the effectiveness of current and upcoming PCVs.

Our study has some limitations as it was conducted in a single region of Portugal using a convenience sample, which could affect the generalizability of the findings. However, to mitigate these limitations, we included day-care centers that reflect the socio-economic diversity found throughout the country. Previous studies that included other regions of Portugal yielded consistent results across them, suggesting that surveillance of a single region is sufficient to obtain a snapshot of the impact of PCV in the country [4]. It has also some strengths. The current report is part of a long-term surveillance initiative launched in 1996, which has consistently used the same study design to monitor the impact of PCVs in carried pneumococci, providing the chance for robust conclusions despite potential secular trends. Also, the consistent serotyping, antibiotyping and genotyping of isolates enables understanding the origins of serotype shifts, tracking of lineages, unveiling potential capsular switch events, and direct comparison with studies conducted in other settings. The observation that several current antimicrobial-resistant lineages express non-vaccine serotypes but were previously associated with vaccine serotypes highlights the need for continuous genomic surveillance as relying solely on the identification of serotype and antibiogram does not reveal these important changes.

In conclusion, pneumococcal carriage did not change following introduction of PCV13 in the NIP. PCV13 serotypes are now infrequent and mostly associated to non-vaccinated and older children. Intermediate resistance to penicillin and resistance to erythromycin have increased significantly and are mainly associated with non-PCV13 serotypes. Some GPSC associated with non-PCV13 serotypes are expanding. These findings underscore the crucial need for continued surveillance to monitor the evolution of resistant lineages, serotype shifts, and the impact of new pneumococcal vaccines, as well as to guide public health strategies. Beyond robust surveillance, it is essential to implement targeted vaccination programs, antimicrobial stewardship, and educational initiatives. Collaborative efforts among researchers, healthcare providers, and policymakers will be vital to address the evolving landscape of pneumococcal colonization and disease and to develop effective long-term solutions.

Author contributions

The study was conceived by RSL, ABA and HL. RSL contributed with reagents and materials. Data acquisition was done by CC, STA, AS, BF, ARC, MQ and TT. Analysis and interpretation was done by CC, STA, ACP and RSL. The manuscript was drafted by STA, CC, ACP and RSL and critically revised by all authors. All authors read and approved the final version of the manuscript.

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CRediT authorship contribution statement

Catarina Candeias: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Sónia T. Almeida:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **A. Cristina Paulo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Alexandra S. Simões:** Writing – review & editing, Project administration, Investigation. **Bárbara Ferreira:** Writing – review & editing, Investigation.

Ana R. Cruz: Writing – review & editing, Investigation. **Miguel Queirós:** Investigation. **Tiago Touret:** Writing – review & editing, Investigation. **António Brito-Avô:** Writing – review & editing, Validation. **Hermínia de Lencastre:** Writing – review & editing, Validation, Resources. **Raquel Sá-Leão:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

RSL has received consulting and speaking fees from Pfizer and Merck Sharp & Dome. RSL has received funds for unrestricted research grants from Pfizer and Merck Sharp & Dome, paid directly to her institution. ABA has received consulting and speaking fees from Pfizer and Merck Sharp & Dome. All other authors declare they have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.126219.

References

[1] Neal EFG, Chan J, Nguyen CD, Russell FM. Factors associated with pneumococcal nasopharyngeal carriage: a systematic review. PLOS Glob Public Health 2022;2:e0000327.
[2] Weiser JN, Ferreira DM, Paton JC. *Streptococcus pneumoniae*: transmission, colonization and invasion. Nat Rev Microbiol 2018;16:355–67.
[3] Wahl B, O'Brien KL, Greenbaum A, Majumder A, Liu L, Chu Y, et al. Burden of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b disease in children in the era of conjugate vaccines: global, regional, and national estimates for 2000-15. Lancet Glob Health 2018;6:e744–57.
[4] Félix S, Handem S, Nunes S, Paulo AC, Candeias C, Valente C, et al. Impact of private use of the 13-valent pneumococcal conjugate vaccine (PCV13) on pneumococcal carriage among Portuguese children living in urban and rural regions. Vaccine 2021;39:4524–33.
[5] Ganaie FA, Saad JS, Lo SW, McGee L, Bentley SD, van Tonder AJ, et al. Discovery and characterization of pneumococcal serogroup 36 capsule subtypes, serotypes 36A and 36B. J Clin Microbiol 2023;61:e0002423.
[6] CDC. Pneumococcal Vaccination. Vaccines and Preventable Diseases. https://www.cdc.gov/vaccines/vpd/pneumo/index.html2023. Accessed on April 23, 2023.
[7] Nunes S, Félix S, Valente C, Simões AS, Tavares DA, Almeida ST, et al. The impact of private use of PCV7 in 2009 and 2010 on serotypes and antimicrobial resistance of *Streptococcus pneumoniae* carried by young children in Portugal: comparison with data obtained since 1996 generating a 15-year study prior to PCV13 introduction. Vaccine 2016;34:1648–56.
[8] Direcção Geral de Saúde. Norma 008/2005-Programa Nacional de Vacinação. Introdução da vacina conjugada de 13 valências contra infeções por *Streptococcus pneumoniae* (Pn13). 2015.
[9] Kislaya I, Rodrigues AP, Sousa-Uva M, Gómez V, Gonçalves P, Froes F, et al. Indirect effect of 7-valent and 13-valent pneumococcal conjugated vaccines on pneumococcal pneumonia hospitalizations in elderly. PLoS One 2019;14:e0209428.
[10] Instituto Nacional de Estatistica (INE). Available at, http://www.ine.pt/. Accessed on July 25, 2024.
[11] Sá-Leão R, Tomasz A, Sanches IS, Nunes S, Alves CR, Avô AB, et al. Genetic diversity and clonal patterns among antibiotic-susceptible and -resistant *Streptococcus pneumoniae* colonizing children: day care centers as autonomous epidemiological units. J Clin Microbiol 2000;38:4137–44.
[12] Sá-Leão R, Nunes S, Brito-Avô A, Frazão N, Simões AS, Crisóstomo MI, et al. Changes in pneumococcal serotypes and antibiotypes carried by vaccinated and unvaccinated day-care Centre attendees in Portugal, a country with widespread use of the seven-valent pneumococcal conjugate vaccine. Clin Microbiol Infect 2009;15:1002–7.
[13] Candeias C, Félix S, Handem S, de Lencastre H, Sá-Leão R. Clonal changes in the pneumococcal population carried by Portuguese children during six years of private use of the 13-valent pneumococcal conjugate vaccine: the relative contribution of clonal expansion, clonal emergence, and capsular switch events. Microbiol Spectr 2023;11:e0290922.
[14] Aguiar SI, Brito MJ, Goncalo-Marques J, Melo-Cristino J, Ramirez M. Serotypes 1, 7F and 19A became the leading causes of pediatric invasive pneumococcal infections in Portugal after 7 years of heptavalent conjugate vaccine use. Vaccine 2010;28:5167–73.
[15] Silva-Costa C, Brito MJ, Aguiar SI, Lopes JP, Ramirez M, Melo-Cristino J, et al. Dominance of vaccine serotypes in pediatric invasive pneumococcal infections in Portugal (2012-2015). Sci Rep 2019;9:6.
[16] Silva-Costa C, Gomes-Silva J, Prados L, Ramirez M, Melo-Cristino J, On Behalf Of The Portuguese Group For The Study Of Streptococcal Infections, et al. Pediatric invasive pneumococcal disease three years after PCV13 introduction in the national immunization plan-the continued importance of serotype 3. Microorganisms 2021;9(7):1428.
[17] Simell B, Auranen K, Kayhty H, Goldblatt D, Dagan R, O'Brien KL. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines 2012;11:841–55.
[18] Watkins ER, Kalizang'Oma A, Gori A, Gupta S, Heyderman RS. Factors affecting antimicrobial resistance in *Streptococcus pneumoniae* following vaccination introduction. Trends Microbiol 2022;30:1135–45.
[19] Simões AS, Pereira L, Nunes S, Brito-Avô A, de Lencastre H, Sá-Leão R. Clonal evolution leading to maintenance of antibiotic resistance rates among colonizing Pneumococci in the PCV7 era in Portugal. J Clin Microbiol 2011;49:2810–7.
[20] DGS. Direção-Geral da Saúde: Programa de Prevenção e Controlo de Infeções e de Resistência aos Antimicrobianos. 2017.
[21] European Centre for Disease Prevention and Control. Antimicrobial resistance in the EU/EEA (EARS-Net) - annual epidemiological report 2022. Stockholm: ECDC; 2023.
[22] WHO. WHO Bacterial Priority Pathogens List, 2024: Bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. Geneva: WHO; 2024.
[23] Ben-Shimol S, Givon-Lavi N, Kotler L, Adriaan van der Beek B, Greenberg D, Dagan R. Post-13-valent pneumococcal conjugate vaccine dynamics in young children of serotypes included in candidate extended-spectrum conjugate vaccines. Emerg Infect Dis 2021;27:150–60.
[24] Cooper D, Yu X, Sidhu M, Nahm MH, Fernsten P, Jansen KU. The 13-valent pneumococcal conjugate vaccine (PCV13) elicits cross-functional opsonophagocytic killing responses in humans to *Streptococcus pneumoniae* serotypes 6C and 7A. Vaccine 2011;29:7207–11.
[25] Satzke C, Turner P, Virolainen-Julkunen A, Adrian PV, Antonio M, Hare KM, et al. Standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*: updated recommendations from the World Health Organization Pneumococcal Carriage Working Group. Vaccine 2013;32:165–79.
[26] CLSI. Performance standards for antimicrobial susceptibility testing; Tenth Edition M02-A12. Wayne, PA, USA: CLSI; 2020.
[27] Epping L, van Tonder AJ, Gladstone RA, The Global Pneumococcal Sequencing Consortium, Bentley SD, Page AJ, et al. SeroBA: rapid high-throughput serotyping of *Streptococcus pneumoniae* from whole genome sequence data. Microb Genom 2018;4.
[28] Francisco AP, Bugalho M, Ramirez M, Carriço JA. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics 2009;10:152.
[29] Gladstone RA, Lo SW, Lees JA, Croucher NJ, van Tonder AJ, Corander J, et al. International genomic definition of pneumococcal lineages, to contextualise disease, antibiotic resistance and vaccine impact. EBioMedicine 2019;43:338–46.
[30] Lees JA, Harris SR, Tonkin-Hill G, Gladstone RA, Lo SW, Weiser JN, et al. Fast and flexible bacterial genomic epidemiology with PopPUNK. Genome Res 2019;29:304–16.
[31] R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available online at https://www.R-project.org/. 2015.
[32] Severiano A, Pinto FR, Ramirez M, Carriço JA. Adjusted Wallace coefficient as a measure of congruence between typing methods. J Clin Microbiol 2011;49:3997–4000.
[33] Desmet S, Wouters I, Heirstraeten LV, Beutels P, Van Damme P, Malhotra-Kumar S, et al. In-depth analysis of pneumococcal serotypes in Belgian children (2015-2018): diversity, invasive disease potential, and antimicrobial susceptibility in carriage and disease. Vaccine 2021;39:372–9.
[34] Kielbik K, Pietras A, Jablonska J, Bakiera A, Borek A, Niedzielska G, et al. Impact of pneumococcal vaccination on nasopharyngeal carriage of *Streptococcus pneumoniae* and microbiota profiles in preschool children in South East Poland. Vaccines (Basel) 2022;10.
[35] Kalizang'oma A, Swarthout TD, Mwalukomo TS, Kamng'ona A, Brown C, Msefula J, et al. Clonal expansion of a *Streptococcus pneumoniae* serotype 3 capsule

variant sequence type 700 with enhanced vaccine escape potential after 13-valent pneumococcal conjugate vaccine introduction. J Infect Dis 2024;230:e189–98.
[36] Sekulovic O, Gallagher C, Lee J, Hao L, Zinonos S, Tan CY, et al. Evidence of reduced virulence and increased colonization among pneumococcal isolates of serotype 3 clade II lineage in mice. J Infect Dis 2024;230:e182–8.
[37] Lo SW, Mellor K, Cohen R, Alonso AR, Belman S, Kumar N, et al. Emergence of a multidrug-resistant and virulent *Streptococcus pneumoniae* lineage mediates serotype replacement after PCV13: an international whole-genome sequencing study. Lancet Microbe 2022;3:e735–43.
[38] Wyres KL, Lambertsen LM, Croucher NJ, McGee L, von Gottberg A, Liñares J, et al. Pneumococcal capsular switching: a historical perspective. J Infect Dis 2013;207:439–49.
[39] Løvlie A, Vestrheim DF, Aaberge IS, Steens A. Changes in pneumococcal carriage prevalence and factors associated with carriage in Norwegian children, four years after introduction of PCV13. BMC Infect Dis 2020;20:29.
[40] Danino D, Ben-Shimol S, van der Beek BA, Givon-Lavi N, Avni YS, Greenberg D, et al. Decline in pneumococcal disease in young children during the coronavirus disease 2019 (COVID-19) pandemic in Israel associated with suppression of seasonal respiratory viruses, despite persistent pneumococcal carriage: a prospective cohort study. Clin Infect Dis 2022;75:e1154–64.
[41] Cleary DW, Jones J, Gladstone RA, Osman KL, Devine VT, Jefferies JM, et al. Changes in serotype prevalence of *Streptococcus pneumoniae* in Southampton, UK between 2006 and 2018. Sci Rep 2022;12:13332.
[42] Fonseca IC, Pereira AI, Barros L. Portuguese parental beliefs and attitudes towards vaccination. Health Psychol Behav Med 2021;9:422–35.
[43] Quirk SJ, Haraldsson G, Erlendsdóttir H, Hjálmarsdóttir M, van Tonder AJ, Hrafnkelsson B, et al. Effect of vaccination on pneumococci isolated from the nasopharynx of healthy children and the middle ear of children with otitis media in Iceland. J Clin Microbiol 2018;56.
[44] Sá-Leão R, Nunes S, Brito-Avô A, Alves CR, Carriço JA, Saldanha J, et al. High rates of transmission of and colonization by *Streptococcus pneumoniae* and *Haemophilus influenzae* within a day care center revealed in a longitudinal study. J Clin Microbiol 2008;46:225–34.
[45] Ricketson LJ, Wood ML, Vanderkooi OG, MacDonald JC, Martin IE, Demczuk WH, et al. Trends in asymptomatic nasopharyngeal colonization with *Streptococcus pneumoniae* after introduction of the 13-valent pneumococcal conjugate vaccine in Calgary. Canada Pediatr Infect Dis J 2014;33:724–30.
[46] Rybak A, Levy C, Angoulvant F, Auvrignon A, Gembara P, Danis K, et al. Association of nonpharmaceutical interventions during the COVID-19 pandemic with invasive pneumococcal disease, pneumococcal carriage, and respiratory viral infections among children in France. JAMA Netw Open 2022;5:e2218959.
[47] Kaur R, Casey JR, Pichichero ME. Emerging *Streptococcus pneumoniae* strains colonizing the nasopharynx in children after 13-valent pneumococcal conjugate vaccination in comparison to the 7-valent era, 2006-2015. Pediatr Infect Dis J 2016;35:901–6.
[48] Galanis I, Lindstrand A, Darenberg J, Browall S, Nannapaneni P, Sjöström K, et al. Effects of PCV7 and PCV13 on invasive pneumococcal disease and carriage in Stockholm. Sweden Eur Respir J 2016;47:1208–18.
[49] Tiley KS, Ratcliffe H, Voysey M, Jefferies K, Sinclair G, Carr M, et al. Nasopharyngeal carriage of pneumococcus in children in England up to 10 years after 13-valent pneumococcal conjugate vaccine introduction: persistence of serotypes 3 and 19A and emergence of 7C. J Infect Dis 2023;227:610–21.
[50] Sheppard CL, Groves N, Andrews N, Litt DJ, Fry NK, Southern J, et al. The Genomics of *Streptococcus pneumoniae* carriage isolates from UK children and their household contacts, pre-PCV7 to post-PCV13. Genes (Basel) 2019;10.
[51] Paulo AC, Sá-Leão R. Periodic cycles of pneumococcal serotypes carried by children before and after 7-valent pneumococcal conjugate vaccine. PLoS One 2017;12:e0176723.
[52] De Wals P. Commentary on paradoxical observations pertaining to the impact of the 13-valent pneumococcal conjugate vaccine on serotype 3 *Streptococcus pneumoniae* infections in children. Vaccine 2018;36:5495–6.
[53] Poolman J, Borrow R. Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines. Expert Rev Vaccines 2011;10:307–22.
[54] Paulo AC, Sá-Leão R. Evidence for the intermediate disturbance hypothesis and exponential decay in replacement in *Streptococcus pneumoniae* following use of conjugate vaccines. Sci Rep 2022;12:7510.
[55] Kovács E, Sahin-Tóth J, Tóthpál A, Kristóf K, van der Linden M, Tirczka T, et al. Vaccine-driven serotype-rearrangement is seen with latency in clinical isolates: comparison of carried and clinical pneumococcal isolates from the same time period in Hungary. Vaccine 2019;37:99–108.
[56] Briles DE, Paton JC, Mukerji R, Swiatlo E, Crain MJ. Pneumococcal Vaccines. Microbiol Spectr 2019;7.
[57] Oliveira GS, Oliveira MLS, Miyaji EN, Rodrigues TC. Pneumococcal vaccines: past findings, present work, and future strategies. Vaccines (Basel) 2021;9.
[58] Aguiar SI, Brito MJ, Horacio AN, Lopes JP, Ramirez M, Melo-Cristino J, et al. Decreasing incidence and changes in serotype distribution of invasive pneumococcal disease in persons aged under 18 years since introduction of 10-valent and 13-valent conjugate vaccines in Portugal, July 2008 to June 2012. Euro Surveill 2014;19:20750.
[59] Henriques-Normark B, Blomberg C, Dagerhamn J, Bättig P, Normark S. The rise and fall of bacterial clones: *Streptococcus pneumoniae*. Nat Rev Microbiol 2008;6:827–37.
[60] Almeida SCG, Lo SW, Hawkins PA, Gladstone RA, Cassiolato AP, Klugman KP, et al. Genomic surveillance of invasive. Genomic surveillance of invasive *Streptococcus pneumoniae* isolates in the period pre-PCV10 and post-PCV10 introduction in Brazil. Microb Genom 2021;7(10):000635.
[61] Lo SW, Gladstone RA, van Tonder AJ, Lees JA, du Plessis M, Benisty R, et al. Pneumococcal lineages associated with serotype replacement and antibiotic resistance in childhood invasive pneumococcal disease in the post-PCV13 era: an international whole-genome sequencing study. Lancet Infect Dis 2019;19:759–69.
[62] Rybak A, Levy C, Ouldali N, Bonacorsi S, Béchet S, Delobbe JF, et al. Dynamics of antibiotic resistance of *Streptococcus pneumoniae* in France: a pediatric prospective nasopharyngeal carriage study from 2001 to 2022. Antibiotics (Basel) 2023;12.
[63] González-Díaz A, Càmara J, Ercibengoa M, Cercenado E, Larrosa N, Quesada MD, et al. Emerging non-13-valent pneumococcal conjugate vaccine (PCV13) serotypes causing adult invasive pneumococcal disease in the late-PCV13 period in Spain. Clin Microbiol Infect 2020;26:753–9.
[64] Bentley S, McGee L, Lo S. The global pneumococcal sequencing project. https://www.pneumogen.net/gps/.
[65] Cao H, Chiu KH, Chiu SS, Jiang S, Chow KH, Ho PL. Genomic investigation of a *Streptococcus pneumoniae* serotype 24F strain causing meningoencephalitis in Hong Kong. Int J Med Microbiol 2021;311:151543.
[66] Kavalari ID, Fuursted K, Krogfelt KA, Slotved HC. Molecular characterization and epidemiology of *Streptococcus pneumoniae* serotype 24F in Denmark. Sci Rep 2019;9:5481.
[67] Southern J, Andrews N, Sandu P, Sheppard CL, Waight PA, Fry NK, et al. Pneumococcal carriage in children and their household contacts six years after introduction of the 13-valent pneumococcal conjugate vaccine in England. PLoS One 2018;13:e0195799.
[68] Hilty M, Wüthrich D, Salter SJ, Engel H, Campbell S, Sá-Leão R, et al. Global phylogenomic analysis of nonencapsulated *Streptococcus pneumoniae* reveals a deep-branching classic lineage that is distinct from multiple sporadic lineages. Genome Biol Evol 2014;6:3281–94.