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Phase I, randomized, observer-blind, placebo-controlled studies to evaluate the safety, reactogenicity and immunogenicity of an investigational non-typeable *Haemophilus influenzae* (NTHi) protein vaccine in adults

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ABSTRACT

Background: Non-typeable *Haemophilus influenzae* (NTHi) is a major cause of various respiratory diseases. The development of an effective vaccine against NTHi mandates new approaches beyond conjugated vaccines as this opportunistic bacterium is non-encapsulated. Here we report on the safety, reactogenicity and immunogenicity of a multi-component investigational vaccine based on three conserved surface proteins from NTHi (proteins D [PD], E [PE] and Pilin A [PilA]) in two observer-blind phase I studies.
Methods: In the first study (NCT01657526), 48 healthy 18–40 year-olds received two vaccine formulations (10 or 30 μg of each antigen [PD and a fusion protein PE-PilA]) or saline placebo at months 0 and 2. In the second study (NCT01678677), 270 50–70 year-olds, current or former smokers, received eight vaccine formulations (10 or 30 μg antigen/dose non-adjuvanted or adjuvanted with alum, $AS01_E$ or $AS04_C$) or saline placebo at months 0, 2 and 6 (plain and alum-adjuvanted groups) and at months 0 and 2 (AS-adjuvanted groups). Solicited and unsolicited adverse events (AEs) were recorded for 7 and 30 days post-vaccination, respectively; potential immune-mediated diseases (pIMDs) and serious AEs (SAEs) throughout the studies. Humoral and antigen-specific T-cell immunity (in study 2 only) responses were assessed up to 12 months post-vaccination.
Results: Observed reactogenicity was highest in the AS-adjuvanted groups but no safety concerns were identified with any of the NTHi vaccine formulations. One fatal SAE (cardiac arrest) not considered related to vaccination, and one pIMD (non-serious psoriasis) in the Placebo group, were reported post-dose 3 in Study 2. All formulations generated a robust antibody response while the AS01-adjuvanted formulations produced the highest humoral and cellular immune responses.
Conclusion: This study confirms that the NTHi vaccine formulations had an acceptable reactogenicity and safety profile and were immunogenic in adults. These results justify further clinical development of this NTHi vaccine candidate.

Abbreviations: AEs, adverse events; ATP, according-to-protocol; CIs, confidence intervals; CMI, cell-mediated immune; COPD, chronic obstructive pulmonary disease; EU, ELISA units; GMCs, geometric mean concentrations; GMTs, geometric mean titers; ICS, intracellular cytokine staining; NTHi, non-typeable *Haemophilus influenzae*; PBMCs, peripheral blood mononuclear cells; PD, protein D; PE, protein E; PilA, Pilin A; pIMDs, potentially immune mediated diseases; SAEs, serious adverse events; TVC, total vaccinated cohort.

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1. Introduction

Non-typeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen and a major cause of various respiratory diseases including otitis media, sinusitis, conjunctivitis, community-acquired pneumonia and exacerbations of chronic obstructive pulmonary disease (COPD) [1,2]. An increase in NTHi prevalence as an etiological factor of invasive infections has been observed, especially among immunocompromised individuals and the adults [3–5]. With older age, comorbidities and immunosenescence are common risk factors for severe infections [6,7]. Another risk factor in adults is smoking, which interferes with mucociliary clearance, diminishes the inflammatory cytokine response and disrupts the epithelial barrier [8,9].

NTHi infections are responsible for considerable morbidity and healthcare costs while NTHi exacerbations of COPD are also associated with significant mortality rates [10–12]. Antibiotics should be the first line treatment of NTHi infections, but increasing antibiotic resistance has been observed [13]. An effective vaccine against NTHi could circumvent this issue, but its development remains a challenge since NTHi is a non-encapsulated bacterium (in contrast to typeable *H. influenzae* strains) that does not present at the surface polysaccharide chains which could be used as vaccine antigens. Therefore, surface-exposed proteins represent important NTHi vaccine and diagnostic targets [14,15].

We developed a new NTHi multi-component investigational vaccine, based on 3 selected conserved surface proteins in the form of 2 vaccine antigens: a free recombinant protein D (PD) and a recombinant fusion protein combining protein E and Pilin A (PE-PilA). PD is a highly conserved lipoprotein among encapsulated and non-encapsulated *H. influenzae* strains [16]. PD is also used as carrier in the licensed pneumococcal polysaccharide PD-conjugate vaccine (PHiD-CV, *Synflorix*™; GSK Vaccines, Rixensart, Belgium) [17]. PE, a highly conserved protein in NTHi, is involved in adhesion and human complement resistance [18–21], while PilA plays a role in biofilm formation, adherence to human epithelial cells and colonization of the upper respiratory tract [22].

On top of the classical aluminum hydroxide (alum)-based approach, we have assessed the potential benefit of including an Adjuvant System (AS01 or AS04) in the investigational vaccine, to eventually overcome immunosenescence or impaired immunity in certain target populations [23].

Here we report the safety, reactogenicity and immunogenicity results of two phase I trials following administration of 2 or 3 doses of different formulations of NTHi investigational vaccine in adults.

2. Methodology

2.1. Studies design and participants

Study 1 was a first-time-in-humans, phase I, randomized, observer-blind, placebo-controlled, single-center, dose-escalation study, conducted in Australia, between 08 August 2012 and 25 November 2013. Healthy 18–40 year-olds were enrolled in a staggered manner in 2 steps and randomized using a centralized randomization system on internet (SBIR) (2:1 per step) to receive 2 doses of an NTHi formulation (10 μg of each antigen [PD and PE-PilA] in step 1 [10-PLAIN group] or 30 μg of each antigen in step 2 [30-PLAIN group]) or saline placebo at months 0 and 2.

Study 2 was a phase I, randomized, observer-blind, placebo-controlled, dose-escalation study, conducted in 3 centers in Belgium, between 31 August 2012 and 30 January 2014. Current and former smokers, 50–70 years old, were enrolled in a staggered manner in 2 steps and randomized using SBIR (2:2:2:2:1 per step) to receive 2 or 3 doses of an NTHi vaccine out of 8 different formulations (10 μg of antigen per dose in step 1 and 30 μg of each antigen per dose in step 2, either non-adjuvanted [plain] or adjuvanted with alum, $AS01_E$ or $AS04_C$ [10-PLAIN, 10-AL, 10-AS01, 10-AS04, 30-PLAIN, 30-AL, 30-AS01 and 30-AS04 groups]) or saline placebo at months 0, 2 and 6 for plain and alum-adjuvanted groups and at months 0 and 2 for AS-adjuvanted groups. The participants from the AS-adjuvanted groups received saline placebo at month 6.

These studies were observer-blind, i.e. the vaccine recipients and those responsible for the evaluation of any study endpoint were blinded to the administered vaccines. Due to differences in the appearance of the study vaccines, vaccines were prepared and administered by authorized medical personnel who did not participate in any of the study clinical evaluations or assays.

The inclusion and exclusion criteria are detailed in Supplementary methods.

The studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. The protocols and associated documents were reviewed and approved by an independent ethics committee. All participants provided written informed consent prior to study entry. These studies have been registered at www.clinicaltrials.gov (NCT01657526 and NCT01678677). Protocol summaries are available at http://www.gsk-clinicalstudyregister.com (GSK studies 116018 and 116647).

2.2. Studies objectives

The primary objective was to evaluate the safety and reactogenicity profile of the NTHi vaccine formulations. The secondary objectives were to evaluate: the antibody and cell mediated immune (CMI) (Study 2 only) responses to PD, PE and PilA induced by the vaccine formulations prior to, and at 30 days after each vaccination (both studies); and the persistence of the immune responses up to 12 months post-dose 2 (i.e. Day 420).

2.3. Study vaccines

Eight formulations were assessed: 10 or 30 μg antigen (PD and PE-PilA) per dose with or without adjuvant. NTHi PD and PE-PilA fusion protein were prepared using recombinant strains of *Escherichia coli*, followed by protein purification and sterile filtration. A detailed description of vaccine presentation and adjuvants is given in Supplementary.

2.4. Reactogenicity and safety

Data were collected in an observer-blind manner (details given in Supplementary).

Diary cards were used to record solicited local (pain, redness and swelling) and general adverse events (AEs) (fatigue, headache, gastrointestinal symptoms, fever) for 7 days after each vaccine dose and unsolicited AEs for 30 days after each dose. AE intensity was graded on a 1–3 scale. Redness or swelling of diameter >100 mm, temperature >39.5 °C and other AEs that prevented normal activities were considered as Grade 3.

Data regarding any pregnancy, new medical condition requiring medical attention, potentially immune mediated diseases (pIMDs) and serious AEs (SAEs) were collected throughout the studies. Hematological and biochemical parameters were measured to assess the subject eligibility at screening, and at Days 7, 60, 67, 180, 187, and 420. Abnormal laboratory findings or other assessments (e.g. abnormal blood pressure) judged by the investigator to be clinically significant were recorded as AE or SAE.

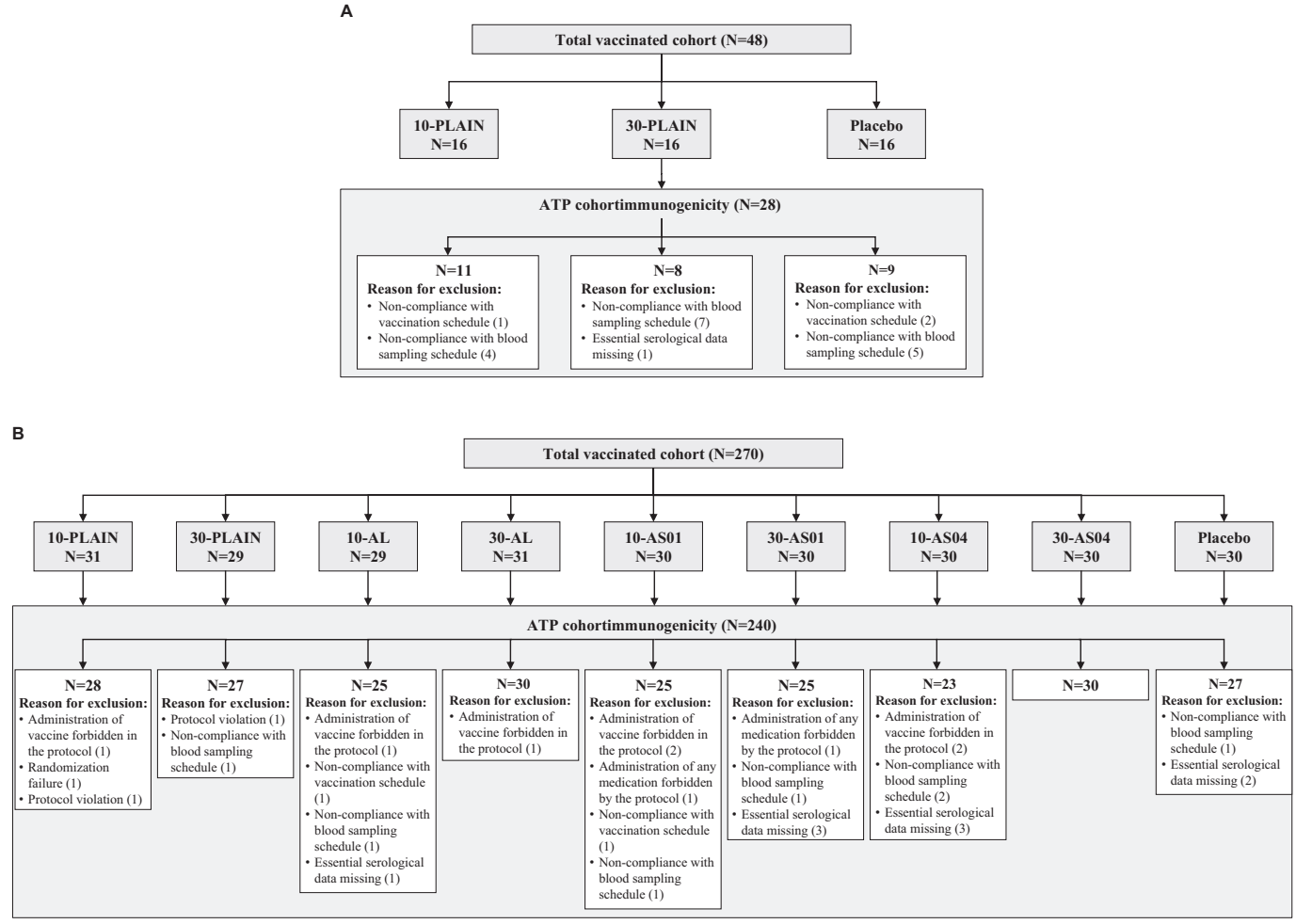

Fig. 1. Disposition of study participants and reasons for exclusion from according-to-protocol cohort for immunogenicity: Study 1 until Day 90 (A) and Study 2 until Day 420 (B). *N*: number of participants; ATP: according-to-protocol.

2.5. Humoral and cellular immunogenicity

Blood samples for immunogenicity were taken prior to, and at 30 days after each vaccination, and at 12 months post-dose 2 (i.e. Days 0, 30, 60, 90, 180, 210 and 420).

The anti-PD, anti-PE and anti-PilA antibody concentrations were measured by ELISA, using standardized procedures. The cut-off of the assays was 100 ELISA units (EU)/mL, 8 EU/mL and 7 EU/mL for anti-PD, anti-PE and anti-PilA, respectively.

CMI responses (antigen-specific $CD4^+$ and $CD8^+$ T-cells) were measured only in Study 2, by flow cytometry using intracellular cytokine staining (ICS) on frozen peripheral blood mononuclear cells (PBMCs), following an adaptation of previously described methods [24]. After PBMC stimulation with the relevant antigens, the frequency of $CD4^+$ and/or $CD8^+$ T-cells expressing a selected set of cytokines (IL-2, IL-13, IL-17, IFN-γ, TNF-α and CD40L) or a selected combination of cytokines was evaluated.

2.6. Statistical analysis

The safety analysis was performed on the total vaccinated cohort (TVC), which included all vaccinated participants. The incidence of AEs per study group was tabulated with exact 95% confidence intervals (CIs) after each vaccine dose and overall. All SAEs, withdrawals due to AE(s), pIMDs, pregnancies and all treatment-related AEs were described in detail.

The immunogenicity analysis was based on the according-to-protocol (ATP) cohort for immunogenicity. Seropositivity rates, geometric mean concentrations (GMCs) and geometric mean titers (GMTs) with their 95% CIs were calculated for each study group. Details on the statistical analysis of antibody responses, GMC ratios and CMI responses (Study 2) are given in Supplementary methods.

3. Results

3.1. Demographics

In Study 1, 48 healthy adults were enrolled and vaccinated, and 46 completed the study (1 consent withdrawal and 1 loss to follow-up). Twenty participants were excluded from the ATP cohort for immunogenicity, mainly because of non-compliance with blood sampling schedule (Fig. 1A). Mean age at first vaccination was 27.0 years; 64.6% were women; the majority (95.8%) was of Caucasian origin (Table 1).

In Study 2, 270 (former) smokers were enrolled and vaccinated, and 257 completed the study (13 withdrawals: 9 consent withdrawals, 3 due to SAEs [1 cardiac arrest, 1 traumatic intracranial hemorrhage and 1 myocardial infarct] and 1 protocol violation [medical history of psoriasis]). Thirty participants were excluded from the ATP cohort for immunogenicity, mainly due to: essential serological data missing (9 participants), administration of vaccine(s) forbidden by the protocol (7 participants) and non-compliance with the blood sampling schedule (7 participants)

Table 1
Demographics of healthy adults (Study 1) (total vaccinated cohort).

Group	10-PLAIN *N* = 16	30-PLAIN *N* = 16	Placebo *N* = 16
Mean age, years (SD)	28.5 (6.85)	26.9 (6.82)	25.7 (7.20)
Females, *n* (%)	9 (56.3%)	12 (75.0%)	10 (62.5%)
Asian/East or South-East Asian Heritage, *n* (%)	0	0	2 (12.5%)
White-Caucasian/European Heritage/, *n* (%)	16 (100%)	16 (100%)	14 (87.5%)

10-PLAIN indicates participants who received 10 μg/antigen/dose; 30-PLAIN indicates participants who received 30 μg/antigen/dose; Placebo indicates participants who received a saline solution; *N*, number of participants; *n* (%), number and percentage of participants in a specific category; SD, standard deviation.

(Fig. 1B). Mean age at first vaccination was 59.4 years; 47.8% were women; 58.9% were current smokers; the majority (98.5%) was of Caucasian origin (Table 2).

3.2. Reactogenicity and safety

In Study 1, placebo and active vaccine recipients had similar levels of reactogenicity. Pain was the most frequently reported solicited local AE during the 7-day post-vaccination period, with only one case of grade 3 pain recorded (Placebo group post-Dose 1) (Supplementary Fig. 1A). In Study 2, pain at the injection site was also the most frequently reported solicited local AE (Fig. 2A), with higher percentages observed in the AS-adjuvanted (point estimates >75%) vs. the plain and alum-adjuvanted groups (point estimates <50%). There were 11 cases of grade 3 pain and 3 cases of grade 3 redness, all recorded post-Dose 2 in AS-adjuvanted recipients.

During the 7-day post-vaccination period, headache and fatigue were the most frequently reported solicited general AEs in both Study 1 (<62.5% of 30-PLAIN participants post-dose 1) (Supplementary Fig. 1B) and Study 2 with high percentages observed in both 10- and 30-AS01 groups and in the 30-AS04 group (point estimates >20% post-Dose 1 or 2) (Fig. 2B). In Study 1, 3 grade 3 general AEs cases were reported: 2 fatigue cases (1 post-Dose 1 in Placebo and 1 post-Dose 2 in the 30-PLAIN group) and 1 grade 3 gastrointestinal symptoms post-Dose 1 in Placebo. In Study 2, there were 19 grade 3 cases (6 fatigue, 7 gastrointestinal symptoms and 6 headache cases) reported in Placebo (6), AS01 (8), AS04 (3) and in Alum groups (2); 11 out of 19 cases (57.9%) occurred post-Dose 2.

In Study 1, at least one unsolicited AE was reported by 42/48 (87.5%) of the participants during the 30-day post-vaccination period. Headache (14/48 [29.2%]) and oropharyngeal pain (8/48 [16.7%]) were the most frequently reported unsolicited AEs both in placebo and vaccine groups. No grade 3 vaccine-related unsolicited AEs were reported. In Study 2, 51.7–80.0% of the participants reported unsolicited AEs post-Dose 1, 2 and/or 3. Nasopharyngitis and headache were the most frequently reported unsolicited AEs. There were 2 cases of grade 3 vaccine-related unsolicited AEs: 1 hypersensitivity case in the 10-AS01 group post-Dose 2 (started 1 day post-vaccination and lasted 4 days) and 1 upper respiratory tract infection in the 30-AS04 group post-Dose 1 (started the day of vaccination and lasted 19 days).

SAEs, unrelated to vaccination, were reported by 2 and 24 participants in Studies 1 and 2, respectively. In Study 2, one fatal SAE was reported in the 30-PLAIN group (cardiac arrest; 50 days post-Dose 3) and 3 other SAE cases did not resolve by Day 420 (1 chronic lymphocytic leukemia, 1 cardiac failure and 1 traumatic intracranial hemorrhage). One pIMD (non-serious psoriasis) considered related to vaccination was reported at 114 days post-Dose 3 in the Placebo group.

No clinically relevant laboratory abnormalities were observed during Study 1. In Study 2, grade 3 changes in hematological and biochemical levels were reported by a maximum of 2 participants per group (details in Supplementary). A grade 4 neutropenia (<500 cells/mm^3) was reported for 1 participant in the 10-PLAIN group at Day 420 (8 months post-Dose 3).

Table 2
Demographics of (former) smokers (Study 2) (total vaccinated cohort).

Group	10-PLAIN *N* = 31	30-PLAIN *N* = 29	10-AL *N* = 29	30-AL *N* = 31	10-AS01 *N* = 30	30-AS01 *N* = 30	10-AS04 *N* = 30	30-AS04 *N* = 30	Placebo *N* = 30
Mean age, years (SD)	57.7 (5.77)	59.8 (4.95)	59.9 (5.23)	60.0 (5.95)	59.3 (5.96)	59.7 (4.85)	59.4 (5.74)	60.0 (6.21)	59.1 (5.06)
Females, *n* (%)	17 (54.8%)	13 (44.8%)	14 (48.3%)	20 (64.5%)	14 (46.7%)	15 (50.0%)	9 (30.0%)	17 (56.7%)	10 (33.3%)
Current smoker	19 (61.3%)	17 (58.6%)	17 (58.6%)	17 (54.8%)	18 (60.0%)	17 (56.7%)	19 (63.3%)	17 (56.7%)	18 (60.0%)
African-American/African Heritage, *n* (%)	0	2 (6.9%)	0	0	0	0	1 (3.3%)	0	0
Asian/Central-South Asian Heritage, *n* (%)	0	0	0	0	1 (3.3%)	0	0	0	0
White-Caucasian/European Heritage, *n* (%)	31 (100%)	27 (93.1%)	29 (100%)	31 (100%)	29 (96.7%)	30 (100%)	29 (96.7%)	30 (100%)	30 (100%)
White-Caucasian/European Heritage, *n* (%)	31 (100%)	27 (93.1%)	29 (100%)	31 (100%)	29 (96.7%)	30 (100%)	29 (96.7%)	30 (100%)	30 (100%)

10-PLAIN indicates participants who received 10 μg/antigen/dose; 30-PLAIN indicates participants who received 30 μg/antigen/dose; 10-AL indicates participants who received 10 μg/antigen/dose adjuvanted with alum; 30-AL indicates participants who received 30 μg/antigen/dose adjuvanted with alum; 10-AS01 indicates participants who received 10 μg/antigen/dose adjuvanted with $AS01_E$; 30-AS01 indicates participants who received 30 μg/antigen/dose adjuvanted with $AS01_E$; 10-AS04 indicates participants who received 10 μg/antigen/dose adjuvanted with $AS04_C$; 30-AS04 indicates participants who received 30 μg/antigen/dose adjuvanted with $AS04_C$; Placebo indicates participants who received a saline solution; *N*, number of participants; *n* (%), number and percentage of participants in a specific category; SD, standard deviation.

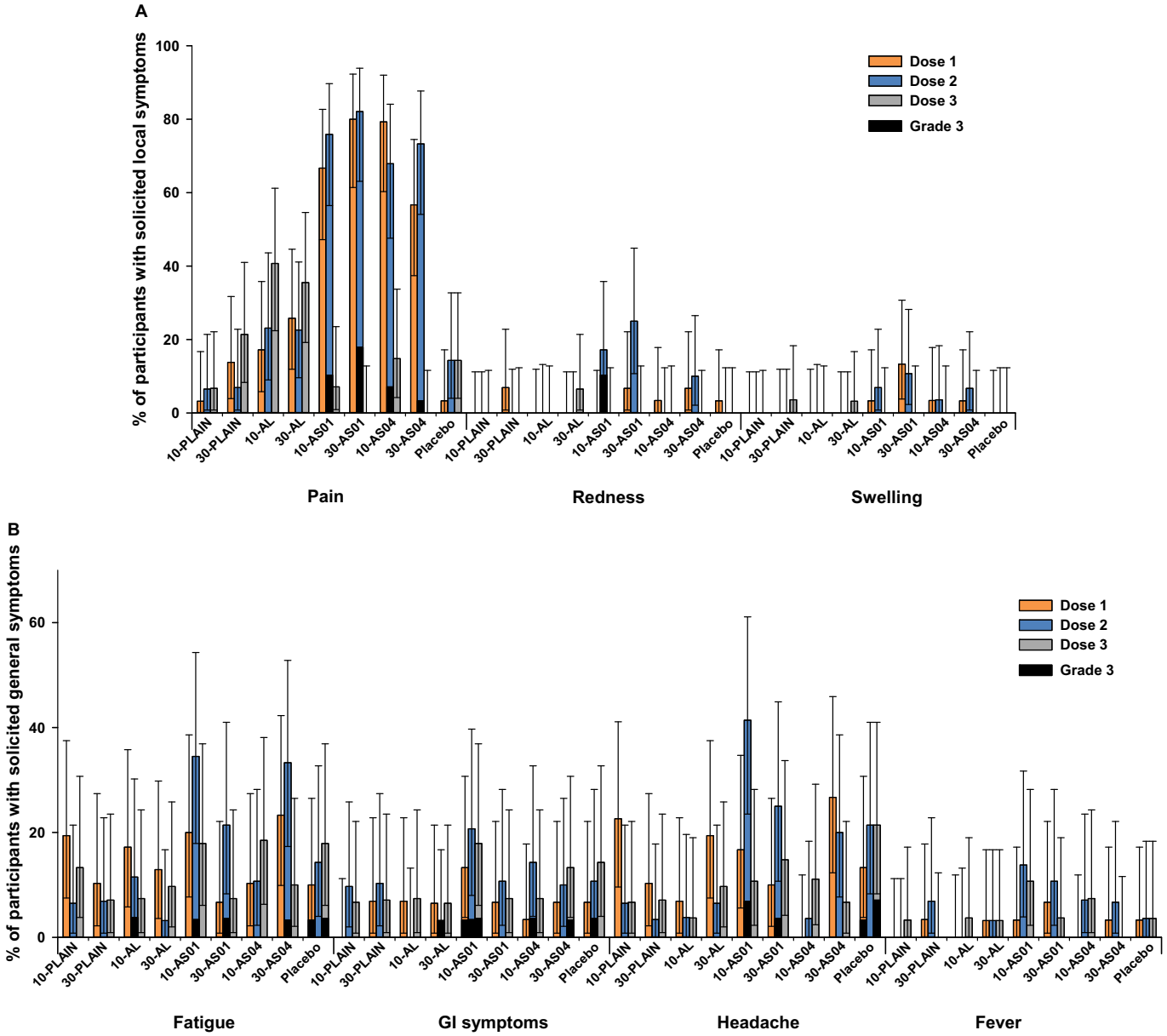

Fig. 2. Solicited local (A) and general (B) symptoms reported during the 7-day post-vaccination period in Study 2 (Total vaccinated cohort). 10-PLAIN indicates participants who received 3 doses of NTHi at 10 μg/antigen/dose; 30-PLAIN indicates participants who received 3 doses of NTHi at 30 μg/antigen/dose; 10-AL indicates participants who received 3 doses of NTHi at 10 μg/antigen/dose adjuvanted with alum; 30-AL indicates participants who received 3 doses of NTHi at 30 μg/antigen/dose adjuvanted with alum; 10-AS01 indicates participants who received 2 doses of NTHi at 10 μg/antigen/dose adjuvanted with $AS01_E$ and one dose of saline placebo; 30-AS01 indicates participants who received 2 doses of NTHi at 30 μg/antigen/dose adjuvanted with $AS01_E$ and one dose of saline placebo; 10-AS04 indicates participants who received 2 doses of NTHi at 10 μg/antigen/dose adjuvanted with $AS04_C$ and one dose of saline placebo; 30-AS04 indicates participants who received 2 doses of NTHi at 30 μg/antigen/dose adjuvanted with $AS04_C$ and one dose of saline placebo; Placebo indicates participants who received a saline solution; PRE, pre-Dose 1; PI(D30), 30 days post-Dose 1; PI(D60), pre-Dose 2; PII(D90), 30 days post-Dose 2; PII(D180), pre-Dose 3; PIII(D210), 30 days post-Dose 3; PIII(D420), 8 months post-Dose 3. *N* = number of documented doses; % = percentage of doses followed by at least one type of symptom; 95% CI = exact 95% confidence interval; GI = gastrointenstinal.

3.3. Immunogenicity

3.3.1. Antibody response

In Study 1, GMCs for anti-PD, anti-PE and anti-PilA antibodies increased up to Day 90 after each active dose compared to placebo (except for anti-PD in PLAIN groups) (Supplementary Fig. 2).

In Study 2, the AS01-adjuvanted formulations generated the highest observed GMCs (Fig. 3). For PE and PilA, antibody responses after 2 doses of the AS01-adjuvanted formulations were higher compared to the responses after 3 doses of the alum-adjuvanted formulations (lower limits of the 95% CIs of GMC ratios were >2). In all AS-adjuvanted groups, no clear antigen-dose effect could be observed in antibody responses. The GMCs at Day 420 remained much higher compared to pre-vaccination levels in all active groups. Results remained similar when the analysis was done on the TVC.

In Study 2, immunogenicity was also presented per group by smoking status: similar immunogenicity responses were observed for current smokers and ex-smokers (data not shown).

3.3.2. Antigen-specific $CD4^+$ and $CD8^+$ T-cells (Study 2)

The specific $CD4^+$ T-cell responses for all NTHi vaccine formulations are presented in Fig. 4. The observed CMI responses in the AS01 groups were the highest. In the AS-adjuvanted groups, there

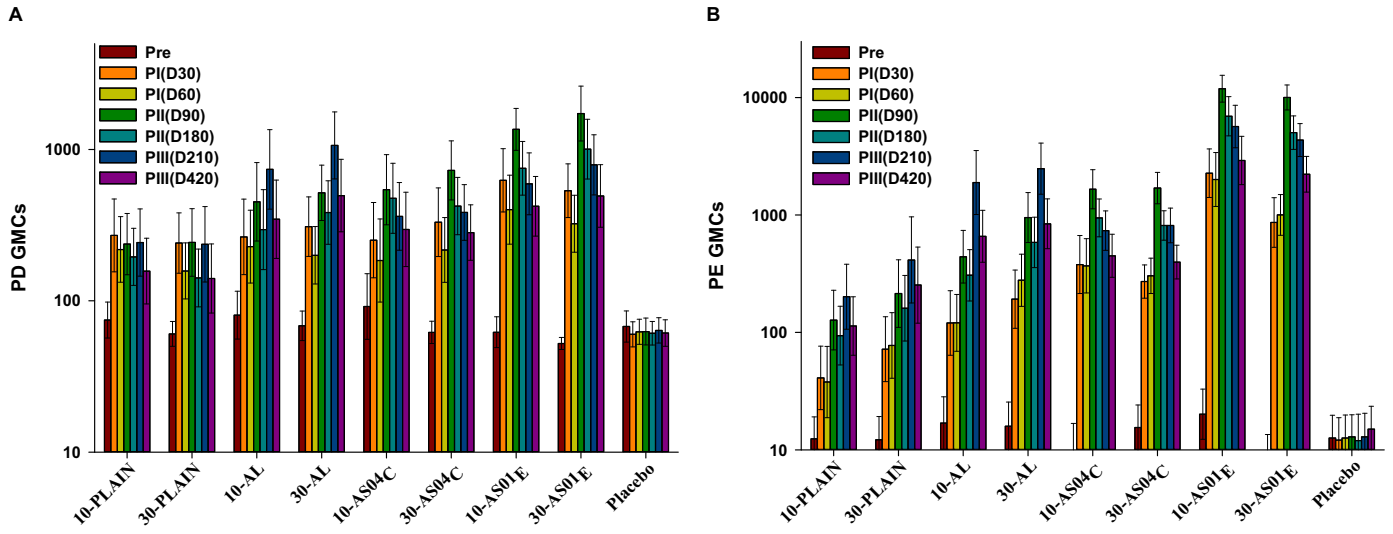

Fig. 3. Geometric mean concentrations of anti-PD (A), anti-PE (B) and anti-PilA (C) (according-to-protocol cohort for immunogenicity). 10-PLAIN indicates participants who received 3 doses of NTHi at 10 μg/antigen/dose; 30-PLAIN indicates participants who received 3 doses of NTHi at 30 μg/antigen/dose; 10-AL indicates participants who received 3 doses of NTHi at 10 μg/antigen/dose adjuvanted with alum; 30-AL indicates participants who received 3 doses of NTHi at 30 μg/antigen/dose adjuvanted with alum; 10-AS01 indicates participants who received 2 doses of NTHi at 10 μg/antigen/dose adjuvanted with $AS01_E$ and one dose of saline placebo; 30-AS01 indicates participants who received 2 doses of NTHi at 30 μg/antigen/dose adjuvanted with $AS01_E$ and one dose of saline placebo; 10-AS04 indicates participants who received 2 doses of NTHi at 10 μg/antigen/dose adjuvanted with $AS04_C$ and one dose of saline placebo; 30-AS04 indicates participants who received 2 doses of NTHi at 30 μg/antigen/dose adjuvanted with $AS04_C$ and one dose of saline placebo; Placebo indicates participants who received a saline solution; PRE, pre-Dose 1; PI(D30), 30 days post-Dose 1; PI(D60), pre-Dose 2; PII(D90), 30 days post-Dose 2; PII(D180), pre-Dose 3; PIII(D210), 30 days post-Dose 3; PIII(D420), 8 months post-Dose 3.

was a trend for CD40L, IL-2, TNF-α and to a lesser extent IFN-γ, IL-13 and IL-17 expression (Supplementary Fig. 3).

No clear antigen-dose effect could be observed. A waning of the response was seen after the second dose in the AS-adjuvanted groups, but the response at Day 420 remained much higher than the pre-vaccination levels. A third dose of plain or alum-adjuvanted vaccine formulation did not change the levels of the observed cellular response (Fig. 4).

No detectable specific $CD8^+$ T-cell responses were observed (data not shown).

4. Discussion

The present studies were primarily designed to assess the safety, reactogenicity and immunogenicity of a new NTHi vaccine candidate based on well conserved surface and virulence factors (PD and PE-PilA) in adults.

Animal studies have shown that PD has induced protection against NTHi otitis media in rat and chinchilla models [14]. While anti-PD antibodies have been linked to clinical protection against otitis media in children [17], the role of anti-PD antibodies in protection against pulmonary or systemic infections caused by NTHi in adults is yet not known despite the fact that anti-PD antibodies may impair bacterial adherence and phosphorylcholine decoration [25]. A recent phase I study indicated that a tri-protein investigational vaccine, including PD and 2 pneumococcal proteins, was immunogenic in healthy adults (18–40 year-olds) [26].

No safety concerns were identified with any of the NTHi vaccine formulations tested. In Study 1, similar levels of reactogenicity (local and systemic) were observed both in placebo and active vaccine recipients. In Study 2, there were higher frequencies of reported AEs in the AS groups.

The higher local and systemic reactogenicity to the AS-adjuvanted formulations may be due to the fact that adjuvants lead to an enhanced migration of monocytes and macrophages to the injection site and an increased production of cytokines and chemokines [27,28]; other adjuvanted formulations have been shown to be more reactogenic than non-adjuvanted vaccine formulations [26,29,30]. Our phase I studies enrolled a limited number of subjects, and thus no formal statistical comparisons of reactogenicity between groups were performed. However, no major differences in the frequency of Grade 3 solicited AEs between adjuvanted and non-adjuvanted vaccine groups and no clear dose-related increases in reactogenicity were observed in Study 2.

Robust and persistent antibody responses post-Dose 1, with a substantial increase post-Dose 2 for the adjuvanted formulations (up to 3.2-fold for PD, 11.6-fold for PE and 10.8-fold for PilA in the 30-AS01 group) and a further increase post-Dose 3 in the alum-adjuvanted groups (up to 2.1-fold for PD, 4.3-fold for PE and 2.0-fold for PilA) were observed. After 2 doses, immune responses in the AS-adjuvanted groups were higher than in the other groups, with the AS01-adjuvanted formulation inducing the highest response. After 3 doses with alum-adjuvanted or plain formulations, the immune responses were lower than those following the administration of 2 doses of the AS01-adjuvanted formulation. Although an AS03-adjuvanted pandemic influenza vaccine has been shown to allow for antigen sparing [31], in our case no clear antigen-dose effect could be observed with the AS01 formulation and the one observed with the other formulations was limited. However, these comparisons were exploratory and have to be interpreted with caution considering that there was no adjustment for multiplicity. As per design, there was no way to dissociate step effect and dose effect (the participants were enrolled in 2 steps and the lower dose formulation was given in the first step), and therefore it was assumed that there was no step effect.

The CMI response in terms of $CD4^+$ T-cells expressing at least two markers amongst IL-2, IFN-γ, IL13, IL17, TNF-α and CD40L was very low for the plain and for the alum-adjuvanted formulations (especially for PD and PilA). A third dose did not impact the CMI responses for these NTHi formulations. The AS-adjuvanted formulations induced the highest CMI response, with a substantial increase post-Dose 2. No clear antigen-dose effect could be observed with any of the NTHi formulations. One of the causes of the low CMI response (especially in the plain formulations) may be the older age of the participants in Study 2. Age-associated immunosenescence is known to cause a decline in the total number of T-cells

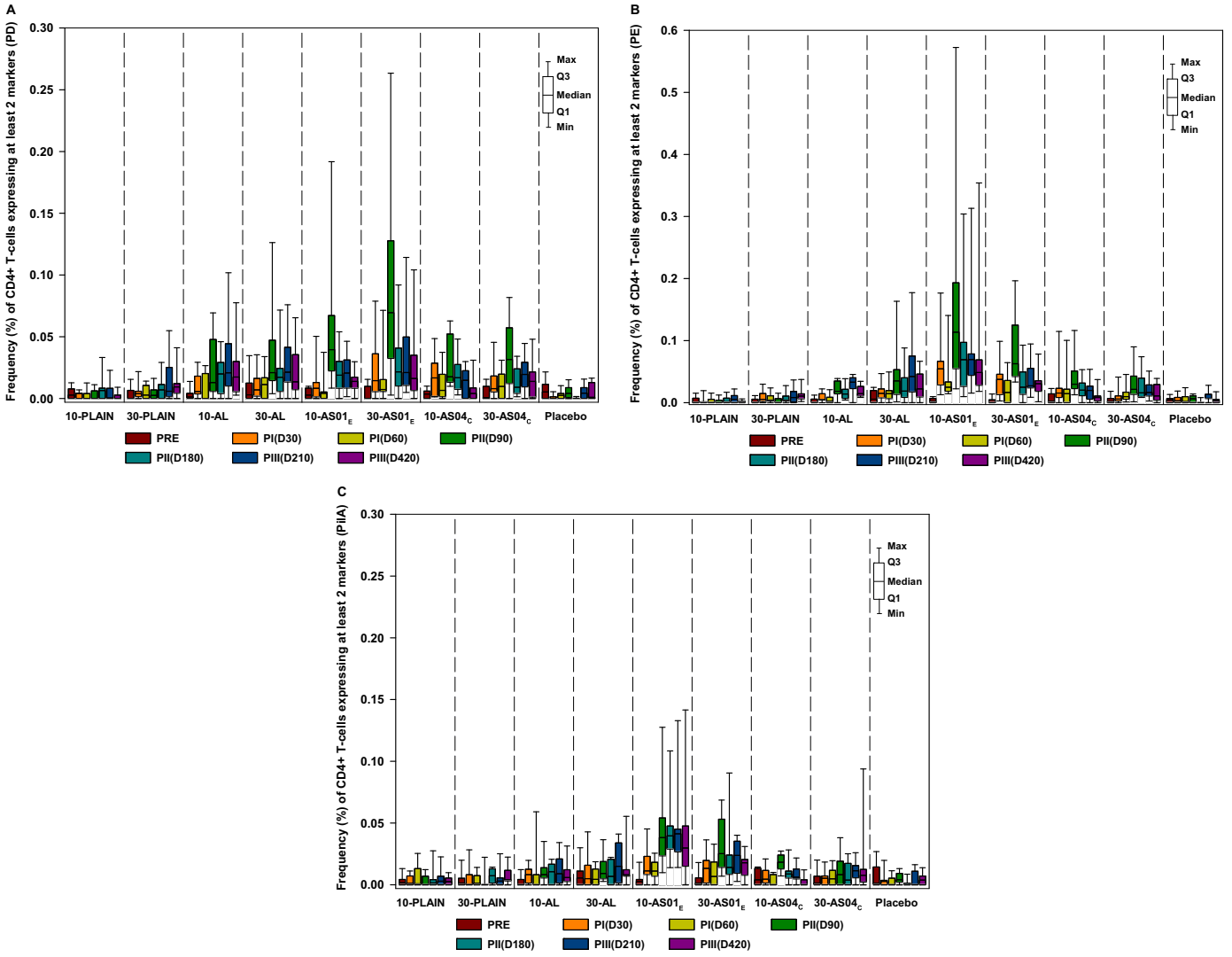

Fig. 4. Frequency (%) of PD (A), PE (B) and PilA (C) specific $CD4^+$ T-cells expressing at least 2 markers amongst IL-2, IFN-γ, IL-13, IL-17, TNF-α and CD40L, prior and after each vaccination (according-to-protocol cohort for immunogenicity). 10-PLAIN indicates participants who received 3 doses of NTHi at 10 μg/antigen/dose; 30-PLAIN indicates participants who received 3 doses of NTHi at 30 μg/antigen/dose; 10-AL indicates participants who received 3 doses of NTHi at 10 μg/antigen/dose adjuvanted with alum; 30-AL indicates participants who received 3 doses of NTHi at 30 μg/antigen/dose adjuvanted with alum; 10-AS01 indicates participants who received 2 doses of NTHi at 10 μg/antigen/dose adjuvanted with $AS01_E$ and one dose of saline placebo; 30-AS01 indicates participants who received 2 doses of NTHi at 30 μg/antigen/dose adjuvanted with $AS01_E$ and one dose of saline placebo; 10-AS04 indicates participants who received 2 doses of NTHi at 10 μg/antigen/dose adjuvanted with $AS04_C$ and one dose of saline placebo; 30-AS04 indicates participants who received 2 doses of NTHi at 30 μg/antigen/dose adjuvanted with $AS04_C$ and one dose of saline placebo; Placebo indicates participants who received a saline solution; PRE, pre-Dose 1; PI(D30), 30 days post-Dose 1; PI(D60), pre-Dose 2; PII(D90), 30 days post-Dose 2; PII(D180), pre-Dose 3; PIII(D210), 30 days post-Dose 3; PIII(D420), 8 months post-Dose 3; Min/Max, minimum/maximum; Q1 and Q3, first and third quartiles.

and to compromise the T-cell repertoire and function [32,33]. Alterations of the immune system of adult smokers may be an additional cause of the low immune responses in our case [8,9]. Nicotine from the cigarette smoke is the main inhibitor of both the innate and adaptive immune responses [8].

As expected [23], the induced response to vaccination was improved by the use of adjuvants. In our case, the highest CMI response was observed for the $AS01_E$-adjuvanted formulations. The high humoral and CMI response for the $AS01_E$-adjuvanted formulations was not surprising since the capacity of AS01-based adjuvants to strongly enhance the immune responses was previously demonstrated in various clinical studies [34,35]. The inclusion of another AS containing MPL and QS-21 ($AS02_V$) in a pneumococcal protein PhtD vaccine was previously shown to partially restore the reduced immune response to vaccines among older adults to the level of vaccine-induced response observed in younger adults [30].

Functional characterization of the T-cells upon vaccination showed a trend for a dominant $CD4^+$ Th0/Th1 cytokine profile, with CD40L, Il-2 and TNF-α expression, and low levels of IFN-γ; very low $CD4^+$ Th2 or Th17 and no $CD8^+$ T-cell induction was observed. For the AS01-adjuvanted formulation, these results are in line with previously shown responses in adults [36].

In conclusion, the new NTHi vaccine formulations have an acceptable reactogenicity, safety and immunogenicity profile in a limited number of adults per vaccine group. All formulations generated a robust antibody response while the AS01-adjuvanted formulations were shown to produce the highest humoral and CMI response. These results justify further clinical assessment of the new NTHi vaccine formulations.

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Conflict of interest: E. A., M. C., J.-M. D., and M. P. are or were employees of the GSK group of companies at the time of the conduct of the studies and manuscript development. E. A., J.-M. D. and M. P. own stock options/restricted shares in the GSK group of companies. G. L.-R., W. H., S. S., and P. V. D. declare that they have no conflicts of interest.

Authors' contribution: E. A., M. C., J.-M. D., P. V. D., M. P. contributed to the conception, design and planning of the study. M. C., J.-M. D., G. L.-R., M. P., S. S., W. H. contributed to the collection of the data. G. L.-R., P. V. D., M. P. contributed to the coordination of center. E. A., M. C., J.-M. D., G. L.-R., P. V. D., M. P., S. S. contributed to the analysis and interpretation of the results. E. A., M. C. provided statistical expertise. J.-M. D., M. P. contributed to the acquisition of funding. J.-M. D., G. L.-R., P. V. D., M. P. contributed to the supervision of the studies. All authors have reviewed the manuscript during its development and approved its final version.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.04.051.

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