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RESEARCH PAPER

Long-term immunogenicity and safety of tetravalent dengue vaccine (CYD-TDV) in healthy populations in Singapore and Vietnam: 4-year follow-up of randomized, controlled, phase II trials

Ngoc Huu Tran[a], Danaya Chansinghakul[b], Chia Yin Chong[c], Chian Yong Low[d], Lynette P. Shek[e], Chan Quang Luong[a], Carina Fargo[f], T. Anh Wartel[f], Sunny Sun[g], Anna Skipetrova[h], and Alain Bouckenooghe[f]

[a]Department for Disease Control and Prevention, Pasteur Institute Ho Chi Minh City, Ho Chi Minh City, Vietnam; [b]Clinical Sciences, Sanofi Pasteur, Pathumwan, Thailand; [c]Infectious Diseases, Department of Pediatrics, KK Women's and Children's Hospital, Singapore; [d]Department of Infectious Disease, Singapore General Hospital, Singapore; [e]Department of Pediatrics, National University of Singapore, Singapore; [f]Clinical Sciences and Medical Affairs Departments, Sanofi Pasteur, Singapore; [g]Biostatistic and Programming Department, Sanofi, Beijing, China; [h]Global Pharmacovigilance, Sanofi Pasteur, Lyon, France

ABSTRACT

Dengue is prevalent in the Asia-Pacific region. Participants of two immunogenicity and safety phase II studies conducted in Singapore and Vietnam (NCT0088089 and NCT00875524, respectively) were followed for up to four years after third vaccine dose of a recombinant, live, attenuated, tetravalent dengue vaccine (CYD-TDV). Participants (2–45 years) received three doses of CYD-TDV or control at 0, 6, and 12 months. Dengue plaque reduction neutralization test ($PRNT_{50}$) antibody titers were measured in both studies. Cytokine-producing antigen-specific CD4+ and CD8+ T-cells were quantified to assess cell-mediated immunity (CMI) in Singapore. *Post-hoc* analyses were carried out for participants aged <9 and ≥9 years old. Related and fatal serious adverse events (SAEs) were collected during long-term follow-up. Of participants who received ≥1 CYD-TDV injection in Singapore (n = 1198) and Vietnam (n = 180), 87% and 92% participants completed long-term follow-up, respectively. At four years, geometric mean titers (GMTs) in participants who received CYD-TDV ranged from 30.2 1/dil (95% CI 23.9–38.3) to 73.7 (49.3–110) 1/dil in Vietnam and 9.73 1/dil (95% CI 8.28–11.4) to 21.8 (18.9–25.1) 1/dil in Singapore. Interferon and interleukin-13 levels were lower at four years than one year post-vaccination but were still present. Tumor necrosis factor-α levels at four years were similar to those after the third vaccine dose. Seropositivity rates were higher at year four in participants who were seropositive vs. seronegative at baseline in both studies. No safety concerns were identified. CYD-TDV demonstrated long-term immunogenicity and was well-tolerated for four years after the third vaccine dose.

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Dengue; CYD-TDV; immunogenicity; safety; cell-mediated immunity; follow-up; Singapore; Vietnam

Introduction

CYD-TDV is a recombinant, live, attenuated, tetravalent dengue vaccine approved for the prevention of symptomatic dengue in individuals aged ≥9 years in several endemic areas.[1] Recent data indicated pre-existing dengue serostatus could be a major determinant in vaccine efficacy, and an increased risk of hospitalized and severe virologically-confirmed dengue (VCD) was observed in seronegative participants, with onset of increased risk from about the third year after first vaccine dose in 9–16-year-olds.[2] Although this potential risk was observed, the vaccine demonstrated a good safety and efficacy profile in pooled analyses of clinical trials in the indicated age group of ≥9 years.[3-5] The safety and immunogenicity of CYD-TDV was previously investigated in two phase II studies in Vietnam and Singapore, countries with high and low dengue endemicity, respectively.[6,7]

We present here the four-year follow-up of these participants (aged <9 and ≥9 years) after the third vaccine dose in two study populations to assess the persistence of immunogenicity and safety of CYD-TDV. In addition, we extended the characterization of the cell-mediated immunity (CMI) induced by the vaccine from one year in the study undertaken in Singapore[8] through to four years after the third vaccine dose.

Results

Study participants

Of the 1198 participants enrolled in the phase II Singapore study, 87% (study vaccine group n = 791; control group n = 255) completed the long-term follow-up (four years after third vaccine dose) (Figure 1). Forty-nine percent (study vaccine group n = 438; control group n = 147) of these participants were included in the full analysis set (FAS), and 100% (study vaccine group n = 898; control group n = 300) in the safety analysis set (SAS). The majority of withdrawals were not vaccine-related but due to employment commitments, overseas relocation, or non-compliance with the protocol as a result of pregnancy, mainly during the long-term follow-up (Figure 1).

CONTACT Danaya Chansinghakul danaya.chansinghakul@sanofi.com Sanofi Pasteur Clinical Sciences, 87/2 CRC Tower 23rd Floor, All Seasons Place, Wireless Road, Lumpini, Pathumwan, Bangkok 10330, Thailand

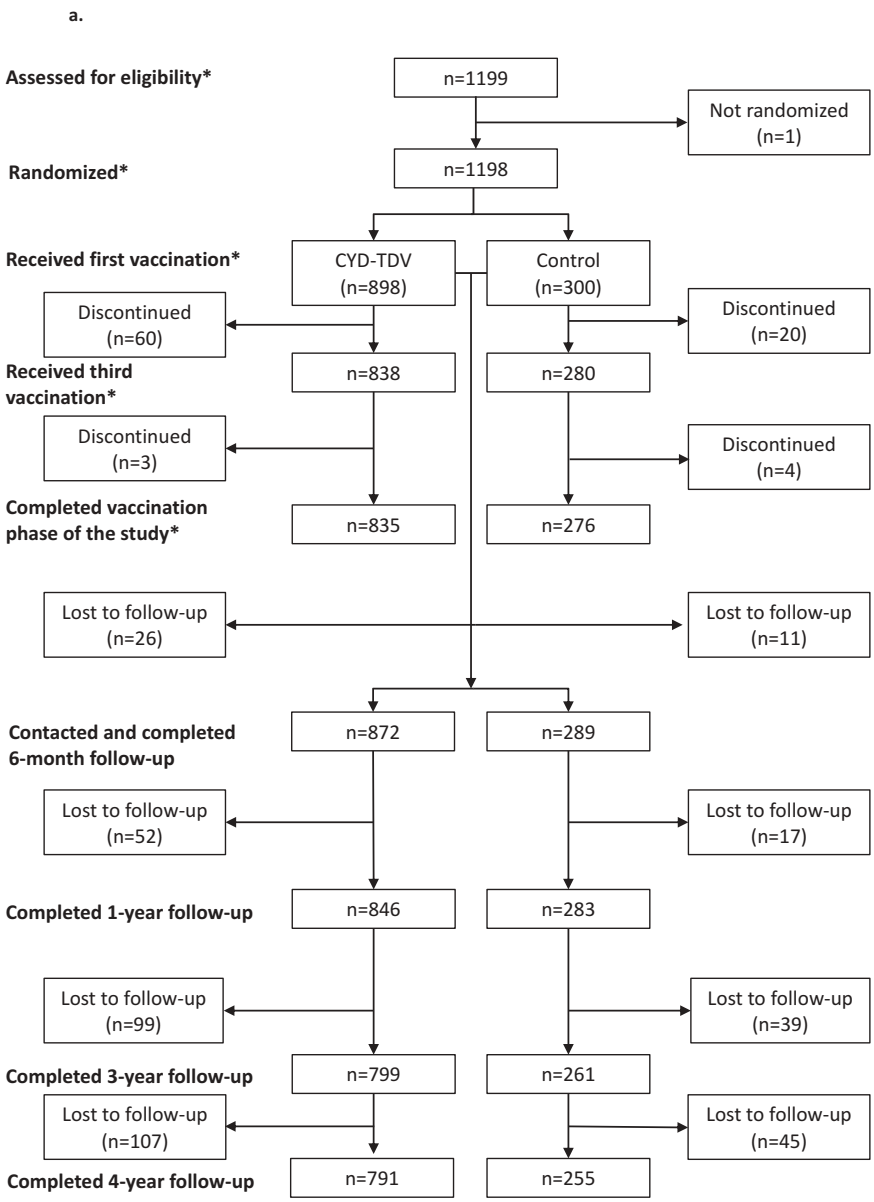

Figure 1. Participant disposition in the Singapore (a) and Vietnam (b) studies.
*Reasons for discontinuations in Vietnam[7] and Singapore[6] were given previously. SAEs that precluded the four participants from completing the study in Singapore were: acute lymphoblastic leukemia, metastatic ovarian cancer, and acute coronary syndrome in the study vaccine group; and thyroid cancer in the control group. These SAEs were considered unrelated to the vaccinations.

There were three discontinuations due to AEs of high fever, rash, or spondylosis in the study vaccine group, and one due to back pain in the control group. There were no discontinuations due to treatment-related serious adverse events (SAEs).

Of the 180 participants enrolled in the phase II Vietnam study, 92% (study vaccine group n = 112; control group n = 54) completed the long-term follow-up (Figure 1). One-hundred and eighty participants (study vaccine group n = 120; control group n = 60) were included in the FAS and SAS. Baseline demographics of both studies have been summarized previously, with 26.5% and 36% of participants seropositive to all four serotypes of dengue in Singapore and Vietnam, respectively.[6,7]

Antibody responses

Geometric mean titers

GMTs (1/dil) were higher at baseline and throughout follow-up in participants in Vietnam compared with those in Singapore, regardless of serotype (Tables 1 and 2). In Singapore, GMTs in the control group were generally similar to those at baseline throughout the four-year follow-up regardless of age group (Table 1). GMTs in the vaccine group generally declined from the levels observed after the third vaccine dose over time but remained higher than baseline, except for serotype 1 which returned to similar baseline values. In both age groups in the vaccine group, GMTs were highest against

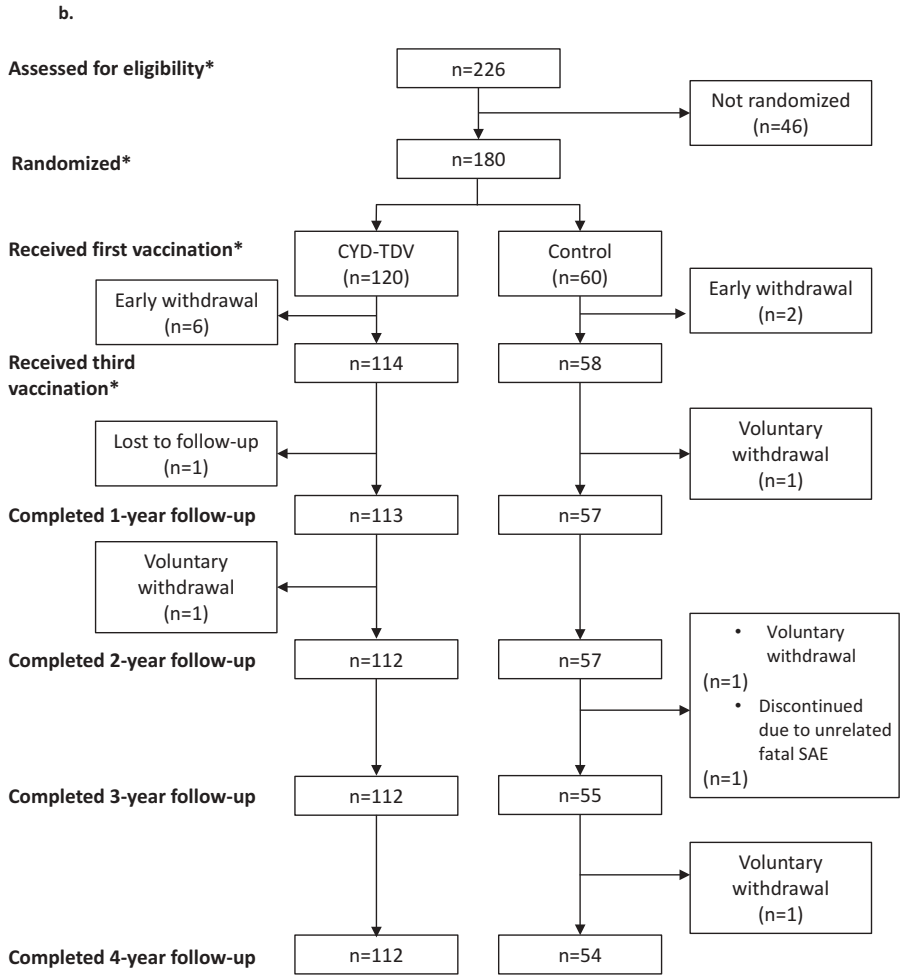

Figure 1. (Continued).

serotype 4 at four-year follow-up (Table 1). In Vietnam, GMTs in the control group were also generally similar to those at baseline throughout the four-year follow-up in those aged <9 years, but appeared to slightly increase over time in those aged ≥9 years (Table 2). GMTs in the vaccine group generally declined from the levels observed after the third vaccine dose during follow-up but remained higher than baseline. The highest GMTs achieved were against serotype 2 in those aged ≥9 years (Table 2).

Seropositivity after vaccination

Baseline dengue seropositivity against at least one dengue serotype was lower in Singapore (25.3%; 71/281) than in Vietnam (69%; 125/180), consistent with the higher dengue endemicity in the latter country. Dengue seropositivity against each serotype at baseline and over time is summarized by age group for participants in the Singapore and Vietnam studies in Figures 2 and 3, respectively. There was a general decline in dengue seropositivity over time in the Singapore study and in the Vietnam study in children aged <9 years but with only a minimal decline in those aged ≥9 years in the latter country. Dengue seropositivity was generally higher in those aged ≥9 years throughout the study, with the difference in the two age groups more marked in the Vietnam study.

Participants seropositive at baseline in the vaccine group generally had higher seropositivity rates throughout follow-up against all dengue serotypes compared with those who were seronegative in both countries (Tables 3 and 4). In addition, seropositivity against all dengue serotypes was higher in those seropositive at baseline aged ≥9 years in the dengue vaccine group than those aged <9 years in both countries; however, the opposite was generally the case in those dengue seronegative at baseline in Vietnam following vaccination with higher seropositivity rates in those aged <9 years (Table 4). Age group differences were not consistently apparent in the Singapore study in those seronegative at baseline following vaccination (Table 3).

Cytokine levels following CYD-TDV in the Singapore study

IFN-γ levels following CYD-TDV re-stimulation were higher after the third vaccine dose compared with other cytokines at the same time point. IL-13 and IFN-γ levels decreased over time and demonstrated no obvious difference between age groups throughout the long-term follow-up. IL-13 levels showed a similar profile as IFN-γ (Figure 4[a,c]). TNF-α levels at four-year follow-up were similar to those after the third vaccine dose (Figure 4[b]). No IL-5 cytokine secretion (GM below the limit of detection of 3.2 pg/mL) was detected (Figure 4[d]). The CMI

Table 1. GMTs against parental dengue virus serotypes during the follow-up period in the Singapore study (Full Analysis Set).

	<9 years				≥9 years			
	CYD-TDV		Control		CYD-TDV		Control	
Time point	M	GMT (95% CI)	M	GMT (95% CI)	M	GMT (95% CI)	M	GMT (95% CI)
Dengue virus serotype 1								
Baseline	89	5.14 (4.94–5.34)	31	5.69 (5.01–6.46)	342	9.17 (7.83–10.7)	114	9.25 (7.05–12.1)
One year after third vaccine dose	85	11.5 (8.97–14.8)	27	5.58 (4.75–6.56)	310	15.1 (12.4–18.5)	102	8.62 (6.65–11.2)
Two years after third vaccine dose	84	8.50 (6.86–10.5)	27	5.00 (5.00–5.00)	301	13.3 (10.9–16.2)	97	8.83 (6.70–11.6)
Three years after third vaccine dose	80	6.80 (5.66–8.17)	27	5.00 (5.00–5.00)	291	12.4 (10.1–15.3)	95	7.95 (6.17–10.2)
Four years after third vaccine dose	83	5.86 (5.20–6.60)	27	5.00 (5.00–5.00)	277	11.3 (9.24–13.9)	90	8.12 (6.23–10.6)
Dengue virus serotype 2								
Baseline	88	5.55 (5.04–6.12)	31	5.00 (5.00–5.00)	341	10.2 (8.55–12.1)	114	9.80 (7.33–13.1)
One year after third vaccine dose	86	19.2 (14.6–25.2)	28	6.10 (4.47–8.31)	308	27.6 (22.3–34.3)	102	9.50 (7.15–12.6)
Two years after third vaccine dose	84	13.7 (10.5–17.9)	27	6.88 (5.18–9.14)	301	31.4 (25.0–39.4)	97	9.93 (7.22–13.7)
Three years after third vaccine dose	81	9.48 (7.54–11.9)	27	5.81 (4.67–7.22)	291	20.9 (16.7–26.2)	95	8.84 (6.71–11.7)
Four years after third vaccine dose	79	12.0 (9.03–16.0)	27	5.16 (4.83–5.52)	272	20.5 (16.4–25.6)	90	8.57 (6.46–11.4)
Dengue virus serotype 3								
Baseline	88	6.29 (5.37–7.38)	31	5.58 (4.71–6.61)	340	9.16 (8.00–10.5)	113	10.6 (7.96–14.1)
One year after third vaccine dose	86	22.8 (16.6–31.4)	27	5.79 (4.69–7.15)	309	29.4 (24.3–35.5)	101	9.04 (6.94–11.8)
Two years after third vaccine dose	82	29.7 (21.4–41.2)	27	6.84 (5.21–8.98)	294	34.7 (28.6–42.1)	95	13.4 (9.63–18.5)
Three years after third vaccine dose	81	12.7 (9.73–16.5)	27	5.70 (4.73–6.87)	288	24.8 (20.1–30.5)	95	8.52 (6.50–11.2)
Four years after third vaccine dose	79	10.3 (8.09–13.2)	27	5.15 (4.85–5.47)	275	18.1 (15.0–21.9)	89	7.95 (6.14–10.3)
Dengue virus serotype 4								
Baseline	89	5.55 (5.00–6.15)	31	5.29 (4.71–5.94)	339	7.30 (6.53–8.16)	113	7.34 (6.08–8.86)
One year after third vaccine dose	86	32.2 (24.5–42.3)	26	5.00 (5.00–5.00)	309	42.8 (36.2–50.5)	102	7.17 (5.98–8.60)
Two years after third vaccine dose	84	33.0 (24.1–45.1)	27	5.59 (4.44–7.03)	301	38.4 (32.4–45.5)	97	7.01 (5.89–8.34)
Three years after third vaccine dose	80	21.7 (16.2–29.1)	27	6.36 (4.51–8.98)	289	30.5 (25.8–36.0)	95	6.70 (5.61–8.00)
Four years after third vaccine dose	83	16.1 (12.3–21.2)	27	5.00 (5.00–5.00)	278	23.8 (20.2–28.1)	90	6.15 (5.41–6.99)

CI, confidence interval; GMT, geometric mean titers; M, number of participants with data available.

Table 2. GMTs against parental dengue virus serotypes during the follow-up period in the Vietnam study (Full Analysis Set).

	<9 years				≥9 years			
	CYD-TDV		Control		CYD-TDV		Control	
Time point	M	GMT (95% CI)	M	GMT (95% CI)	M	GMT (95% CI)	M	GMT (95% CI)
Dengue virus serotype 1								
Baseline	67	12.9 (8.40–19.7)	30	8.84 (5.23–14.9)	53	107 (56.1–203)	30	43.3 (20.6–90.7)
One year after third vaccine dose	63	64.7 (38.0–110)	29	15.6 (7.15–34.2)	50	183 (104–324)	28	52.9 (20.8–134)
Two years after third vaccine dose	62	38.5 (23.0–64.5)	29	12.8 (5.79–28.3)	50	161 (90.7–285)	28	53.0 (21.7–130)
Three years after third vaccine dose	62	26.2 (15.0–224)	28	10.5 (5.08–21.7)	50	144 (75.5–274)	27	87.2 (31.7–240)
Four years after third vaccine dose	62	26.1 (14.8–46.0)	27	11.9 (5.42–26.3)	50	125 (68.1–231)	27	77.7 (29.8–203)
Dengue virus serotype 2								
Baseline	67	11.4 (7.94–16.3)	30	8.56 (5.35–13.7)	53	134 (76.0–235)	30	86.1 (35.5–209)
One year after third vaccine dose	63	56.6 (37.9–84.5)	29	11.8 (6.84–20.2)	50	389 (225–672)	28	121 (43.9–331)
Two years after third vaccine dose	62	43.2 (27.7–67.4)	29	13.7 (7.13–26.4)	50	412 (241–705)	28	138 (50.0–378)
Three years after third vaccine dose	62	35.1 (21.4–57.5)	28	13.9 (6.96–27.9)	50	210 (123–361)	27	153 (60.7–385)
Four years after third vaccine dose	62	30.1 (18.5–48.8)	27	14.8 (7.67–28.7)	50	224 (130–386)	27	173 (67.9–443)
Dengue virus serotype 3								
Baseline	67	19.1 (12.9–28.2)	29	9.67 (6.52–14.3)	53	63.6 (39.8–102)	30	42.3 (20.7–86.3)
One year after third vaccine dose	63	70.1 (46.9–105)	29	10.5 (6.51–16.9)	50	361 (230–568)	28	100 (43.0–234)
Two years after third vaccine dose	62	49.6 (33.4–73.7)	29	10.5 (6.29–17.6)	50	180 (117–277)	28	67.1 (30.8–146)
Three years after third vaccine dose	62	45.9 (28.8–73.3)	28	10.0 (6.58–15.3)	50	106 (67.2–167)	27	61.5 (27.9–136)
Four years after third vaccine dose	62	26.1 (17.2–39.7)	27	8.87 (5.68–13.8)	50	78.7 (51.2–121)	27	43.9 (22.0–87.6)
Dengue virus serotype 4								
Baseline	67	9.26 (7.01–12.2)	30	6.90 (5.36–8.87)	53	37.0 (23.2–58.8)	29	28.8 (14.2–58.7)
One year after third vaccine dose	63	53.3 (39.2–72.5)	29	9.09 (5.52–15.0)	50	163 (110–243)	28	41.0 (20.1–83.3)
Two years after third vaccine dose	62	36.7 (28.3–47.4)	29	8.83 (5.32–14.7)	50	98.1 (66.9–144)	28	35.4 (16.9–74.0)
Three years after third vaccine dose	62	32.6 (23.6–45.1)	28	9.65 (6.14–15.2)	50	74.9 (52.8–106)	27	50.4 (23.1–110)
Four years after third vaccine dose	62	21.6 (16.5–28.4)	27	8.36 (5.12–13.6)	50	45.8 (31.0–67.7)	27	39.5 (19.8–78.8)

CI, confidence interval; FU, follow-up; GMT, geometric mean titers; M, number of participants with data available.

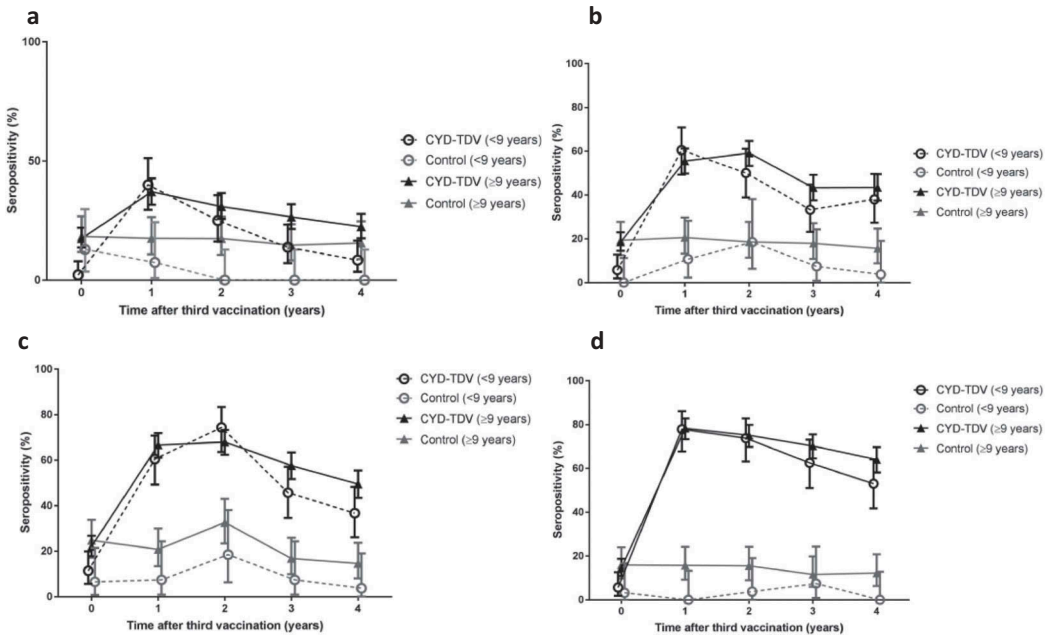

Figure 2. Persistence of dengue virus antibody seropositivity over time against serotype 1 (a), 2 (b), 3 (c), and 4 (d) following CYD-TDV primary immunization versus control in children and adults in Cohorts 1 and 2 in the Singapore study (Full Analysis Set).

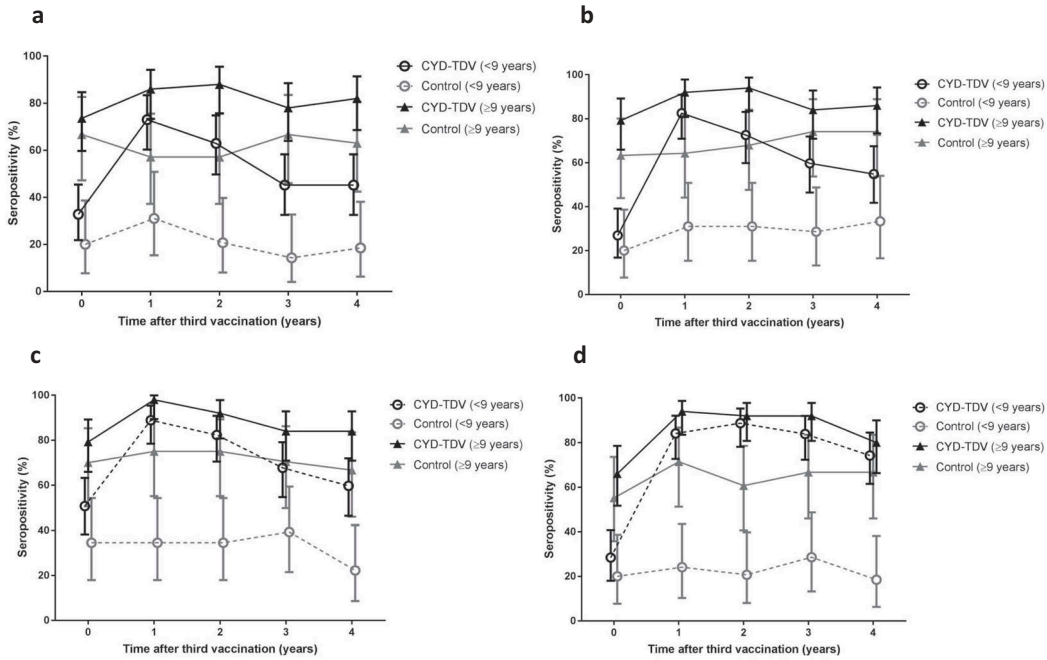

Figure 3. Persistence of dengue virus antibody seropositivity over time against serotype 1 (a), 2 (b), 3 (c), and 4 (d) following CYD-TDV primary immunization versus control in children and adults in the Vietnam study (Full Analysis Set).

responses tended to be strongest against serotype 2 and the weakest against serotype 1.

Dengue- and YF17-NS3-specific $CD3^+CD8^+$ and $CD3^+CD8^-$ (CD4) T cell cytokines

CD8+ T-cell and CD4+ T-cell responses to dengue and YF17 NS3 peptides in the vaccine and control groups are summarized in Figures 5(a–c) and 6(a–c), respectively. Both CD8 + T and CD4+ T-cells from the vaccine group secreted IFN-γ and TNF-**α** at comparable levels for each cytokine at both time points assessed. Overall, both CD8+ T-cell and CD4 + T-cell TNF-**α**, IFN-γ, and IL-2 responses generally remained constant in both study groups from one year to four years after third vaccine dose.

Safety

In the Singapore study, SAEs were observed at a low frequency in both the CYD-TDV and control group after

Table 3. Percentage of participants ≥10 1/dil against each serotype following CYD-TDV primary immunization versus control in participants aged <9 and ≥9 years according to baseline dengue status in the Singapore study (Full Analysis Set).

	<9 years								≥9 years							
	CYD-TDV				Control				CYD-TDV				Control			
	Immune*		Naïve†		Immune		Naïve		Immune		Naïve		Immune		Naïve	
Time point	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)
Dengue virus serotype 1																
Baseline	2/17	11.8 (1.5–36.4)	0/71	0.0 (0.0–5.1)	4/7	57.1 (18.4–90.1)	0/24	0.0 (0.0–14.2)	60/97	61.9 (51.4–71.5)	0/242	0.0 (0.0–1.5)	21/40	52.5 (36.1–68.5)	0/74	0.0 (0.0–4.9)
One year after third vaccine dose	13/17	76.5 (50.1–93.2)	20/67	29.9 (19.3–42.3)	0/7	0.0 (0.0–41.0)	2/20	10.0 (1.2–31.7)	59/81	72.8 (61.8–82.1)	55/222	24.8 (19.2–31.0)	14/32	43.8 (26.4–62.3)	4/68	5.9 (1.6–14.4)
Two years after third vaccine dose	10/17	58.8 (32.9–81.6)	11/66	16.7 (8.6–27.9)	0/7	0.0 (0.0–41.0)	0/20	0.0 (0.0–16.8)	56/76	73.7 (62.3–83.1)	36/217	16.6 (11.9–22.2)	14/31	45.2 (27.3–64.0)	3/64	4.7 (1.0–13.1)
Three years after third vaccine dose	5/17	29.4 (10.3–56.0)	6/62	9.7 (3.6–19.9)	0/7	0.0 (0.0–41.0)	0/20	0.0 (0.0–16.8)	53/78	67.9 (56.4–78.1)	24/207	11.6 (7.6–16.8)	13/30	43.3 (25.5–62.6)	1/63	1.6 (0.0–8.5)
Four years after third vaccine dose	4/17	23.5 (6.8–49.9)	3/65	4.6 (1.0–12.9)	0/7	0.0 (0.0–41.0)	0/20	0.0 (0.0–16.8)	47/70	67.1 (54.9–77.9)	15/201	7.5 (4.2–12.0)	13/28	46.4 (27.5–66.1)	1/60	1.7 (0.0–8.9)
Dengue virus serotype 2																
Baseline	5/17	29.4 (10.3–56.0)	0/71	0.0 (0.0–5.1)	0/7	0.0 (0.0–41.0)	0/24	0.0 (0.0–14.2)	63/97	64.9 (54.6–74.4)	0/242	0.0 (0.0–1.5)	22/40	55.0 (38.5–70.7)	0/74	0.0 (0.0–4.9)
One year after third vaccine dose	13/17	76.5 (50.1–93.2)	39/68	57.4 (44.8–69.3)	1/7	14.3 (0.4–57.9)	2/21	9.5 (1.2–30.4)	72/81	88.9 (80.0–94.8)	96/220	43.6 (37.0–50.5)	17/32	53.1 (34.7–70.9)	4/68	5.9 (1.6–14.4)
Two years after third vaccine dose	12/17	70.6 (44.0–89.7)	30/66	45.5 (33.1–58.2)	2/7	28.6 (3.7–71.0)	3/20	15.0 (3.2–37.9)	64/76	84.2 (74.0–91.6)	110/217	50.7 (43.8–57.5)	16/31	51.6 (33.1–69.8)	2/64	3.1 (0.4–10.8)
Three years after third vaccine dose	8/17	47.1 (23.0–72.2)	19/63	30.2 (19.2–43.0)	1/7	14.3 (0.4–57.9)	1/20	5.0 (0.1–24.9)	62/78	79.5 (68.8–87.8)	63/207	30.4 (24.2–37.2)	15/30	50.0 (31.3–68.7)	2/63	3.2 (0.4–11.0)
Four years after third vaccine dose	8/17	47.1 (23.0–72.2)	22/61	36.1 (24.2–49.4)	0/7	0.0 (0.0–41.0)	1/20	5.0 (0.1–24.9)	58/71	81.7 (70.7–89.9)	59/196	30.1 (23.8–37.0)	13/28	46.4 (27.5–66.1)	1/60	1.7 (0.0–8.9)
Dengue virus serotype 3																
Baseline	10/17	58.8 (32.9–81.6)	0/71	0.0 (0.0–5.1)	2/7	28.6 (3.7–71.0)	0/24	0.0 (0.0–14.2)	75/97	77.3 (67.7–85.2)	0/242	0.0 (0.0–1.5)	28/39	71.8 (55.1–85.0)	0/74	0.0 (0.0–4.9)
One year after third vaccine dose	15/17	88.2 (63.6–98.5)	36/68	52.9 (40.4–65.2)	0/7	0.0 (0.0–41.0)	2/20	10.0 (1.2–31.7)	71/81	87.7 (78.5–93.9)	130/221	58.8 (52.0–65.4)	16/32	50.0 (31.9–68.1)	5/67	7.5 (2.5–16.6)
Two years after third vaccine dose	14/17	82.4 (56.6–96.2)	46/64	71.9 (59.2–82.4)	0/7	0.0 (0.0–41.0)	5/20	25.0 (8.7–49.1)	70/76	92.1 (83.6–97.0)	125/210	59.5 (52.6–66.2)	19/31	61.3 (42.2–78.2)	11/62	17.7 (9.2–29.5)
Three years after third vaccine dose	9/17	52.9 (27.8–77.0)	27/63	42.9 (30.5–56.0)	1/7	14.3 (0.4–57.9)	1/20	5.0 (0.1–24.9)	67/78	85.9 (76.2–92.7)	97/204	47.5 (40.5–54.6)	15/30	50.0 (31.3–68.7)	1/63	1.6 (0.0–8.5)
Four years after third vaccine dose	8/15	53.3 (26.6–78.7)	20/63	31.7 (20.6–44.7)	0/7	0.0 (0.0–41.0)	1/20	5.0 (0.1–24.9)	57/70	81.4 (70.3–89.7)	77/199	38.7 (31.9–45.8)	12/28	42.9 (24.5–62.8)	1/59	1.7 (0.0–9.1)
Dengue virus serotype 4																
Baseline	5/17	29.4 (10.3–56.0)	0/71	0.0 (0.0–5.1)	1/7	14.3 (0.4–57.9)	0/24	0.0 (0.0–14.2)	49/97	50.5 (40.2–60.8)	0/242	0.0 (0.0–1.5)	18/39	46.2 (30.1–62.8)	0/74	0.0 (0.0–4.9)
One year after third vaccine dose	14/17	82.4 (56.6–96.2)	52/68	76.5 (64.6–85.9)	0/7	0.0 (0.0–41.0)	0/19	0.0 (0.0–17.6)	80/81	98.8 (93.3–100.0)	157/221	71.0 (64.6–76.9)	13/32	40.6 (23.7–59.4)	3/68	4.4 (0.9–12.4)
Two years after third vaccine dose	11/17	64.7 (38.3–85.8)	51/66	77.3 (65.3–86.7)	0/7	0.0 (0.0–41.0)	1/20	5.0 (0.1–24.9)	73/76	96.1 (88.9–99.2)	148/217	68.2 (61.6–74.3)	10/31	32.3 (16.7–51.4)	5/64	7.8 (2.6–17.3)
Three years after third vaccine dose	9/17	52.9 (27.8–77.0)	41/62	66.1 (53.0–77.7)	1/7	14.3 (0.4–57.9)	1/20	5.0 (0.1–24.9)	77/78	98.7 (93.1–100.0)	124/205	60.5 (53.4–67.2)	10/30	33.3 (17.3–52.8)	1/63	1.6 (0.0–8.5)
Four years after third vaccine dose	10/17	58.8 (32.9–81.6)	34/65	52.3 (39.5–64.9)	0/7	0.0 (0.0–41.0)	0/20	0.0 (0.0–16.8)	69/71	97.2 (90.2–99.7)	106/201	52.7 (45.6–59.8)	9/28	32.1 (15.9–52.4)	2/60	3.3 (0.4–11.5)

CI, confidence interval; M, number of participants with data available. Participants who were flavivirus (FV) positive were seropositive to dengue or JE. Participants may have been seropositive to other FVs but these were not tested for. The definition of dengue status was based on detection of dengue neutralizing antibodies collected at baseline, assuming that no other dengue virus was circulating in Singapore at the time points studied. *Dengue immune participants at baseline were defined as those participants with titers ≥10 1/dil against at least one dengue serotype at baseline. †Dengue naïve participants were defined as those participants with titers <10 1/dil against all dengue serotypes at baseline.

Table 4. Percentage of participants ≥10 1/dil against each serotype following CYD-TDV primary immunization versus control in participants aged <9 and ≥9 years according to baseline dengue status in the Vietnam study (Full Analysis Set).

	<9 years								≥9 years							
	CYD-TDV				Control				CYD-TDV				Control			
	Immune		Naïve		Immune		Naïve		Immune		Naïve		Immune		Naïve	
Time point	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)	n/M	% (95% CI)
Dengue virus serotype 1																
Baseline	22/41	53.7 (37.4–69.3)	0/26	0.0 (0.0–13.2)	6/16	37.5 (15.2–64.6)	0/14	0.0 (0.0–23.2)	39/44	88.6 (75.4–96.2)	0/9	0.0 (0.0–33.6)	20/24	83.3 (62.6–95.3)	0/6	0.0 (0.0–45.9)
One year after third vaccine dose	30/38	78.9 (62.7–90.4)	16/25	64.0 (42.5–82.0)	5/15	33.3 (11.8–61.6)	4/14	28.6 (8.4–58.1)	39/41	95.1 (83.5–99.4)	4/9	44.4 (13.7–78.8)	15/22	68.2 (45.1–86.1)	1/6	16.7 (0.4–64.1)
Two years after third vaccine dose	26/37	70.3 (53.0–84.1)	13/25	52.0 (31.3–72.2)	4/15	26.7 (7.8–55.1)	2/14	14.3 (1.8–42.8)	39/41	95.1 (83.5–99.4)	5/9	55.6 (21.2–86.3)	16/22	72.7 (49.8–89.3)	0/6	0.0 (0.0–45.9)
Three years after third vaccine dose	18/37	48.6 (31.9–65.6)	10/25	40.0 (21.1–61.3)	3/15	20.0 (4.3–48.1)	1/13	7.7 (0.2–36.0)	37/41	90.2 (76.9–97.3)	2/9	22.2 (2.8–60.0)	17/22	77.3 (54.6–92.2)	1/5	20.0 (0.5–71.6)
Four years after third vaccine dose	19/37	51.4 (34.4–68.1)	9/25	36.0 (18.0–57.5)	4/14	28.6 (8.4–58.1)	1/13	7.7 (0.2–36.0)	38/41	92.7 (80.1–98.5)	3/9	33.3 (7.5–70.1)	17/22	77.3 (54.6–92.2)	0/5	0.0 (0.0–52.2)
Dengue virus serotype 2																
Baseline	18/41	43.9 (28.5–60.3)	0/26	0.0 (0.0–13.2)	6/16	37.5 (15.2–64.6)	0/14	0.0 (0.0–23.2)	42/44	95.5 (84.5–99.4)	0/9	0.0 (0.0–33.6)	19/24	79.2 (57.8–92.9)	0/6	0.0 (0.0–45.9)
One year after third vaccine dose	31/38	81.6 (65.7–92.3)	21/25	84.0 (63.9–95.5)	5/15	33.3 (11.8–61.6)	4/14	28.6 (8.4–58.1)	40/41	97.6 (87.1–99.9)	6/9	66.7 (29.9–92.5)	18/22	81.8 (59.7–94.8)	0/6	0.0 (0.0–45.9)
Two years after third vaccine dose	29/37	78.4 (61.8–90.2)	16/25	64.0 (42.5–82.0)	6/15	40.0 (16.3–67.7)	3/14	21.4 (4.7–50.8)	40/41	97.6 (87.1–99.9)	7/9	77.8 (40.0–97.2)	18/22	81.8 (59.7–94.8)	1/6	16.7 (0.4–64.1)
Three years after third vaccine dose	22/37	59.5 (42.1–75.2)	15/25	60.0 (38.7–78.9)	6/15	40.0 (16.3–67.7)	2/13	15.4 (1.9–45.4)	39/41	95.1 (83.5–99.4)	3/9	33.3 (7.5–70.1)	19/22	86.4 (65.1–97.1)	1/5	20.0 (0.5–71.6)
Four years after third vaccine dose	22/37	59.5 (42.1–75.2)	12/25	48.0 (27.8–68.7)	7/14	50.0 (23.0–77.0)	2/13	15.4 (1.9–45.4)	40/41	97.6 (87.1–99.9)	3/9	33.3 (7.5–70.1)	19/22	86.4 (65.1–97.1)	1/5	20.0 (0.5–71.6)
Dengue virus serotype 3																
Baseline	34/41	82.9 (67.9–92.8)	0/26	0.0 (0.0–13.2)	10/15	66.7 (38.4–88.2)	0/14	0.0 (0.0–23.2)	42/44	95.5 (84.5–99.4)	0/9	0.0 (0.0–33.6)	21/24	87.5 (67.6–97.3)	0/6	0.0 (0.0–45.9)
One year after third vaccine dose	33/38	86.8 (71.9–95.6)	23/25	92.0 (74.0–99.0)	5/15	33.3 (11.8–61.6)	5/14	35.7 (12.8–64.9)	41/41	100.0 (91.4–100.0)	8/9	88.9 (51.8–99.7)	19/22	86.4 (65.1–97.1)	2/6	33.3 (4.3–77.7)
Two years after third vaccine dose	31/37	83.8 (68.0–93.8)	20/25	80.0 (59.3–93.2)	6/15	40.0 (16.3–67.7)	4/14	28.6 (8.4–58.1)	39/41	95.1 (83.5–99.4)	7/9	77.8 (40.0–97.2)	20/22	90.9 (70.8–98.9)	1/6	16.7 (0.4–64.1)
Three years after third vaccine dose	26/37	70.3 (53.0–84.1)	16/25	64.0 (42.5–82.0)	8/15	53.3 (26.6–78.7)	3/13	23.1 (5.0– 53.8)	39/41	95.1 (83.5–99.4)	3/9	33.3 (7.5–70.1)	18/22	81.8 (59.7–94.8)	1/5	20.0 (0.5–71.6)
Four years after third vaccine dose	24/37	64.9 (47.5–79.8)	13/25	52.0 (31.3–72.2)	5/14	35.7 (12.8–64.9)	1/13	7.7 (0.2–36.0)	40/41	97.6 (87.1–99.9)	2/9	22.2 (2.8–60.0)	17/22	77.3 (54.6–92.2)	1/5	20.0 (0.5–71.6)
Dengue virus serotype 4																
Baseline	19/41	46.3 (30.7–62.6)	0/26	0.0 (0.0–13.2)	6/16	37.5 (15.2–64.6)	0/14	0.0 (0.0–23.2)	35/44	79.5 (64.7–90.2)	0/9	0.0 (0.0–33.6)	16/23	69.6 (47.1–86.8)	0/6	0.0 (0.0–45.9)
One year after third vaccine dose	33/38	86.8 (71.9–95.6)	20/25	80.0 (59.3–93.2)	4/15	26.7 (7.8–55.1)	3/14	21.4 (4.7–50.8)	41/41	100.0 (91.4–100.0)	6/9	66.7 (29.9–92.5)	19/22	86.4 (65.1–97.1)	1/6	16.7 (0.4–64.1)
Two years after third vaccine dose	32/37	86.5 (71.2–95.5)	23/25	92.0 (74.0–99.0)	3/15	20.0 (4.3–48.1)	3/14	21.4 (4.7–50.8)	40/41	97.6 (87.1–99.9)	6/9	66.7 (29.9–92.5)	17/22	77.3 (54.6–92.2)	0/6	0.0 (0.0–45.9)
Three years after third vaccine dose	32/37	86.5 (71.2–95.5)	20/25	80.0 (59.3–93.2)	6/15	40.0 (16.3–67.7)	2/13	15.4 (1.9–45.4)	40/41	97.6 (87.1–99.9)	6/9	66.7 (29.9–92.5)	17/22	77.3 (54.6–92.2)	1/5	20.0 (0.5–71.6)
Four years after third vaccine dose	27/37	73.0 (55.9–86.2)	19/25	76.0 (54.9–90.6)	4/14	28.6 (8.4–58.1)	1/13	7.7 (0.2–36.0)	38/41	92.7 (80.1–98.5)	2/9	22.2 (2.8–60.0)	17/22	77.3 (54.6–92.2)	1/5	20.0 (0.5–71.6)

CI, confidence interval; M, number of participants with data available. Participants who were flavivirus (FV) positive were seropositive to dengue or JE. Participants may have been seropositive to other FVs but these were not tested for. The proportion of participants who were seropositive to each dengue serotype at baseline ranged from 59.3% (54/91) for serotype 4 to 83.5% (76/91) for serotype 3.

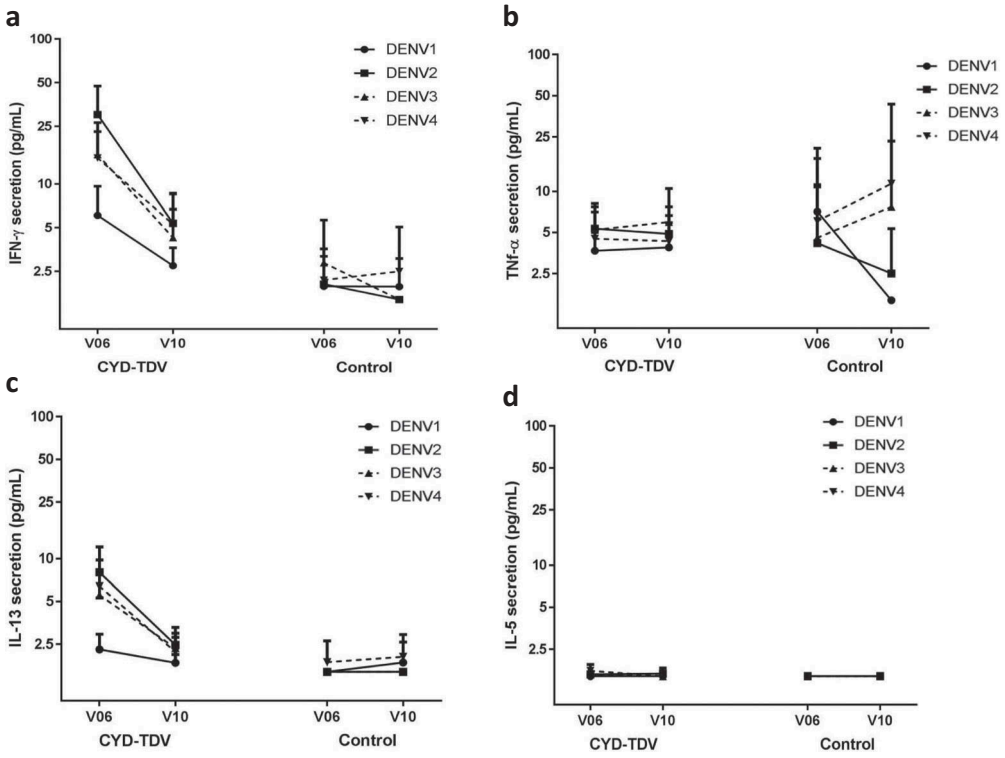

Figure 4. Specific Th1 and Th2 cytokine secretion (IFN-γ [a], TNF-**α** [b], IL-13 [c], and IL-15 [d]; pg/mL) by purified peripheral blood mononuclear cells after stimulation with live vaccines of each dengue serotype in all participants in the Singapore study (Luminex assay).

CMI, cell-mediated immunity; CYD, dengue serotype; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; V, visit. The lower limit of quantification of the assay was 10 (1/dil). Data are presented on a Log 2 scale. Visit 6, one year after the third vaccine dose; Visit 10, four years after the third vaccine dose. Participants were split into two randomized cohorts for CMI analyses. Cohort 1: in the study vaccine group, n (%) of immune vs. naïve at baseline was 48 (22.1%) vs. 159 (73.3%), respectively; in the control group, n (%) of immune vs. naïve at baseline was 23 (31.5%) vs. 48 (65.8%), respectively. Cohort 2: in the study vaccine group, n (%) of immune vs. naïve at baseline was 66 (29.9%) vs. 154 (69.7%), respectively; in the control group, n (%) of immune vs. naïve at baseline was 24 (32.4%) vs. 50 (67.6%), respectively.

any vaccination (4.8% [3.5–6.4] vs. 4.3% [2.3–7.3], respectively) and were considered unrelated to the vaccine. SAEs following CYD-TDV administration were observed in 6.3% (95% CI 2.9–11.5) of participants aged <9 years compared with 4.5% (95% CI 3.1–6.2) in the ≥9 years age group. There were three cases of dengue during the four-year follow-up, all of which occurred in the vaccine group; of these, two were serologically confirmed only and one virologically confirmed. The serologically confirmed (IgM/IgG ELISA) cases included a hospitalized dengue fever reported at the four-year follow-up that occurred 152 days following the third vaccination in a 42-year-old female. The other was a case of dengue fever in a 12-year-old male that occurred more than three years after receiving the third dose. The virologically confirmed case (dengue NS1 antigen positive) was a hospitalized dengue hemorrhagic fever reported 339 days following the third dose of the study vaccine in a 23-year-old male. Three participants died, all were in the study vaccine group; the causes of death were acute lymphoblastic leukemia (18-year-old male), metastatic ovarian cancer (45-year-old female), or acute coronary syndrome (37-year-old male). The deaths were considered unrelated to the vaccine.

In Vietnam, SAEs were also observed at a low frequency in both the study vaccine and control groups after any vaccination (2.5% [95% CI 0.5–7.1] vs. 6.7% [95% CI 1.8–16.2], respectively) and were considered unrelated to the vaccine by the Investigator. SAEs following CYD-TDV administration were observed in 1.5% (95% CI 0.0–8.0) of participants aged <9 years compared with 3.8% (95% CI 0.5–13.0) in the ≥9 years age group. There were no dengue cases observed during the four-year follow-up. One 13-year-old male participant in the control group died due to varicella two years and nine months after receiving typhoid vaccine as a third vaccine dose in Vietnam. There were no deaths in the study vaccine group.

Discussion

Dengue neutralizing antibody GMTs and seropositivity rates after CYD-TDV vaccination were higher in Vietnam than Singapore, consistent with difference in dengue endemicity in the two countries. The decline in GMTs and seropositivity rates during follow-up was more pronounced in Singapore and in those aged <9 years, in particular. Studies undertaken with CYD-TDV in dengue endemic settings with high seropositive rates at baseline tend to report higher GMTs and seropositivity rates than those undertaken in non-/lower endemicity settings. The presence of higher GMTs in participants who were dengue seropositive at baseline may be due to higher neutralizing antibody titers associated with a stronger immunogenicity response post-vaccination.[9] A pre-existing antibody response to the dengue virus may be beneficial to the vaccine-induced antibody response. In an integrated immunogenicity analysis of ten phase II and six phase III trials that

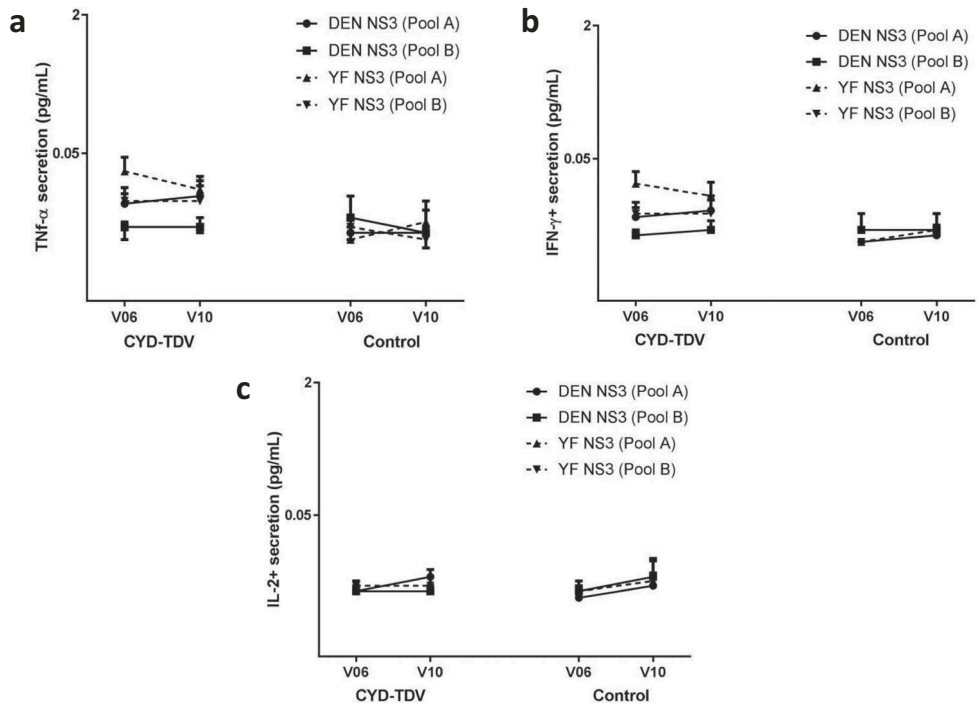

Figure 5. Cytokine-positive secreting CD3+ CD8 + T-cells (TNF-**α** [a], IFN-γ+ [b], and IL-2+ [c]) after stimulation of whole blood with pools of NS3 peptides from dengue and YF 17D in all participants in the Singapore study (Intracellular Cytokine Staining).

CMI, cell-mediated immunity; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; V, visit. The positive control used was CytoStim. The lower limit of detection for cytokine secretion was 0.01%. Data are presented on a Log 2 scale. Visit 6, one year after the third vaccine dose; Visit 10, four years after the third vaccine dose. Participants were split into two randomized cohorts for CMI analyses. Cohort 1: in the study vaccine group, n (%) of immune vs. naïve at baseline was 48 (22.1%) vs. 159 (73.3%), respectively; in the control group, n (%) of immune vs. naïve at baseline was 23 (31.5%) vs. 48 (65.8%), respectively. Cohort 2: in the study vaccine group, n (%) of immune vs. naïve at baseline was 66 (29.9%) vs. 154 (69.7%), respectively; in the control group, n (%) of immune vs. naïve at baseline was 24 (32.4%) vs. 50 (67.6%), respectively.

administered CYD-TDV in Asia Pacific and Latin America – including the initial Vietnam and Singapore studies – participants who were seropositive to dengue at baseline demonstrated higher GMTs up to four years after the third dose, irrespective of region.[10]

The GMTs against all four serotypes at the four-year follow-up were generally lower to those observed at one year post-vaccination in both countries.[6,7] This is in contrast with another four-year immunogenicity and safety follow-up of CYD-TDV in participants aged 2–45 years in the Philippines, a country considered highly endemic for dengue, where GMTs remained similar to those observed at one year post-vaccination.[11] It is possible that exposure to circulating wild-type dengue may have boosted antibody levels across all serotypes during longer-term follow-up in the later study. Our study demonstrated a decrease in neutralizing antibody at one year after third vaccine dose and through follow-up, but the antibody level persistence was sustained above baseline levels.

The GMTs at four years after the third study vaccine dose were higher against serotype 4 in both studies. A strain of serotype 4 of dengue has previously been circulating at low levels in the northern-eastern part of Singapore, and these cases were scattered with no clear clustering effect. Silent transmission of this serotype may be occurring in Singapore,[12] which may be why this serotype appeared to be dominant above others at four years after the third vaccine dose. In Southern Vietnam, serotype 4 was dominant in 2001–2002[13] and seasonality and short-term cross-protection may affect these dengue dynamics.[14] In addition, there may be a time-lagged correlation between serotype dynamics and disease incidence rates,[15] possibly fluctuating the frequency and dominance of serotypes year-by-year observed in the present study in Singapore. As such, it is possible that immunodominance of serotype 4 after CYD-TDV administration may be a potential explanation for the higher GMTs and seroconversion rates against this serotype, especially in dengue seronegatives.

An outbreak of dengue was recorded during 2013–14 in Singapore at a time when the study was ongoing, possibly due to a switch in the dominant circulating serotype, from serotype 2 to 1.[16,17] The outbreak incidence rate was 410.6 and 335.0 per 100,000 population in 2013 and 2014, respectively, compared with 17 per 100,000 in 2000.[17,18] The dengue outbreak may have boosted seropositivity rates to the relevant serotype as well as to the other serotypes in the Singapore study, thereby slowing the rate of decline than would otherwise be expected in a low dengue endemic country.

GMTs were highest against serotype 2 in those aged ≥9 years in Vietnam. In southern Vietnam, a complete genotype replacement event within serotype 2 was observed throughout the 1990s and 2000s and increased the disease incidence. In addition, serotype 2 was the most prevalent

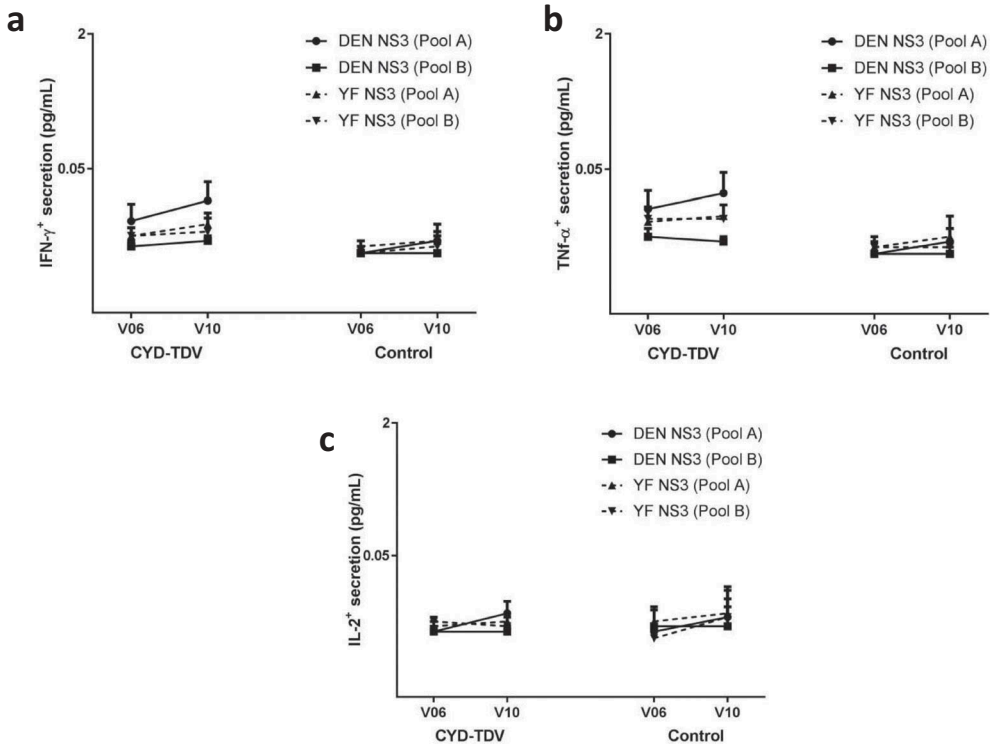

Figure 6. Cytokine-positive secreting CD3+ CD8 – (CD4) T-cells (IFN-γ+ [a], TNF-**α**+ [b], and IL-2+ [c]) after stimulation of whole blood with pools of NS3 peptides from dengue and YF 17D in all participants in the Singapore study (Intracellular Cytokine Staining).

CMI, cell-mediated immunity; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; V, visit. The positive control used was CytoStim. The lower limit of detection for cytokine secretion was 0.01%. Data are presented on a Log 2 scale. Visit 6, one year after the third vaccine dose; Visit 10, four years after the third vaccine dose. Participants were split into two randomized cohorts for CMI analyses. Cohort 1: in the study vaccine group, n (%) of immune vs. naïve at baseline was 48 (22.1%) vs. 159 (73.3%), respectively; in the control group, n (%) of immune vs. naïve at baseline was 23 (31.5%) vs. 48 (65.8%), respectively. Cohort 2: in the study vaccine group, n (%) of immune vs. naïve at baseline was 66 (29.9%) vs. 154 (69.7%), respectively; in the control group, n (%) of immune vs. naïve at baseline was 24 (32.4%) vs. 50 (67.6%), respectively.

serotype as detected by surveillance and its circulation was temporally associated with increased disease incidence during 1999–2002 in this region.[19] Annual dengue incidence rates were estimated to be 113.9 per 100,000 from 1980 to 2010 (an annual average percent change of 10.4%).[20]

Earlier studies have suggested that previous Japanese encephalitis (JE) or yellow fever (YF) exposure may enhance the immunogenicity of CYD-TDV,[21,22] though this could not be clearly established or ruled out in the comprehensive integrated immunogenicity analysis of CYD-TDV data.[10]

A recent analysis by Sridhar S, *et al.* further elucidated the importance of dengue pre-exposure in determining vaccine efficacy against hospitalized and severe dengue, but conversely increases the risk of these events in those dengue seronegative.[2] The risk of severe VCD was lower among those who were seropositive vaccine recipients at baseline compared with seropositive controls.[2] Among those aged ≥9 years, this increased risk of hospitalized and severe dengue was observed from year three onwards in Sridhar S, *et al.*, so participants in this age group who were seronegative in the present study may have been affected. There were three dengue cases (including one dengue hemorrhagic fever) in vaccine group in the Singapore study, two of which required hospitalization. However, two of these cases were only serologically confirmed, which lacks specificity and bias towards false positives in CYD-TDV recipients.[23] These participants could have been seronegative at baseline, but as the influence of pre-vaccination serostatus was not part of the original study design, the serostatus of these participants with serologically confirmed cases was not assessed. It was not part of the original study design to split immunogenicity data by immune versus naïve, so performing sub-analyses based on pre-vaccination serostatus was not possible. Although the present study did not investigate efficacy, this potential increased risk of severe dengue should be noted in the dengue seronegative participants who were included in these analyses. It is of importance that the dengue serostatus of people who plan to receive CYD-TDV is known. Vaccine label variations have been proposed in light of the risk observed in people who were seronegative to dengue. The WHO Strategic Advisory Group of Experts (WHO-SAGE) on Immunization preferred approach for CYD-TDV use in endemic settings is to screen for previous dengue infection to ensure that only those with previous dengue infection are vaccinated.[24]

To our knowledge, this is the first study to demonstrate long-term follow-up CMI data in addition to humoral response following CYD-TDV vaccination. The present study provided some insight into the CMI of CYD-TDV but was not as in-depth as previous studies investigating T-cell responses and the efficacy against VCD.[25-27] Harenberg *et al.* previously demonstrated that a higher level of IFN-γ than TNF-**α** was produced in response to CYD-TDV re-stimulation irrespective of age,[8] with the difference in levels decreasing at one year after vaccination and no

longer apparent at four years after the third vaccine dose in our study. IL-13 response appeared to follow the same pattern as IFN-γ, but IL-5 response was not different to that observed in controls. These differences in CMI responses need to interpreted with caution as the CMI profile may be dependent on dengue serostatus at baseline,[8,28] and as such, may not be extrapolated to regions with high dengue endemicity. There is no real change in intracellular cytokines assessed. In Harenberg *et al*., CYD-TDV was associated with YF-17D-NS 3-specific CD8/IFN-γ responses, without significant TNF-α, and a CYD-specific Th1/Tc1 cellular response in adolescents and adults.[8] For secreted cytokines (IFN and IL-13), responses seemed to be at lower levels at four years than one year after the third dose for both <9 and ≥9 years. Infections eliciting a dominant humoral immune response induced a higher expression of Th2-related cytokines, whereas those characterized by delayed-type hypersensitivity response showed a higher expression of Th1 cytokines.[29] As such, Th1 and Th2-related cytokines levels were measured in the present study to assess the humoral immune response of CYD-TDV.

The present study had some limitations, notably the considerable difference in total sample size between the Singapore and Vietnam studies. The CMI results acquired in the Singapore study were not representative as only a small subset of participants were included in this analysis, and so not as in-depth as previous studies, but as the first CMI data with long-term follow-up they provide us with additional understanding of the effect of the vaccine over time. The studies also lacked positive (e.g. phytohemagglutinin stimulation) and negative (e.g. an irrelevant pool of peptides) controls. In addition, a more sensitive assay such as enzyme-linked immunospotting (ELISPOTs) and/or tetramers may have allowed a more accurate detection of T–cell memory in the CMI analyses.[8,30] The low number of participants in the immunogenicity subset may not have been representative of the overall study population in the two countries.

In conclusion, these phase II studies showed that CYD-TDV induced persistent anti-dengue antibodies over five years in the Singapore and Vietnam studies, with no new safety concerns, supporting the use of a three-dose regimen with a 0-, 6-, and 12-month schedule in these countries in those with prior dengue exposure. Despite a gradual decrease in neutralizing antibody levels over time, a broad and lasting T-cell response against all four serotypes was observed. Booster vaccine studies may facilitate understanding of the T-cell memory response in populations with low and high endemicity. Other ongoing immunogenicity and safety follow-ups may help to further confirm these results in other endemic regions.

Materials and methods

Study design and participants

Data were obtained from a long-term follow-up of two randomized, controlled, observer-blind phase II trials in participants aged 2–45 years who received CYD-TDV (Singapore: NCT0088089; Vietnam: NCT00875524), the methodology had previously been described.[6,7] Data were presented separately and not pooled. The Singapore study was observer-blind for the first vaccine dose and single-blind for the second and third vaccine doses. Participants were randomized 3:1 to receive CYD-TDV or control. The Vietnam study was observer-blind for the first and second vaccinations, with the third administered in a single-blind manner. Participants were randomized 2:1 to receive three CYD-TDV doses or control. Data obtained from healthy participants were split according to age group (<9 and ≥9 years) for *post-hoc* analyses. The study period was from April 2009 to October 2014 in the Singapore study, and March 2009 to July 2014 in the Vietnam study.

Both trials were undertaken in compliance with the International Conference on Harmonization (ICH) guidelines for Good Clinical Practice and the principles of the Declaration of Helsinki. The protocol and amendments were approved by an independent ethics committee and institutional review board. Parents or legal guardians provided informed consent before participation.

Participants or their parents/guardians received follow-up visits or calls. In both studies, all participants had yearly visits after the third vaccine dose.

Study outcomes

Antibody responses

In the Singapore study, antibody titers against dengue were measured before and 28 days following the first and third vaccine doses in a randomized subset of 300 participants (dengue n = 225; control n = 75 [Cohort 1]). These were also measured before and 28 days following the second and third vaccine dose in another randomized subset of 300 other participants (dengue n = 225; control n = 75 [Cohort 2]). A blood sample was taken pre-vaccination from all eligible participants in either Cohort 1 or 2 to determine baseline dengue serostatus.

In the Vietnam study, dengue plaque reduction neutralization test ($PRNT_{50}$; Sanofi Pasteur GCI, Swiftwater, USA) antibody titers were measured as previously described by Kim *et al*.,[31] before and 28 days after each vaccination. The lower limit of quantification of the assay was 10 (1/dil). The use of the $PRNT_{50}$ test was recommended in the WHO guidelines in 2008.[32] A blood sample was also taken at the screening visit in all participants to test for neutralizing antibody level against JE and dengue. Further blood samples were taken yearly up to four years after third vaccine dose.

CMI in the Singapore study

The cellular response against dengue was measured using two complementary tests that evaluated the Th1 and Th2 balance, and CD4 versus CD8 responses. In a subset of adult and adolescent participants, CMI responses to each dengue serotype following the first and third vaccine dose were assessed. In Cohort 1, 80 (dengue n = 60; control n = 20) participants were analyzed for CMI against each serotype of the vaccine parental strains.

For assessment of CMI, cytokine Th1 and Th2-producing antigen-specific $CD4^+$ and $CD8^+$ T-cells were quantified following stimulation *in vitro* of whole blood samples and

peripheral blood mononuclear cells (PBMCs) with pools of NS3 peptides from dengue and YF viruses, as described previously by Harenberg *et al.*[8] Th1 and Th2 cytokines secreted by purified PBMCs were quantified following *in vitro* stimulation with live vaccines of each serotype. Cytokine levels (pg/mL) were analyzed using a multiplex assay (Merck Millipore, Germany). Intracellular cytokine staining was used to quantify antigen-specific cytokine secreting $CD4^+$ and $CD8^+$ cells by flow cytometry following stimulation of whole blood with pools of NS3 peptides from dengue and YF 17D. Specific Th1 and Th2 cytokine secretion by purified peripheral blood mononuclear cells after stimulation with live vaccines of each dengue serotype were assessed in all participants by a Luminex assay.

Safety assessments

The occurrence of any SAEs was reported up to 6 months after the third vaccine dose. Information on related and fatal SAEs was collected throughout the long-term follow-up.

An Independent Data Monitoring Committee (IDMC) reviewed VCD cases for severity assessment and general safety data throughout the two studies. Assessment of dengue infection in the event of fever (≥38°C for 48 hours [Vietnam]/or on at least two consecutive days [Singapore]) consisted of dengue NS1 ELISA, and dengue RT-PCR.

IgM and IgG ELISAs were also used to assess samples from all dengue suspected cases, regardless of time of event after vaccination.

Statistical analysis

In both studies, planned statistical analyses were descriptive with no hypothesis testing performed. *Post-hoc* analyses were undertaken by age subgroup: <9 and ≥9 years age groups, respectively. Data were presented as point estimates with 95% confidence intervals (CIs) calculated using Clopper-Pearson method for proportions in both studies.[33] Antibody analyses were performed on the full analysis set (FAS), defined as the participants who received at least one dose of trial or control vaccine, and had at least one blood sample taken with a valid post-vaccination serology result. Safety analysis were performed on the safety analysis set (SAS) defined as those participants who received at least one dose of trial or control vaccine.

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Disclosure of Potential Conflicts of Interest

DC, CF, TAW, SS, AS, and AB are employed by Sanofi Pasteur. The authors have no other conflicts of interest to declare.

Ethics Statement

Both trials were undertaken in compliance with the International Conference on Harmonization (ICH) guidelines for Good Clinical Practice and the principles of the Declaration of Helsinki. The protocol and amendments were approved by an independent ethics committee and institutional review board. Parents or legal guardians provided informed consent before participation.

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