How nasopharyngeal pneumococcal carriage evolved during and after a PCV13-to-PCV10 vaccination programme switch in Belgium, 2016 to 2018

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Citation style for this article: Wouters Ine , Desmet Stefanie , Van Heirstraeten Liesbet , Herzog Sereina A , Beutels Philippe , Verhaegen Jan , Goossens Herman , Van Damme Pierre , Malhotra-Kumar Surbhi , Theeten Heidi , NPcarriage Study Group . How nasopharyngeal pneumococcal carriage evolved during and after a PCV13-to-PCV10 vaccination programme switch in Belgium, 2016 to 2018. Euro Surveill. 2020;25(5):pii=1900303. https://doi.org/10.2807/1560-7917.ES.2020.25.5.1900303

Article submitted on 16 May 2019 / accepted on 10 Dec 2019 / published on 06 Feb 2020

Background: The current carriage study was set up to reinforce surveillance during/after the PCV13-to-PCV10 switch in Belgium. Aim: This observational study monitored carriage of Streptococcus pneumoniae (Sp) serotypes, particularly those no longer covered (3, 6A, 19A), as well as Haemophilus influenzae (Hi), because PCV10 contains the non-typeable Hi protein D. Methods: A total of 2,615 nasopharyngeal swabs from children (6–30 months old) attending day care were collected in three periods over 2016–2018. Children’s demographic and clinical characteristics and vaccination status were obtained through a questionnaire. Sp and Hi were identified by culture and PCR. Pneumococcal strains were tested for antimicrobial (non-)susceptibility by disc diffusion and sero-typed by Quellung-reaction (Quellung-reaction and PCR for serotypes 3, 6A, 19A). Results: The carriage prevalence of Sp (> 75%) remained stable over the successive periods but that of Hi increased (87.4%, 664 Hi-carriers/760 in 2016 vs 93.9%, 895/953 in 2017–2018). The proportion of non-PCV13 vaccine serotypes decreased (94.6%, 438 isolates/463 in 2016 vs 89.7%, 599/668 in 2017–2018) while that of PCV13-non-PCV10 vaccine serotypes (3 + 6A + 19A) increased (0.9%, 4 isolates/463 in 2016 vs 7.8%, 52/668 in 2017–2018), with serotype 19A most frequently identified (87.9%, 58/66 isolates). Non-susceptibility of pneumococci against any of the tested antibiotics was stable over the study period (> 44%).

Conclusions: During and after the PCV13-to-PCV10 vaccine switch, the proportion of non-PCV13 serotypes decreased, mainly due to a serotype 19A carriage prevalence increase. These results complement invasive pneumococcal disease surveillance data, providing further basis for pneumococcal vaccination programme policy making.

Introduction

Nasopharyngeal carriage of Streptococcus pneumoniae (Sp) frequently occurs asymptotically [1-5]. Nevertheless, it may evolve to respiratory infections such as otitis media and pneumonia or even invasive diseases including bacteremia and meningitis [2,3,5]. Besides the elderly, young children are prone to (invasive) pneumococcal diseases (IIPD) [6-10]. Before pneumococcal conjugate vaccines (PCVs) were introduced, the global annual number of serious pneumococcal disease cases (pneumonia, meningitis, and bacteremia) in children under 5 years of age was estimated to be 14.5 million [11].

The primary virulence factor of Sp is its polysaccharide capsule, which also determines the serotype. More than 95 serotypes exist and they vary in their capacity to activate the host immune system and to invade [12-15]. PCVs provide direct protection to the vaccinated individuals against a number of clinically relevant serotypes [12]. In addition, the wider population experiences indirect protection against pneumococcal...
disease through reduced nasopharyngeal carriage of pneumococcal vaccine serotypes (VTs). However, the observed magnitude of this indirect effect varies in different contexts, and it is eroded by the rising incidence of non-VT-(NVT-)related diseases [16]. Several studies on carriage or IPD in the pre- and post-PCV era reported on serotype replacement, i.e. VTs being largely replaced by NVTs [17,18]. Furthermore, co-colonisation with other pathogens such as *Haemophilus influenzae* (Hi), *Moraxella catarrhalis* (Mc), *Staphylococcus aureus* (Sa), and *Streptococcus pyogenes* (GAS) may be changed after PCV-introduction because of mutual interactions [19-21].

Belgium initiated a universal childhood PCV-programme according to a two plus one schedule in 2007 (at 8 weeks, 16 weeks, and 12 months of age). The seven-valent vaccine (PCV7, including serotypes 4, 6B, 9V, 14, 18C, 19F, 23F) was superseded by the 13-valent vaccine (PCV13, including PCV7 serotypes plus 1, 5, 7F, 3, 6A, 19A, same 2+1 schedule) in 2011, which was in turn replaced by the 10-valent vaccine (PCV10, including PCV7 serotypes plus 1, 5, 7F, same 2+1 schedule) in 2015–2016. The implementation of immunisation programmes constitutes a regional responsibility in Belgium. PCV10 was introduced in the Flemish (Northern) region in July 2015 and in the Walloon (Southern) region in May 2016 [22]. In the Brussels (Capital) region either the Flemish or the Walloon programme was followed, depending on the consulting physician. The pneumococcal vaccination programme rapidly achieved high three-dose coverage in children (coverage in Belgium; > 80% in all regions in 2008–2009 vs > 94% in all regions in 2015–2016 [23-26]) and the overall incidence of IPD in Belgium significantly decreased after implementation of the vaccination programme; post-PCV7 period (2007–2010) vs pre-PCV7 period (pre 2007): decrease of 35%; post-PCV13 (2015) vs PCV7-era (2007–2010): decrease of 42% [22].

The current carriage study was set up to reinforce surveillance after the PCV13-to-PCV10 vaccination programme switch, in order to monitor the three pneumococcal serotypes that were no longer covered (3, 6A, 19A), as well as Hi, because PCV10 contains the non-typeable Hi (NTHi) protein D. To this end, we studied nasopharyngeal carriage of Sp and Hi in children between 6 and 30 months of age attending day care centres (DCCs) during three consecutive periods between 2016 and 2018. High pneumococcal carriage rates (range: 21–89%) have been reported in young children attending day care [17,27-29]. As such, the

### Table 1

Demographic and clinical characteristics of the healthy child population in day care per period, Belgium, 2016–2018 (n = 760 children in 2016, 902 in 2016–2017, 953 in 2017–2018)

| Characteristics | Period 1 (N = 760) | Period 2 (N = 902) | Period 3 (N = 953) | p value chi² for trend |
|-----------------|-------------------|-------------------|-------------------|-----------------------|
| **Region**      |                   |                   |                   |                       |
| Wallonia        | 353 (46.4)        | 287 (31.8)        | 282 (29.6)        | <0.001                |
| Flanders        | 332 (43.7)        | 488 (54.1)        | 552 (57.9)        |                       |
| Brussels        | 75 (9.9)          | 127 (14.1)        | 119 (12.5)        |                       |
| **Age in months** |                 |                   |                   |                       |
| 6–12            | 98 (12.9)         | 217 (24.1)        | 209 (21.9)        | <0.001                |
| 13–24           | 415 (54.6)        | 457 (50.7)        | 528 (55.4)        |                       |
| 25–30           | 247 (32.5)        | 228 (25.3)        | 216 (22.7)        |                       |
| **Sex**         |                   |                   |                   |                       |
| Male            | 387 (50.9)        | 455 (50.4)        | 469 (49.2)        | 0.474                 |
| **Preterm delivery** |               |                   |                   |                       |
| Yes             | 60 (8.0)          | 71 (7.9)          | 78 (8.2)          | 0.872                 |
| **Breastfeeding** |                 |                   |                   |                       |
| Yes             | 230 (30.4)        | 289 (32.1)        | 336 (35.4)        | 0.026                 |
| **Parental smoking** |              |                   |                   |                       |
| Yes             | 170 (22.4)        | 183 (20.4)        | 190 (20.0)        | 0.231                 |
| **Siblings**    |                   |                   |                   |                       |
| Yes             | 459 (62.9)        | 548 (61.0)        | 599 (63.3)        | 0.813                 |
| **Common cold symptoms** |           |                   |                   |                       |
| Yes             | 169 (22.4)        | 344 (38.2)        | 429 (45.0)        | <0.001                |
| **AOM-history** |                   |                   |                   |                       |
| Yes             | 258 (34.8)        | 225 (25.5)        | 199 (21.8)        | <0.001                |
| **AB > 3 months** |                 |                   |                   |                       |
| Yes             | 248 (33.4)        | 254 (30.5)        | 217 (23.5)        | <0.001                |

AB: antibiotic; AOM: acute otitis media.

* Due to missing information on some characteristics for some children, the denominator can at times differ from 'N' in the column heading.

* Child was breastfed for more than 6 months.

* At least one parent smokes.

* Child with a history of AOM based on parental recall.

* Use of antibiotics in the 3 months before sampling.

Significant p values (<0.05) are indicated in bold.
**Figure 1**

(A) Vaccination status of healthy children in day care (n = 760 in 2016, 902 in 2016–2017, 952 in 2017–2018) and (B) proportions of vaccine and non-vaccine serotypes among *Streptococcus pneumoniae* carriers (n = 463 carriers in 2016, 613 in 2016–2017, 668 in 2017–2018), Belgium, 2016–2018

### A. Vaccination status of healthy children in day care

- **Period 1** 2016 (n = 760 children)
  - Incomplete: 286 (11.9%)
  - Mixed schedule: 96 (12.6%)
  - PCV13-schedule: 558 (73.4%)

- **Period 2** 2016–2017 (n = 902 children)
  - Incomplete: 231 (14.5%)
  - Mixed schedule: 288 (31.9%)
  - PCV13-schedule: 260 (28.8%)

- **Period 3** 2017–2018 (n = 952 children)
  - Incomplete: 26 (2.7%)
  - Mixed schedule: 223 (23.9%)
  - PCV13-schedule: 731 (77.5%)

### B. Proportions of vaccine and non-vaccine serotypes among *Streptococcus pneumoniae* carriers

- **Period 1** 2016 (n = 463 carriers)
  - STs 1+5+7F: 6 (0.9%)
  - PCV7-VTs: 43 (9.3%)
  - non-PCV7-VTs: 324 (70.6%)

- **Period 2** 2016–2017 (n = 613 carriers)
  - STs 1+5+7F: 9 (1.5%)
  - PCV7-VTs: 33 (5.4%)
  - non-PCV7-VTs: 571 (93.7%)

- **Period 3** 2017–2018 (n = 668 carriers)
  - STs 1+5+7F: 12 (1.8%)
  - PCV7-VTs: 21 (3.2%)
  - non-PCV7-VTs: 646 (96.7%)

*PCV7/10/13: 7/10/13-valent pneumococcal conjugate vaccine; STs: serotypes; VTs: vaccine serotypes.*

- **Note:** Complete results are available in the full report. A total of 952 is used because vaccination status was missing for one of the 953 children in 2017–2018.
- **Note:** Serotypes are determined by Quellung reaction.
- **Note:** Incomplete schedule: children who were not or incompletely vaccinated.
- **Note:** Mixed schedule: children vaccinated with a combination of PCV13 and PCV10.
- **Note:** STs 3+6A+19A: serotypes included in PCV13, but not in PCV10.
- **Note:** STs 1+5+7F: serotypes included in PCV10 but not in PCV7.
- **Note:** PCV7-VTs: vaccine serotypes included in PCV7 (4, 6B, 9V, 14, 18C, 19F, 23F).
- **Note:** Non-PCV13-VTs: vaccine serotypes not included in PCV13.

Children in the study were 6–30 months old. Vaccination status was based on vaccination documentation or parental reporting.
impact of the PCV-programme change was monitored in a random sample of this target population, to complement sentinel laboratory-reported IPD-surveillance. In this paper, we focus on pneumococcal serotype distribution and antimicrobial (non-)susceptibility during and after the PCV13-to-PCV10 vaccination programme switch.

Methods

Ethical statement
The current study was in line with the Declaration of Helsinki, as revised in 2013. Approval to conduct the current study with ID 15/45/471 was obtained from the University of Antwerp and University Hospital of Antwerp ethics committee (Commissie voor Medische Ethiek van UZA/UA) on 30 November 2015.

Study design
The design of this observational study was previously described in detail and is summarised here for the complete study period (from Period 1 in 2016 up to Period 3 in 2017–2018) [30,31].

Nasopharyngeal sampling was performed between March and July in Period 1 (2016) and between November and March in the consecutive periods (Period 2: 2016–2017 and Period 3: 2017–2018). Healthy children were recruited in DCCs randomly selected over the three Belgian regions (Wallonia, Flanders, Brussels), according to a population-proportionate distribution at regional level based on Belgian Federal Government Statistics for the 0–4 year population. In the consecutive periods, 85, 112, and 102 DCCs participated in the study, of which 66 DCCs participated in all three periods, 44 in two periods, and 24 in one period. A population-proportionate sample at the regional level was achieved from 2016 to 2017 onwards, after deliberate over-recruitment in Wallonia in 2016, in order to include a maximum of children who received PCV13 for both primary vaccine doses and their booster, since at that time, PCV10 was not yet introduced in Wallonia. The inclusion criteria were: no treatment with oral antibiotics (ABs) in the 7 days before sampling and age between 6 and 30 months included. An additional age criterion (date of birth before 1 January 2015) was applied in Flanders and Brussels for 2016, in order to exclusively include children who received PCV13 for their primary vaccine doses [30]. In this way, children recruited in the first period were on average older compared with children recruited in the subsequent periods.

Trained nurses and physicians conducted a questionnaire collecting demographic and clinical characteristics of the study participants. The vaccination status of the participating child was based on vaccination documentation or parental reporting. A single nasopharyngeal swab was taken with a flocked nylon fibre swab, transported in 1 mL skim milk-tryptone-glucose-glycerol (STGG) and cultured or stored at −80°C within 24 hours.

Culture analyses
At the National Reference Centre (NRC) for pneumococci, nasopharyngeal samples were plated on blood agar plates for identification of Sp, Mc, Sa, GAS and a selective plate for identification of Hi, following overnight enrichment in brain-heart infusion (BHI) broth (entire study period for blood agar plates, 2016 only for selective plate) and directly (from 2016–2017 onwards for blood agar plates and selective plate). Sp-strains were serotyped via Quellung-reaction. Antimicrobial (non-)susceptibility of the Sp-strains for erythromycin, levofloxacin, penicillin, tetracycline and trimethoprim/sulfamethoxazole was determined by disc diffusion according to the guidelines of Clinical and Laboratory Standards Institute (CLSI; 2016 and 2016–2017) [32] and European Committee on Antimicrobial Susceptibility Testing (EUCAST; 2017–2018) [33]. If non-susceptibility for penicillin or levofloxacin was identified by disc diffusion, the minimum inhibitory concentration (MIC) was determined by Etest (Biomérieux, Craponne, France). A MIC of ≥0.06 mg/L for penicillin or ≥2 mg/L for levofloxacin was interpreted as non-susceptible.

Molecular analyses
DNA was extracted from 200 μL of nasopharyngeal sample and tested in real-time PCR targeting lytA (for Sp) or P6 (for Hi) [30,34,35]. Real-time PCR was performed for Sp on all samples and for Hi on culture-negative samples. Samples were classified as positive for Sp or Hi when cycle threshold (CT) values were ≤40 or ≤35, respectively. LytA-positive samples were pooled and screened for presence of the three pneumococcal serotypes included in PCV13, but not in PCV10 (PCV13-non-PCV10 VTs: 3, 6A, 19A). If found positive for serotype 3, 6A, or 19A, pooled samples were unpoled and positivity of the individual sample was determined. Serotype-specific PCRs were performed in a 20 μL reaction volume containing 2× Taqman Universal PCR Master Mix (Applied Biosystems), 200 nM concentrations of primer and probe and 2 μL of DNA template (pooled PCR-reaction contains four times 2 μL of DNA). Samples positive for 6A real-time PCR were further subjected to 6C real-time PCR targeting wciN to discriminate between serotypes 6A and 6C [38]. The serotype-specific 6C assay was performed as described above, with the exception that higher primer concentrations of 500 nM were used. Amplification was carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, California, United States) using the following cycling parameters: 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 59.5°C (3, 19A) or 60°C (6A, 6C). Serotype-specific PCRs were classified as positive when C values were ≤35.

Samples were considered positive for any of the respective pathogens if either culture or PCR was positive.
The presented carriage prevalences of Sp and Hi were based on culture and PCR-results, whereas overall serotype distribution and antimicrobial (non-)susceptibility were based on Quellung-reaction and culture results respectively. The reported carriage prevalence of serotypes 3, 6A, and 19A was based on Quellung-reaction and PCR-results.

Statistics
Sample size and power were calculated using the R-package ‘power’ [30]. A sample size of 700 children in 2016 and 900 children from the second period onwards allows the detection of 4% changes in carriage prevalence of Sp-serotypes 19A or 6A over the observation period of the study period with 80% power and assuming a starting carriage prevalence below 2%.

In IBM SPSS Statistics 25, the chi-squared (Chi²) or Fisher’s Exact Test (FET) and the Mann–Whitney U Test (MWU) were used to test for significance at a level of 5%. To identify predictors of carrying Sp, PCV13-non-PCV10-VTs, and Hi in DCC-children (three periods pooled), univariate and multiple binary logistic regressions were performed and adjusted using generalised estimating equations (GEEs) with an exchangeable correlation structure since 148 children (303 isolates) contributed more than one sample over the 3-year study period. The GEE model analyses were performed using the statistical software R (version 3.6.1) with the geepack package (version 1.2–1). Variables with a p-value < 0.1 in the univariate analysis were included in the multiple regression analysis. Since no children were sampled in the youngest age category in Flanders and in Brussels in 2016, no adjustments can be made for the different sampling probabilities in the different study years. A continuity correction was applied for 95% confidence intervals (95% CI) on proportions. Missing values were not replaced.

Results

Study population
Over the three successive periods, nasopharyngeal samples from 2,883 children attending DCCs were collected. In total, 2,615 samples (760 in 2016, 902 in 2016–2017, 953 in 2017–2018) – corresponding to 2,621 pneumococcal isolates, as more than one serotype could be found per child (761 in 2016, 904 in 2016–2017, 956 in 2017–2018), were included in the final analyses, i.e. after exclusion of a random selection of 194 samples collected in 2016–2017 (not analysed by PCR as a consequence of over-recruitment), and after exclusion of 74 samples not fulfilling the inclusion criteria regarding age or use of ABs. Of the 2,615 nasopharyngeal samples, 148 (5.7%) originated from children who contributed more than one sample, but never in the same period. The univariate and multiple binary logistic regression models were adjusted for this through GEEs with an exchangeable correlation structure.

The main demographic and clinical characteristics of the child population over the study period are shown in Table 1, with the majority of these characteristics remaining similar over the study period. Nevertheless, a decreasing trend (p < 0.001) was observed for history of acute otitis media (AOM-history) and AB-use in the 3 months before sampling. The proportions of children being breastfed for more than 6 months (p = 0.026) and with symptoms (runny nose and/or cough) of common cold (p < 0.001) increased over the study period. As a result of the recruitment strategy to include older children in 2016 (mean age: 21.0 months in 2016 vs 18.4 months in 2016–2017 vs 18.4 months in 2017–2018), the majority of the Period 1 population was vaccinated with PCV13 only (Figure 1: 73.4%; 558/760 children). The proportion of PCV13-vaccinated children decreased over the study period to 2.7% (26/952 children; vaccination status was missing for one of the 953 children in 2017–2018), whereas the proportion of PCV10-vaccinated children increased from 0.6% to 75.9% (723/952 children).

The presented carriage prevalences of Sp and Hi were based on culture and PCR-results, whereas overall serotype distribution and antimicrobial (non-)susceptibility were based on Quellung-reaction and culture results respectively. The reported carriage prevalence of serotypes 3, 6A, and 19A was based on Quellung-reaction and PCR-results.

Hi: Haemophilus influenzae; Sp: Streptococcus pneumoniae.
\(^*\) Prevalence was inferred from results of culture and PCR combined.
\(^*\) SpHi-carriers: carriers of Sp and Hi.
\(^*\) Hi-carriers: carriers of Hi.
\(^*\) Sp-carriers: carriers of Sp.

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### Table 2A

Predictors through binary logistic regression of *Streptococcus pneumoniae* carriage\(^a\) (n = 2,615 nasopharyngeal samples) and of PCV13-non-PCV10 vaccine serotype carriage\(^b\) (n = 1,744) among children attending day care (pooled over study periods), Belgium, 2016–2018

| Characteristic | Number of samples/isolates | Univariate regression | Multiple regression |
|---------------|---------------------------|-----------------------|---------------------|
|               | n  | %   | OR  | 95% CI\(^d\) | OR  | 95% CI\(^d\) |
| **Predictors of Sp-carriage\(^a\)** | | | | | | |
| **Study period** | | | | | | |
| 2016 | 760 | 29.1 | REF | REF | REF | REF |
| 2016–2017 | 902 | 34.5 | 0.78 | 0.62–0.99 | 0.64 | 0.47–0.87 |
| 2017–2018 | 953 | 36.4 | 0.92 | 0.73–1.16 | 0.71 | 0.52–0.96 |
| **Region** | | | | | | |
| Wallonia | 922 | 35.3 | REF | REF | REF | REF |
| Flanders | 1,372 | 52.5 | 1.29 | 1.06–1.57 | 1.09 | 0.83–1.42 |
| Brussels | 321 | 12.3 | 1.42 | 1.04–1.95 | 1.16 | 0.81–1.66 |
| **Sex** | | | | | | |
| Female | 1,304 | 49.9 | REF | REF | REF | REF |
| Male | 1,311 | 50.1 | 0.75 | 0.63–0.91 | 0.76 | 0.62–0.93 |
| **Common cold symptoms\(^e\)** | | | | | | |
| Yes | 942 | 36.1 | REF | REF | REF | REF |
| No | 1,668 | 63.9 | 0.65 | 0.53–0.80 | 0.64 | 0.51–0.80 |
| **Sa-carriage\(^f\)** | | | | | | |
| Yes | 116 | 4.4 | REF | REF | REF | REF |
| No | 2,499 | 95.6 | 1.71 | 1.14–2.55 | 1.79 | 1.13–2.85 |
| **Hi-carriage\(^g\)** | | | | | | |
| Yes | 2,402 | 91.9 | REF | REF | REF | REF |
| No | 213 | 8.1 | 0.58 | 0.43–0.79 | 0.64 | 0.45–0.90 |
| **Mc-carriage\(^g\)** | | | | | | |
| Yes | 2,382 | 91.1 | REF | REF | REF | REF |
| No | 233 | 8.9 | 0.26 | 0.19–0.34 | 0.31 | 0.23–0.42 |
| **Siblings\(^h\)** | | | | | | |
| Yes | 1,606 | 62.4 | REF | REF | REF | REF |
| No | 969 | 37.6 | 0.72 | 0.59–0.87 | 0.73 | 0.61–0.89 |
| **AOM-history\(^i\)** | | | | | | |
| Yes | 682 | 26.9 | REF | REF | REF | REF |
| No | 1,855 | 73.1 | 1.36 | 1.11–1.67 | 1.14 | 0.91–1.44 |

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\(^a\) Knowledge of Sp carriage was through results of culture and PCR combined.

\(^b\) Knowledge of PCV13-non-PCV10 vaccine serotype carriage was based on culture or Quellung-reaction results.

\(^c\) The number of samples was used for the analyses regarding Sp-carriage predictors; the number of isolates was used for the analyses regarding PCV13-non-PCV10-VT-carriage predictors.

\(^d\) Confidence intervals that do not overlap the null value of OR = 1 are indicated in bold.

\(^e\) Information on common cold symptoms was not available for five children.

\(^f\) Information on common cold symptoms was not available for five children.

\(^g\) Based on culture-results or Quellung-reaction.

\(^h\) Based on the combination of culture and PCR-results.

\(^i\) Information on siblings was not available for 40 children.

\(^j\) Child with a history of AOM.

\(^k\) Information on history of AOM was not available for 78 children.

\(^l\) Use of antibiotics in the 3 months before sampling.

\(^m\) Information on use of antibiotics in the 3 months before sampling was not available for 157 children.

\(^n\) Information on vaccination status was missing for one child.

Besides the shown confounders, other variables were assessed, but not significant in univariate analysis: preterm delivery, previous hospitalisation, age-appropriate vaccination, carriage of *Streptococcus pyogenes* (based on culture-results or Quellung-reaction), parental smoking, breastfeeding.
## Table 2b
Predictors through binary logistic regression of *Streptococcus pneumoniae* carriage\(^a\) (n = 2,615 nasopharyngeal samples) and of PCV13-non-PCV10 vaccine serotype carriage\(^b\) (n = 1,744) among children attending day care (pooled over study periods), Belgium, 2016–2018

| Characteristic | n   | %   | OR  | 95% CI\(^d\) | OR  | 95% CI\(^d\) |
|---------------|-----|-----|-----|-------------|-----|-------------|
| **AB-use < 3 months**\(^k, l\) |     |     |     |             |     |             |
| Yes           | 719 | 29.3| REF | REF         | REF | REF         |
| No            | 1,739 | 70.7| 1.79| 1.47–2.18  | 1.63| 1.30–2.05  |
| **Age (months)** | |   |   |   |   |   |
| 6–12          | 524 | 20.0| 0.81| 0.64–1.03 | 0.94| 0.73–1.22  |
| 13–24         | 1,400 | 53.5| REF | REF         | REF | REF         |
| 25–30         | 691 | 26.4| 0.79| 0.64–0.99 | 0.84| 0.65–1.07  |
| **Sampled during influenza-peak** | |   |   |   |   |   |
| Yes           | 1,072 | 41.0| REF | REF         | REF | REF         |
| No            | 1,543 | 59.0| 1.23| 1.02–1.48 | 1.01| 0.77–1.33  |
| **Predictors of PCV13-non-PCV10-VT-carriage**\(^e\) |     |     |     |             |     |             |
| **Study period** | |   |   |   |   |   |
| 2016          | 463 | 26.5| REF | REF         | REF | REF         |
| 2016–2017     | 613 | 35.1| 1.90| 0.59–6.11 | 1.36| 0.36–5.07  |
| 2017–2018     | 668 | 38.3| 9.69| 3.48–27.00 | 5.88| 1.56–22.19 |
| **Vaccination schedule**\(^m\) | |   |   |   |   |   |
| PCV13         | 486 | 27.9| REF | REF         | REF | REF         |
| PCV10         | 707 | 40.6| 6.85| 2.70–17.35 | 1.79| 0.53–6.01  |
| Incomplete    | 222 | 12.7| 1.77| 0.47–6.64 | 0.92| 0.22–3.87  |
| Mix           | 328 | 18.8| 3.03| 1.02–8.93 | 1.71| 0.50–5.82  |
| **Sampled during RSV-peak** | |   |   |   |   |   |
| Yes           | 601 | 34.5| REF | REF         | REF | REF         |
| No            | 1,143 | 65.5| 0.512| 0.313–0.838 | 0.88| 0.52–1.50  |

AB: antibiotic; AOM: acute otitis media; CI: confidence interval; Hi: Haemophilus influenza; Mc: Moraxella catarrhalis; OR: odds ratio; PCV: pneumococcal conjugate vaccine; PCV13-non-PCV10-VT: vaccine serotypes included in PCV13, but not in PCV10 (serotypes: 3, 6A, 19A); REF: reference group for the regression analysis; RSV: respiratory syncytial virus; Sa: Staphylococcus aureus; Sp: Streptococcus pneumoniae.

\(^a\) Knowledge of Sp carriage was through results of culture and PCR combined.

\(^b\) Knowledge of PCV13-non-PCV10 vaccine serotype carriage was based on culture or Quellung-reaction results.

\(^c\) The number of samples was used for the analyses regarding Sp-carriage predictors; the number of isolates was used for the analyses regarding PCV13-non-PCV10-VT-carriage predictors.

\(^d\) Confidence intervals that do not overlap the null value of OR = 1 are indicated in bold.

\(^e\) Information on common cold symptoms was not available for five children.

\(^f\) Based on culture-results or Quellung-reaction.

\(^g\) Based on the combination of culture and PCR-results.

\(^h\) Information on siblings was not available for 40 children.

\(^i\) Child with a history of AOM.

\(^j\) Information on history of AOM was not available for 78 children.

\(^k\) Use of antibiotics in the 3 months before sampling.

\(^l\) Information on use of antibiotics in the 3 months before sampling was not available for 157 children.

\(^m\) Information on vaccination status was missing for one child.

Besides the shown confounders, other variables were assessed, but not significant in univariate analysis: preterm delivery, previous hospitalisation, age-appropriate vaccination, carriage of *Streptococcus pyogenes* (based on culture-results or Quellung-reaction), parental smoking, breastfeeding.
Carriage prevalence of Streptococcus pneumoniae and Haemophilus influenzae

As determined by PCR, very few children carried neither Sp, nor Hi, namely 3.8% (29/760 children), 2.3% (21/902 children), 1.8% (17/953 children) in the consecutive periods (Figure 2). The carriage prevalence of Sp was stable and high, ranging from 75.7% Sp-carriers (683/902 children) in 2016–2017 to 80.0% Sp-carriers (608/760 children) in 2016. The carriage prevalence of Hi increased significantly (p < 0.001) over the study period (87.4% Hi-carriers, 664/760 in 2016 vs 93.5% Hi-carriers, 895/953, in 2017–2018). Co-colonisation with Sp and Hi was frequent and did not change over the study period; it ranged from 71.2% (541/760 Sp and Hi-carriers) in 2016 to 74.3% (708/953 Sp and Hi-carriers) in 2017–2018.

In a multiple regression analysis using pooled data of the three periods, positive predictors for Sp-carriage in a multiple regression analysis using pooled data of Hi-carriers) in 2017–2018.

Based on Quellung-results, non-PCV13-VTs dominated Sp-carriage over the entire study period. The serotypes 23B, 23A, 11A, 15B, 15A, and 10A constituted nearly 50% of the total non-PCV13-VTs among Sp-carriers in all three periods. The separate proportions of the different serotypes identified among Sp-carriers fluctuated over the study period except for three serotypes. The proportions of serotypes 19A and 6C consistently increased (p < 0.001); from 0.4% (2/463 isolates) in 2016, to 1.5% (9/613 isolates) in 2016–2017, to 7.0% (47/668 isolates) in 2017–2018 for serotype 19A and from 0.9% (4/463 isolates) in 2016, to 1.5% (9/613 isolates) in 2016–2017, to 5.8% (39/668 isolates) in 2017–2018 for serotype 6C. The proportion of serotype 15A consistently decreased (p = 0.042); from 6.7% (31/463 isolates) in 2016 to 5.2% (32/613 isolates) in 2016–2017 to 3.4% (23/668 isolates) in 2017–2018.

Streptococcus pneumoniae and its antimicrobial non-susceptibility

The proportion of Sp-strains that were non-susceptible against any of the five tested antibiotics remained stable over the study period (47.1%, 218/463 isolates in 2016 vs 49.3%, 299/607 isolates in 2016–2017 vs 44.6%, 295/662 isolates in 2017–2018), whereas non-susceptibility against more than one antibiotic increased (18.6%, 86/462 isolates in 2016 vs 26.3%, 160/609 isolates in 2016–2017, vs 30.5%, 203/662 isolates in 2017–2018; p < 0.001). Non-susceptibility against levofloxacin (cut-off MIC > 2.00 mg/L) was nonexistent. Non-susceptibility against penicillin (cut-off MIC > 0.06 mg/L; 13.4%, 62/463 isolates in 2016 vs 19.2%, 117/609 isolates in 2016–2017 vs 18.5%, 123/666 isolates in 2017–2018; p = 0.043), erythromycin (17.3%, 80/463 isolates in 2016 vs 16.1%, 98/608 isolates in 2016–2017 vs 22.0%, 146/664 isolates in 2017–2018; p = 0.028), and tetracycline (11.7%, 54/463 isolates in 2016 vs 12.6%, 77/610 isolates in 2016–2017 vs 20.0%, 133/666 isolates in 2017–2018; p < 0.001) increased over the study period, whereas non-susceptibility against trimethoprim/sulfamethoxazole fluctuated over the study period (35.2%, 163/463 isolates in 2016 vs 40.3%, 246/610 isolates in 2016–2017 vs 30.3%, 202/666 isolates in 2017–2018; p = 0.042). Nevertheless, trimethoprim/sulfamethoxazole was the antibiotic against which most strains (35.1%; 611/1,739)
were non-susceptible over the entire study period. The serotypes (based on Quellung-reaction) that were most often found to be non-susceptible against at least one of the tested antibiotics among Sp-carriers are shown in Figure 4.

Over the 3-year study period, 23.2% (13/56 19A-strains) of the 19A-strains were non-susceptible to more than one of the tested antibiotics, whereas two 19A-strains (3.6%; 2/56 19A-strains) were non-susceptible to only one of the tested antibiotics; erythromycin and trimethoprim/sulfamethoxazole for the respective strains. The isolated 19A-strains were most frequently non-susceptible to erythromycin (93.3%; 14/15 19A-strains), followed by tetracycline (86.7%; 13/15 19A-strains), trimethoprim/sulfamethoxazole (46.7%; 7/15 19A-strains), and penicillin (20.0%; 3/15 19A-strains).

Discussion
In this pneumococcal carriage study, we evaluated during 3 years any changes in the nasopharyngeal carriage prevalence, serotype distribution and antimicrobial (non-)susceptibility of Sp in healthy children (aged 6–30 months) attending DCCs in Belgium, from 2016 onwards, i.e. during and immediately after a PCV13-to-PCV10 vaccination programme switch. Common co-colonising bacteria were followed as well, with a special focus on Hi.

Demographics and clinical characteristics of the study population showed higher percentages of common cold symptoms in 2016–2017 and 2017–2018 compared with 2016, which might be due to differences in sampling period. In 2016, nasopharyngeal samples were taken during spring (March–July), whereas the subsequent periods encompassed autumn and winter (November–March), during which common cold frequently occurs. The higher percentages of AOM-history in 2016 compared with the other periods might be due to recruitment of (on average) older children in this year compared with the subsequent periods and in 2016 more AB-treatments in the 3 months before sampling were observed. With regard to breastfeeding, it is unclear why it is more frequent in 2017–2018 compared with the previous periods. It is likely that several factors contributed to fluctuations in breastfeeding practices, one of which may be the coinciding extensive campaigns (personal communication: Marc Hainaut, 24 Oct 2018) on the importance of breastfeeding in Brussels’ maternity hospitals (in 2017–2018, >54% of the included Brussels children were breastfed for more than 6 months).

During the study period, the vaccination status of the child populations gradually changed; from mainly PCV13-vaccinated children in 2016 to mainly PCV10-vaccinated children in 2017–2018. Sp-carriage prevalence was consistently high (77.5%) over the study period. Real-life carriage in DCC might be slightly lower since children who were treated with oral ABs in the 7 days before sampling could not take part in the study. In contrast, the carriage prevalence of Hi increased significantly over the study period (especially in the two oldest age categories), despite the increasing number of children vaccinated with PCV10, containing the NTHi Serotypes.
protein D. Other reports also indicated the absence of PCV10-induced protection against NTHi [39-41].

The predictors of Sp-carriage identified in our study include study period, sex, siblings, common cold symptoms, use of antibiotics, Hi-carriage, Mc-carriage, Sa-carriage and confirm the findings of other reports (besides study period and sex) [5,19,20,42,43]. Based on Quellung-reaction, the proportion of non-PCV13-VTs decreased significantly over the study period, associated with an increase in the proportion of the three PCV13-non-PCV10-VTs 3, 6A, 19A (mainly 19A). We verified for confounders (preterm delivery, previous hospitalisation, age-appropriate vaccination, GAS-carriage, parental smoking, breastfeeding, and the variables shown in Table 2) that could have caused increased PCV13-non-PCV10-VT-carriage, but could not identify any besides study period, which strengthens the hypothesis that the increase was caused by the vaccine switch.

Serotype 19A became the most frequent vaccine serotype in 2017–2018 and its PCR-based prevalence rose from 0.4% in 2016 to 6.4% in 2017–2018. According to surveillance data on IPD from the NRC in 2017, serotype 19A was the second most frequent serotype (after serotype 12F) among IPD-isolates of children younger than 2 years of age. While no increase in serotype 19A frequency had been noted since the introduction of PCV13 in 2011, an increase was observed for the first time in 2016, when the frequency changed from 2.1% in 2016 to 14.2% in 2017 [44]. These findings were confirmed in 2018, 2 to 3 years after the switch from PCV13 to PCV10 [45]. In addition to being reported as an invasive serotype, 19A has also been reported as a serotype that is frequently non-susceptible to antimicrobials [46]. Nevertheless, in our study other serotypes dominated
among the non-susceptible strains (23B, 11A, 15B) as they were more prevalent than 19A. Since we excluded children who were treated with oral ABs in the 7 days before sampling, we possibly missed some non-susceptible strains (including 19A). Besides for serotype 19A, a consistently increasing proportion was also observed for the carriage of serotype 6C, which increased from 0.9% in 2016 to 5.8% in 2017–2018. Sweden, where PCV13 is used in some regions, while PCV10 is used in others, also reported an increase in serotype 6C in PCV10-regions [47]. This could have implications for the non-vaccinated elderly, as reported in Finland, where serotypes 19A and 6C are frequently isolated from IPD in adults older than 65 years [48].

Our results should be interpreted in the context of several limitations. First, a decreasing age trend was introduced by recruiting older children in 2016 in order to include a maximum of children vaccinated with PCV13 and for the same reason, Wallonia was over-recruited in 2016. Since this is intrinsic to our study design, we cannot adjust for this: the sampling probabilities did not allow re-weighting for any of the analyses because no children were sampled in the youngest age category in Flanders and in Brussels in 2016.

Furthermore, the Sp-carriage prevalence was stable within the different age categories (6–12, 13–24, 25–30 months) over the study period, but the Hi-carriage prevalence in the two oldest age categories increased over the study period. Second, we over-recruited in Wallonia in 2016 to enlarge the PCV13-vaccinated population, but in the first season no regional differences in overall Hi-carriage or pneumococcal carriage, and vaccine type carriage were found. Third, a comparative analysis based on the children’s vaccination schedule was not performed due to the small size of these subpopulations in either 2016 (few PCV10-vaccinated children) or 2017–2018 (few PCV13-vaccinated children). Finally, a 3-year study period is short to completely exclude natural fluctuations in the carriage prevalence of specific serotypes; a follow-up study should confirm whether or not a new equilibrium is reached.

Despite these limitations, our study allowed to monitor the impact of the PCV13-to-PCV10 vaccine switch on nasopharyngeal carriage, serotype distribution, and antimicrobial (non-)susceptibility of Sp. This is a complementary activity to the analysis of IPD-surveillance data, thus providing further basis for policy making on pneumococcal vaccination programme options.

Conclusions
As the proportion of children vaccinated exclusively with PCV10 increased, the proportion of the serotypes not included in PCV13 decreased over the 3-year study period, mainly due to an increase in the carriage prevalence of serotype 19A.

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Acknowledgements

Funding statement
Research grant from Research Foundation Flanders (1150017N); investigator-initiated research grant from Pfizer.

Conflict of interest
Investigator-initiated research grant from Pfizer.

Authors’ contributions
HT designed and supervised the study including contributions to collection, analysis and interpretation of the data and writing; IW, LVH and SD contributed to the collection, analysis and interpretation of data and writing; SH contributed to the analysis and interpretation of the data; IW initiated and handled the manuscript; PB, JV, HG, PVD and SMK contributed to writing; NPcarrier Study Group contributed to study design and data interpretation.

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