

Immunogenicity and Tolerability of an MF59[®]-Adjuvanted, Egg-Derived, A/H1N1 Pandemic

Influenza Vaccine in Children Aged 6–35 Months

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3
4 Markus Knuf MD Zentrum für Kinder-und Jugendmedizin, Universitätsmedizin, Rheinland-Pfalz,
5
6
7 Germany

8
9 Geert Leroux-Roels MD PhD, Center for Vaccinology, Ghent University and University Hospital,
10
11 Ghent, Belgium

12
13
14 Hans. C. Rümke MD PhD, Vaxinostics BV, University Vaccine Center Rotterdam Nijmegen,
15
16
17 Rotterdam, The Netherlands

18
19 Katia Abarca MD, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago de
20
21 Chile, Chile

22
23
24 Luis Rivera MD, Hospital Maternidad Ntra Sra. de la Altagracia, Gazcue, Santo Domingo, The
25
26
27 Dominican Republic

28
29 Maria Lattanzi MD PhD, Novartis Vaccines and Diagnostics, Inc., Cambridge, MA, USA

30
31 Paola Pedotti PhD, Novartis Vaccines and Diagnostics, Inc., Cambridge, MA, USA

32
33
34 Ashwani Arora MD, Novartis Vaccines and Diagnostics, Inc., Cambridge, MA, USA

35
36 Dorothee Kieninger-Baum MD, Zentrum für Kinder-und Jugendmedizin, Universitätsmedizin,
37
38
39 Rheinland-Pfalz, Germany

40
41 Giovanni Della Cioppa MD Novartis Vaccines and Diagnostics, Inc., Cambridge, MA, USA

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45
46 **Correspondence to:** Dr Maria Lattanzi. Novartis Vaccines and Diagnostics S.r.l., Via Fiorentina, 1,
47
48 I-53100 Siena, Italy. Tel: +39 0577 245273; Fax: +39 0577 245420; Email:
49
50 maria.lattanzi@novartis.com

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58 trials. All authors were involved in the interpretation of analyzed data and the decision to submit for
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60 publication.
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Key Words: Pandemic influenza, A/H1N1, vaccine, MF59, paediatric

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Abstract

Background: Vaccines against pandemic A/H1N1 influenza should provide protective immunity in children, because they are at greater risk of disease than adults. This study was conducted to identify the optimal dose of an MF59[®]-adjuvanted, egg-derived, A/H1N1 influenza vaccine for young children.

Methods: Children aged 6–11 months (N=144) and 12–35 months (N=186) received vaccine formulations containing either 3.75 µg antigen with half the standard dose of MF59, or 7.5 µg antigen with a standard dose MF59, or a non-adjuvanted formulation containing 15 µg antigen (children 12-35 months only). Participants were given two primary vaccine doses three weeks apart, followed by one booster dose of MF59-adjuvanted seasonal influenza vaccine one year later. Immunogenicity was assessed by haemagglutination inhibition and microneutralization assays.

Results: All vaccine formulations were highly immunogenic and met all three European licensure criteria after two doses. MF59-adjuvanted vaccines met all licensure criteria after one dose in both age cohorts, while non-adjuvanted vaccine did not meet all criteria after one dose in children 12-35 months. A single booster dose was highly immunogenic and stable antibody persistence was observed in response to all vaccines. All vaccines were well tolerated.

Conclusions: In this study a single dose of 3.75µg antigen with half the standard dose of MF59 was shown to be optimal, providing adequate levels of immediate and long-term antibodies in paediatric subjects aged 6–35 months. These data demonstrated that MF59 adjuvant allowed for reduced antigen content and promoted significant long-term antibody persistence in children, with a satisfactory safety profile.

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5 Markus Knuf MD Zentrum für Kinder-und Jugendmedizin, Universitätsmedizin, Rheinland-Pfalz,
6
7 Germany

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10 Geert Leroux-Roels MD PhD, Center for Vaccinology, Ghent University and University Hospital,
11
12 Ghent, Belgium

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15
16 Rotterdam, The Netherlands

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47
48 I-53100 Siena, Italy. Tel: +39 0577 245273; Fax: +39 0577 245420; Email:

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51 maria.lattanzi@novartis.com

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2 children, because they are at greater risk of disease than adults. This study was conducted to
3 identify the optimal dose of an MF59[®]-adjuvanted, egg-derived, A/H1N1 influenza vaccine for
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10 formulations containing either 3.75 µg antigen with half the standard dose of MF59, or 7.5 µg
11 antigen with a standard dose MF59, or a non-adjuvanted formulation containing 15 µg antigen
12 (children 12-35 months only). Participants were given two primary vaccine doses three weeks apart,
13 followed by one booster dose of MF59-adjuvanted seasonal influenza vaccine one year later.
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25 criteria after two doses. MF59-adjuvanted vaccines met all licensure criteria after one dose in both
26 age cohorts, while non-adjuvanted vaccine did not meet all criteria after one dose in children 12-35
27 months. A single booster dose was highly immunogenic and stable antibody persistence was
28 observed in response to all vaccines. All vaccines were well tolerated.
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37 **Conclusions:** In this study a single dose of 3.75µg antigen with half the standard dose of MF59 was
38 shown to be optimal, providing adequate levels of immediate and long-term antibodies in paediatric
39 subjects aged 6–35 months. These data demonstrated that MF59 adjuvant allowed for reduced
40 antigen content and promoted significant long-term antibody persistence in children, with a
41 satisfactory safety profile.
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Introduction

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3 The emergence of a novel strain of influenza A/H1N1 virus in the spring of 2009 led to the first
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5 pandemic of the 21st century being declared by the World Health Organization.¹
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8 Current evidence suggests that mass immunization is important for optimal disease control, with the
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10 vaccination of children essential in reducing infection rates within families and consequently
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12 throughout the wider population.²⁻⁴ Massive effort has been put into developing vaccines based on
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14 the A/California/7/2009 strain and disseminating them to large proportions of the population.
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16 The A/H1N1 pandemic strain disproportionately affected children and younger adults.⁵ Children
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18 may have been particularly affected because of close mixing at schools allowing for increased
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20 transmission,⁶ and because of the lack of previous exposure to this viral strain. Thus, it was
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22 important that any candidate vaccine was effective in all age groups, including children.
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30 The oil-in-water adjuvant MF59[®] (Novartis Vaccines and Diagnostics) has been shown to be
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32 effective in pandemic A/H1N1 vaccines for adults and children, and has an acceptable safety
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34 profile.⁷⁻¹¹ Adjuvants help to increase the immune response to vaccines, achieving higher antibody
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36 titres that lead to protection with less amount of antigen.¹²⁻¹⁵ The ability to generate adequate
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38 immunity in the individual while using as little antigen as possible is crucially important during a
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40 pandemic, when the global capacity for vaccine production is stretched to the limit.
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47 The MF59-adjuvanted, egg-based, monovalent vaccine, Focetria[®] (Novartis Vaccines and
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49 Diagnostics), is based on the A/H1N1 California/7/2009 strain, and was approved for use by the
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51 European Medicines Agency with an antigen content of 7.5 µg per dose.¹⁶ A previous clinical trial
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53 demonstrated a single dose of this vaccine was sufficient to meet the European licensure criteria for
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55 pandemic influenza vaccines in both adults and elderly.¹⁷
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The aim of this study was to identify priming antigen and adjuvant doses resulting in optimal antibody levels shortly after primary immunization and one-year booster immunizations in children aged 6–35 months. In addition, long-term antibody persistence and vaccine safety were assessed up to 18 months after vaccination.

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Materials and Methods

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3 This multinational, single-blind, randomized, Phase III, dose-ranging study was conducted across
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5 eleven sites in Germany, two sites in Belgium, one site in The Netherlands, two sites in The
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7 Dominican Republic, and two sites in Chile between September 2009 and July 2011. One German
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9 site was excluded from analyses due to noncompliance with the protocol requirements for safety
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11 reporting for a different study. The trial was performed in accordance with Good Clinical Practice
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13 guidelines and the Declaration of Helsinki. The protocol was reviewed and approved by the
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15 Institutional Review Board or Ethics Committee of each participating institution. Written informed
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17 consent was obtained from the parent or legal guardian of all subjects before their enrolment in the
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19 study. This clinical trial was registered at ClinicalTrials.gov: NCT00971542.
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Subjects

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29 A total of 684 healthy children 6 months to 17 years of age entered this study, of which 330
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31 children were included in one of the younger age cohorts (6-11 months; 12-35 months) covered in
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33 this paper. The results of the older age cohorts (3-8 years; 9-17 years) will be reported separately.
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36 Exclusion criteria included a history of, or ongoing serious illness; a history of anaphylaxis;
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38 hypersensitivity or previous adverse reactions to vaccination or to influenza viral proteins, eggs, or
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40 chicken protein; receipt of any adjuvanted influenza vaccine within three months prior to
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42 enrolment; influenza disease within three months prior to enrolment; receipt of any investigational
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44 agent within four months prior to enrolment; receipt of any other vaccine within four months prior
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46 to enrolment, with the exception of non-adjuvanted seasonal influenza vaccine one week before and
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48 after study vaccinations; an impaired immune system; and the receipt of blood or plasma derivatives
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51 within twelve weeks prior to study enrolment.
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Study procedures

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Subjects were divided into 6–11 and 12–35 month-old cohorts. Subjects in the 6–11 months age group were randomized in a 1:1 ratio to receive vaccine containing either 7.5 µg antigen with a full dose of MF59 (7.5-Full MF59) or 3.75 µg antigen with half a dose of MF59 (3.75-Half MF59). Subjects in the 12–35 months age group were randomized in a 2:2:1 ratio to receive 3.75-Half MF59 vaccine, 7.5-Full MF59 vaccine, and a non-adjuvanted formulation containing 15 µg antigen (15-No MF59), respectively. When the study was designed at the very beginning of the pandemic, it was thought based on previous A/H5N1 data, that the immunogenicity of the nonadjuvanted formulation would be suboptimal.^{18, 19} Therefore, to minimize the number of subjects that were supposed to receive this formulation, it was limited to the 12-35 months age cohort (and the 3-8 years age cohort, results reported elsewhere).

Subjects were randomly assigned to study groups using a system of sealed envelopes. The identity of the study vaccine was not revealed to the subject or their parent/legal guardian. All subjects received two doses of vaccine given three weeks apart. A booster dose of MF59-adjuvanted, seasonal, trivalent influenza vaccine (MF59-TIV) was administered one year after primary immunization. In addition, subjects who had never previously received seasonal vaccine were given a second dose of MF59-TIV three weeks later to complete the seasonal immunization course according to WHO recommendations.²⁰ Therefore, subjects received one dose on Day 1, a second dose on Day 22 (primary doses), a third dose on Day 366 (booster), and, where applicable, a fourth dose on Day 387 (**Figure 1**). Vaccines were administered either in the deltoid muscle of the non-dominant arm (subjects ≥24 months-old) or in the anterolateral thigh muscle (subjects <24 months-old). Blood samples (~5 mL per sample) were collected for immunogenicity analyses on Day 1 (baseline, pre-vaccination), Day 22 (three weeks after first primary dose, before the second primary dose), Day 43 (three weeks after second primary dose), Day 366 (one year after primary immunization, before the booster), and Day 387 (three weeks after booster vaccination). The immunogenicity of the second seasonal immunization was not investigated. Safety data were collected up to 18 months after study entry.

Vaccines

The investigational, egg-derived, monovalent, MF59-adjuvanted, pandemic vaccine, Focetria[®] (Novartis Vaccines and Diagnostics), contained haemagglutinin and neuraminidase surface antigens derived from the A/H1N1/California/7/2009 influenza strain. The vaccine seed A/H1N1 virus was prepared from the reassortant virus, NYMC X-179A (New York Medical College, New York, USA), generated from the A/California/7/2009 strain, as recommended by the World Health Organization.²¹ A full/standard dose of MF59, as used in the commercial seasonal influenza vaccine, Flud[®] (Novartis Vaccines and Diagnostics), contains 9.75 mg squalene, 1.18 mg polysorbate 80, 1.18 mg sorbitan trioleate, 0.66 mg sodium citrate dehydrate, and 0.04 mg citric acid monohydrate. The booster dose of the seasonal, MF59-adjuvanted, trivalent influenza vaccine (MF59-TIV), Flud, contained a standard dose of MF59, and 15 µg of each of the World Health Organization reference strains recommended for the 2010/11 influenza season: A/California/7/2009 (H1N1); A/Perth/16/2009 (H3N2); and B/Brisbane/60/2008. Thus, the A/H1N1 antigen strain contained in both priming and booster vaccines were identical. Vaccines were supplied in pre-filled syringes. Single doses of 7.5-Full MF59 and 15-No MF59 vaccines were administered in a volume of 0.5 mL. A single dose of the 3.75-Half MF59 formulation was administered in a volume of 0.25 mL. Single doses of seasonal vaccine were administered in volumes of 0.5 mL or 0.25 mL for subjects aged ≥ 36 months and < 36 months at the time of booster administration, respectively.

Immunogenicity assessments

Blood samples were obtained by venipuncture and centrifuged at 1500 g for 10 minutes; sera were stored at a temperature of -18°C or below and shipped to the Novartis Vaccines Clinical Serology Laboratory in Marburg, Germany, where antibody responses were assessed by haemagglutination (HI) and microneutralization (MN) assays. The HI assay was based on the method of Stephenson and colleagues;²² HI titre was expressed as the reciprocal of the highest dilution at which haemagglutination was totally inhibited. MN assays were performed according to a method

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3 previously described by Nicholson and colleagues, serial dilutions of serum started at 1:20; the
4 reciprocals of two-fold dilutions that achieved $\geq 50\%$ neutralization of viral growth were considered
5 a positive result.²³ Titers were reported in terms of Geometric Mean Titers (GMT). Geometric Mean
6 Ration (GMR) were calculated as GMT Day 22/GMT Day 1, GMT Day 43/GMT Day 1, GMT Day
7 366/GMT Day 1 and GMT Day 387/GMT Day 366. In addition, HI GMT titers greater or equal
8 than 1: 40 were assessed. Seroconversion, as assessed by HI assay, was defined as a negative pre-
9 vaccination antibody titre of $< 1:10$ to a positive post-vaccination titre of $\geq 1:40$, or a minimum
10 four-fold increase where pre-vaccination titres were $\geq 1:10$. HI titres below the detection limit of
11 1:10 were arbitrarily assigned to half that limit (1:5) for the purpose of analysis. The HI titer of
12 1:40, which has been determined as an immunologic correlate corresponding to a 50% reduction in
13 the risk of contracting influenza, is based on studies in adults and may not be generalizable to
14 children. Indeed, a recent study in a large pediatric population indicated that the conventional HI
15 titer of 1:40 was only associated with 22% protection.²⁴ Therefore, percentages of subjects with HI
16 titers $\geq 1:330$ were also calculated, as this cut-off titer has been shown to predict an 80% clinical
17 protective level in young children. Homologous HI and MN assays were performed using the
18 vaccine antigen strain A/California/7/2009 (H1N1).
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39 *Safety assessment*

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42 Subjects were observed for 30 minutes after receipt of each vaccine dose to monitor for immediate
43 adverse reactions. The frequency and severity of solicited local and systemic reactions occurring
44 within one week of each vaccination were recorded on diary cards and summarized according to the
45 Brighton collaboration case definition.²⁵ Solicited local reactions were ecchymosis, erythema,
46 induration, swelling, (categorized as none, $1 \leq 10$ mm, 11 to ≤ 25 mm, 26 to ≤ 50 mm, 51 to ≤ 100
47 mm and >100 mm [severe local reactions]) and tenderness (categorized as none, minor light
48 reaction to touch, cried or protested to touch, cried when injected limb was moved [severe]).
49 Solicited systemic reactions were sleepiness, diarrhoea, vomiting, irritability, altered eating habits,
50 shivering, unusual crying, and fever ($\geq 38^\circ\text{C}$). A high, grade 3 fever was defined as an axillary body
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1 temperature of $\geq 40^{\circ}\text{C}$. Solicited systemic reactions were collected as present/not present. In
2 addition, use of analgesic/antipyretic was collected as measure of reactogenicity. All unsolicited
3 adverse events (AE) were recorded for three weeks after each vaccination. The onset of new chronic
4 diseases, serious adverse events (SAEs), and AEs leading to withdrawal were recorded throughout
5 the entire study period (Day 1–546). A physician rated AEs as mild, moderate, or severe if resulting
6 in no limitation of, some limitation of, or inability to perform normal daily activities, respectively.
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15 *Statistical analyses*

16 Sample sizes were chosen to meet or exceed the minimum requirements of the European guidelines
17 for influenza vaccine clinical trials. No formal statistical hypothesis was tested, immunogenicity
18 endpoints being based on HI licensure criteria established by the EU Committee for Medicinal
19 Products for Human Use (CHMP).²⁶ There are currently no pre-defined CHMP criteria for children,
20 therefore, the following adult licensure criteria applied: the number of subjects achieving
21 seroconversion or significant increase (defined as $\text{HI} \geq 1:40$ for subjects with a pre-vaccination HI
22 titer $< 1:10$; a minimum 4-fold increase HI titer for subjects with a pre-vaccination HI titer $\geq 1:10$)
23 should be $> 40\%$; geometric mean ratios (GMRs) should be > 2.5 ; and the proportion of subjects
24 achieving an HI titre $\geq 1:40$ should be $> 70\%$.
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39 Percentages of subjects with HI titers $\geq 1:330$ were also calculated.²⁴ Immunogenicity data
40 reflecting the above endpoints as well as the corresponding two-sided 95% confidence intervals
41 (CIs) were calculated for each vaccine group and age cohort using two-way analysis of variance
42 (ANOVA) with factors for vaccine group and center. Safety data were evaluated descriptively and
43 expressed as the proportion and number of subjects with AEs in each group. Immunogenicity
44 analyses were run on the per-protocol (PP) set, which consisted of subjects who received all the
45 relevant doses of vaccine correctly, provided evaluable serum sample at the relevant time points,
46 and had no major protocol violations. Safety was analyzed for all subjects exposed to at least one
47 study vaccination and provided safety data. All statistical analyses were performed by employees of
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Novartis Vaccines and Diagnostics using SAS 9.1[®] software.

Results

After the removal of one study site, 330 subjects were included in the two age cohorts (6-11 months and 12-35 months) covered in this paper. In this study, 64-78% across groups completed the study protocol (**Figure 1**). An average of 93% and 72% of subjects across study groups completed the primary immunization schedule (Day 43) and received the booster dose (Day 366), respectively. A second dose of seasonal vaccine at Day 387 was administered to about 47% of subjects across vaccine groups in both age cohorts.

Demographic and other baseline population characteristics are shown in **Table 1**. The majority of children were of Hispanic ethnic origin. In the youngest age cohort 6-11 months, 144 subjects were enrolled from 10 study sites, i.e. 4 in Germany (n=13), 2 in Belgium (n=17), 2 in Chilli (n=70) and 2 sites in the Dominican Republic (n=44). In the age cohort 12-35 months, a total of 186 subjects were enrolled from 8 study sites, i.e. 2 in Germany (n=10), 1 in the Netherlands (n=4), 2 in Belgium (n=69), 2 in Chile (n=81) and 1 site in the Dominican Republic (n=22). Mean age of the subjects was 8.5 months and 24.2 months in cohorts 6-11 months and 12-35 months, respectively

Immunogenicity

Immunogenicity analyses were performed on the PP data set, which included 68-76% of subjects across groups after the primary vaccination schedule (Day 43), 49-53% of subjects for persistence analyses (Day 366), and 32-55% of subjects for post-booster analyses (Day 387).

HI assay GMTs were low at baseline, with only 18–26% of subjects displaying HI titres of $\geq 1:40$ (**Table 2**). In the 6–11 months cohort, 7.5-Full MF59 vaccine induced greater GMTs than the 3.75-Half MF59 formulation three weeks after both first and second doses (**Figure 2A**). One dose of either MF59-adjuvanted vaccine was sufficient to meet the CHMP criterion for GMR (**Table 2**). In the 12–35 months cohort, the 7.5-Full MF59 vaccine induced greater GMTs than the 3.75-Half MF59 formulation after first and second doses (**Figure 2B**). GMTs were greater in response to both MF59-adjuvanted vaccines compared with the 15 μ g non-adjuvanted formulation (**Figure 2B**). All

1 three vaccine formulations met the licensure criterion for GMRs as early as 3 weeks after the first
2 vaccination (**Table 2**). Subjects in the 6–11 months cohort demonstrated high HI seroconversion
3 rates for both formulations after the first and second vaccinations (91–98%); the same was true in
4 subjects in the 12–35 months cohort who received adjuvanted formulations (97–100%; **Table 2**).
5 Seroconversion rates were lower in subjects who received the 15-No MF59 formulation (52% on
6 Day 22 and 74% on Day 43) than those who received adjuvanted formulations. For both age
7 cohorts, seroconversion rates for all vaccines were above the CHMP licensure criterion of > 40%
8 after both the first and second vaccinations. Rates of subjects with HI titers \geq 1:40 were high after
9 first and second doses in response to both adjuvanted formulations in both age cohorts, ranging
10 from 91% to 100% (**Figure 3A and B**), exceeding the CHMP criterion of > 70%. Rates of subjects
11 with HI titers \geq 1:40 in the 15-No MF59 vaccination group of the 12–35 months cohort failed to
12 meet licensure criterion after the first dose (52%); a second dose was required for this criterion to be
13 achieved (81%) (**Figure 3B**). Analyses by MN assay supported HI data (**Figure 2C and D, Table**
14 **3**).
15 Long-term antibody persistence was demonstrated in subjects receiving MF59-adjuvanted vaccines;
16 95–100% of subjects across age groups achieved HI titers \geq 1:40 one year after immunization,
17 which is considerably higher than in those receiving the non-adjuvanted formulation (62%) (**Figure**
18 **3A and B**). Antibody titres were significantly higher after booster vaccination compared with
19 GMTs after priming doses (**Figure 2**). The licensure criteria for GMR and seroconversion were met
20 by all priming groups following booster vaccination in both age cohorts (**Table 2**) and all subjects
21 achieved HI titers \geq 1:40 in all vaccine groups in both age cohorts, regardless of priming
22 formulations.. Analyses by MN assay supported HI data (**Figure 2C and D, Table 3**).
23 Percentages of subjects with HI titers \geq 1:330 are reported in **Table 4**. In the 6–11 months cohort,
24 35% (3.75-Half MF59), 39% (7.5-Full MF59) and 82% (3.75-Half MF59), 100% (7.5-Full MF59)
25 of subjects achieved HI titers \geq 1:330 after the first and second primary doses, respectively, and all
26 subjects in both vaccine groups had HI titers \geq 1:330 after the booster vaccination.
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3 In children 12-35 months of age receiving adjuvanted formulations, percentages of subjects
4 achieving HI titers \geq 1:330 were higher compared with subjects receiving the nonadjuvanted
5 formulation. HI titers \geq 1:330 were achieved in 37% (3.75-Half MF59), 47% (7.5 Full MF59), 23%
6 (15-No MF59), and 85% (3.75-Half MF59), 91% (7.5-Full MF59), 39% (15-No MF59) after the
7 first and second primary doses, respectively and in 100% (both adjuvanted formulations) and 91%
8 (15-No MF59) of subjects after the booster dose.
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10 *Safety and tolerability*

11 All subjects were exposed to at least one study vaccination and contributed to the safety analyses.

12 In the 6–11 months cohort, the percentage of subjects with solicited local reactions were similar
13 between first and second primary doses, and between the two adjuvanted formulations (**Table 5**).
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15 The most frequently reported local reactions after primary immunizations were erythema (15–25%
16 of subjects) and tenderness (14–25%). There were no cases of severe local reactions after primary
17 vaccinations. Local reactions were generally more frequent after the booster dose than the primary
18 doses. Tenderness was the most frequently reported local reaction after the booster (34–39%) and
19 second (23–30%) seasonal dose, followed by erythema (28–31% and 22–30%, respectively). Severe
20 reactions were reported in up to 8% and 6% across groups after booster dose and second seasonal
21 dose, respectively, with tenderness as the most frequently reported severe local reaction. The
22 incidence of solicited systemic reactions was generally lower after the second primary vaccination
23 compared with the first primary vaccination in the 6–11 months cohort, no substantial differences
24 were observed between the vaccine groups. The incidence of systemic reactions after the booster
25 was similar to that after the second primary vaccination. The incidence of local and systemic
26 reactions tended to be lower after the second seasonal vaccination compared to the booster (**Table**
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54 In the 12–35 months cohort (**Table 6**), solicited local reactions were less frequent in the non-
55 adjuvanted formulation compared with the adjuvanted formulations and occurred at a similar rate
56 after the first and second primary doses.
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Tenderness was the most frequently reported local reaction after the primary immunizations (22–35%), increasing in frequency after the booster (34–48%), but decreasing in frequency after the second seasonal dose (15–29%). Other local reactions occurred at a similar rate after the primary and booster vaccinations, but tended to occur at a lower rate after the second seasonal vaccination. After primary immunizations, severe tenderness was reported in 1-3% of subjects across adjuvanted groups, and severe induration and erythema occurred in 1% of subjects receiving 7.5-full MF59. Following booster and second seasonal dose, severe tenderness was reported in up to 5% of subjects across adjuvanted formulations. Systemic reactions tended to be similar between the vaccine formulations after the primary immunizations in the 12–35 months cohort. There were three reports of fever $\geq 40^{\circ}\text{C}$: one in the 6-11 months cohort (3.75-Half MF59) and two in the 12-35 months cohort (n=1: 3.75-Half MF59, n=1: 7.5-Full MF59), all following the first vaccination. Cases occurred between 3-5 days postvaccination and all resolved within 1-2 days without sequelae. For spontaneously reported AEs, no differences in overall frequency of AEs were observed between the two adjuvanted formulations and between adjuvanted and nonadjuvanted vaccine groups. Between study Days 1 and 43, AEs were spontaneously reported for 51–60% of subjects across age and vaccine groups; 5–16% of these cases were considered at least possibly related to vaccination. In the 6-11 months cohort, the most commonly reported AEs were nasopharyngitis (23–31%), followed by diarrhea (12-13%); in the 12-35 months cohort, the most commonly reported AEs were nasopharyngitis (25-30%) and bronchitis (7-16%). Selected unsolicited AEs from Day 1-Day 546 are presented in **Table 7**. Between Days 1-366, there were 25 subjects with SAEs and 8 subjects with new onset chronic diseases. None of these SAEs and new onset of chronic diseases was considered as related to study vaccination. There were 3 AEs leading to premature study withdrawal, one AE was considered at least possibly related to study vaccination (6-11 months cohort, 3.75-Half MF59 group; subject experienced mild allergic dermatitis on day 2, the AE resolved in 6 days).

1 After the booster vaccination, the most frequently reported unsolicited AEs were nasopharyngitis
2 (12–13%), and diarrhoea (2–6%) in the 6–11 months cohort, and nasopharyngitis (6–9%) and
3 conjunctivitis (0–5%) in the 12-15 months cohort. Nasopharyngitis was also the most frequently
4 reported AE after the second dose of the seasonal MF59 vaccine in both the 6–11 months (15%)
5 and 12–35 months (12%) cohorts.
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9 One 6–11 month-old subject in the 3.75-Half MF59 group experienced one SAE, and one 12–35
10 month-old subject in the 3.75-Half MF59 group experienced two SAEs between booster
11 administration and the end of the study (Day 366–546). No SAEs were related to either the booster
12 or the second seasonal vaccination. There was one new onset of chronic disease in 6-11 month
13 (asthmatic crisis) and 12-35 month (allergic rhinitis) age cohorts, and one AE leading to premature
14 study withdrawal in a 6-11 month-old subject (food allergy), none of which related to study
15 vaccination between booster administration and the end of the study.
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Discussion

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3 All the investigational vaccines used in this study were shown to be highly immunogenic and well
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5 tolerated in children from 6 to 35 months of age. A single dose of either the 3.75-Half MF59 or 7.5-
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7 Full MF59 vaccines was sufficient to meet all three European licensure criteria for influenza
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9 vaccines in both age cohorts; two doses of the non-adjuvanted vaccine were required to achieve
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11 similar results in subjects aged 12-35 months. The ability of MF59 to allow for reduced antigen
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13 content would potentially ensure a higher coverage during a pandemic outbreak, which is an
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15 important public health issue to consider in periods of heightened demand.
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19 The findings of this study are supported by similar trials. A cell culture-derived, MF59-adjuvanted
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21 A/H1N1 vaccine has been demonstrated to provide adequate levels of antibodies after a single dose
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23 in British adults⁹ and Japanese adults and children.^{12, 27} Adults were found to require one 3.75-Half
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25 MF59 vaccine dose, while a single 7.5-Full MF59 dose was optimal for children in the Japanese
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27 study.²⁷ Also, a single dose of 3.75-Half MF59 and 7.5-Full MF59 fulfilled the licensure criteria
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29 for adult and elderly subjects 3 months after seasonal vaccination, or concomitantly with seasonal
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31 vaccination, without compromising the tolerability or immunogenicity of either vaccine.¹¹
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35 A trial of A/H1N1 vaccine was conducted in Costa Rican children to compare an MF59-adjuvanted
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37 formulation containing 7.5 µg of A/California/7/2009 antigen with 15 µg and 30 µg non-adjuvanted
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39 vaccines.⁷ All three vaccines met the US Center for Biologics Evaluation and Research (CBER) and
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41 CHMP licensure criteria after one dose in subjects aged 9–17 years; only the MF59-adjuvanted
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43 formulation met these criteria after one dose in younger subjects of 3 to 8 years.
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47 Paediatric and adult clinical trials using the oil-in-water adjuvant AS03[®]-adjuvanted
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49 (GlaxoSmithKline Biologicals, Wavre, Belgium) provided further evidence of the benefits of
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51 adjuvanted A/H1N1 vaccines.²⁸⁻³¹
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55 It has recently been demonstrated that the conventional titer of 1:40 correlate may not be
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57 appropriate in a pediatric population.²⁴ A titer of 1:40 was only associated with 22% protection,
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59 whereas titers of 1:110 or 1:330 could predict a 50% or an 80% of clinical protective level.²⁴ Our
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1 results demonstrate that in children receiving MF59 adjuvanted formulations, a high proportion
2 reached the stringent HI cut-off titer $\geq 1:330$ postvaccination, i.e. 82-100% and 100% after primary
3 and booster doses respectively, as compared with 39% in children receiving the non-adjuvanted
4 vaccine. The seasonal MF59 vaccine was able to boost the immune responses also in the group
5 primed with the nonadjuvanted formulation: indeed, 91% of subjects primed with the non-
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14 use of MF59 adjuvanted vaccines in the pediatric population.

15 In the present study, significant antibody persistence was observed in response to the MF59-
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In the present study, significant antibody persistence was observed in response to the MF59-
adjuvanted vaccine formulations, with HI titers $\geq 1:40$ detected in 95–100% of subjects one year
after immunization. The ability of MF59 to promote long-term antibody persistence has also been
demonstrated in previous studies.^{32, 33} All subjects in the current study received two priming doses;
as such, further investigations are required to confirm that long-term antibody persistence and
response to booster vaccination is not negatively affected by a one-dose priming schedule. The
production of cross-reactive antibodies able to provide heterologous immunity was not analysed
during this study. However, the ability of MF59 to promote the production of cross-reactive
antibodies is well documented.^{32, 34-36}

The overall safety and tolerability were as expected in this age group and were in line with previous
studies using MF59-adjuvanted vaccines in young children.^{15, 27, 37, 38} Solicited local reactions were
more common in adjuvanted formulations compared with nonadjuvanted vaccine, but there were no
MF59-related increase in the frequency of solicited systemic reactions or unsolicited adverse
events. This study demonstrated an acceptable safety profile for the adjuvanted vaccine over an 18-
month period; although subjects were repeatedly vaccinated with up to four doses of MF59-
adjuvanted vaccine, no vaccine-related SAEs were reported during the study period.

In conclusion, a single dose of 3.75 μ g antigen together with half the standard dose of MF59 was
identified as optimal, provided adequate levels of immediate and long-term antibodies and an
acceptable safety profile in children 6-35 months of age. These data support the suitability of
MF59-adjuvanted vaccines for pandemic immunization in the paediatric population.

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Author contributions

All authors participated in the conception, design and implementation of the trials. All authors were involved in the interpretation of analyzed data and the decision to submit for publication.

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Figure legends

1
2 **Figure 1:** Design of clinical trial and subject disposition.
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7 **Figure 2:** GMTs by HI (A and B) and MN (C and D) assay at baseline (Day 1), three weeks after
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9 first (Day 22) and second (Day 43) priming doses, and pre- and post-booster vaccination in children
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11 aged 6–11 months- (A and C) and 12–35 months old (B and D).
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16 **Figure 3:** Percentages (95% CI) of subjects aged 6–11 months (A) and 12–35 months (B) achieving
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18 HI titer \geq 1:40 after primary and booster immunizations. Broken line represents the CHMP
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20 licensure criteria.
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Introduction

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3 The emergence of a novel strain of influenza A/H1N1 virus in the spring of 2009 led to the first
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5 pandemic of the 21st century being declared by the World Health Organization.¹ Mass immunization
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7 is currently the most effective way to prevent disease in the individual and limit human-to-human
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9 viral transmission. Massive effort has been put into developing vaccines based on the
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11 A/California/7/2009 strain and disseminating them to large proportions of the population. The
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13 A/H1N1 pandemic strain disproportionately affected children and younger adults.² Children may
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15 have been particularly affected because of close mixing at schools allowing for increased
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17 transmission,³ and because of the lack of previous exposure to this viral strain. Thus, it was
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19 important that any candidate vaccine was effective in all age groups, including children.
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27 The oil-in-water adjuvant MF59[®] (Novartis Vaccines and Diagnostics) has been shown to be
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29 effective in pandemic A/H1N1 vaccines for adults and children, and has an acceptable safety
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31 profile.⁴⁻⁷ Adjuvants help to increase the immune response to vaccines, achieving higher antibody
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33 titres that lead to protection with less amount of antigen. The ability to generate adequate immunity
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35 in the individual while using as little antigen as possible is crucially important during a pandemic,
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37 when the global capacity for vaccine production is stretched to the limit.
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44 The MF59-adjuvanted, egg-based, monovalent vaccine, Focetria[®] (Novartis Vaccines and
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46 Diagnostics), is based on the A/H1N1 California/7/2009 strain, and was approved for use by the
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48 European Medicines Agency with an antigen content of 7.5 µg per dose.⁸ A previous clinical trial
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50 demonstrated a single dose of this vaccine was sufficient to meet the European licensure criteria for
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52 pandemic influenza vaccines in both adults and elderly.⁹ In the interest of conserving antigen and
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54 adjuvant, it is essential to investigate whether a single dose of MF59-adjuvanted A/H1N1 vaccine
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56 of reduced antigen content can provide adequate levels of antibodies in young children.
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The aim of this study was to identify priming antigen and adjuvant doses resulting in optimal antibody levels shortly after primary immunization and one-year booster immunizations in children aged 6–35 months. In addition, long-term antibody persistence and vaccine safety were assessed up to 18 months after vaccination.

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Materials and Methods

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3 This multinational, single-blind, randomized, Phase III, dose-ranging study was conducted across
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5 eleven sites in Germany, two sites in Belgium, one site in The Netherlands, two sites in The
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7 Dominican Republic, and two sites in Chile between September 2009 and July 2011. One German
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9 site was excluded from analyses due to noncompliance with the protocol requirements for safety
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11 reporting for a different study. The trial was performed in accordance with Good Clinical Practice
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13 guidelines and the Declaration of Helsinki. The protocol was reviewed and approved by the
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15 Institutional Review Board or Ethics Committee of each participating institution. Written informed
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17 consent was obtained from the parent or legal guardian of all subjects before their enrolment in the
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19 study. This clinical trial was registered at ClinicalTrials.gov: NCT00971542.
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Subjects

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28 A total of 684 healthy children 6 months to 17 years of age entered this study, of which 330
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30 children were included in one of the younger age cohorts (6-11 months; 12-35 months) covered in
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32 this paper. The results of the older age cohorts (3-8 years; 9-17 years) will be reported separately.
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34 Exclusion criteria included a history of, or ongoing serious illness; a history of anaphylaxis;
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36 hypersensitivity or previous adverse reactions to vaccination or to influenza viral proteins, eggs, or
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38 chicken protein; receipt of any adjuvanted influenza vaccine within three months prior to
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40 enrolment; influenza disease within three months prior to enrolment; receipt of any investigational
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42 agent within four months prior to enrolment; receipt of any other vaccine within four months prior
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44 to enrolment, with the exception of non-adjuvanted seasonal influenza vaccine one week before and
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46 after study vaccinations; an impaired immune system; and the receipt of blood or plasma derivatives
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48 within twelve weeks prior to study enrolment.
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Study procedures

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Subjects were divided into 6–11 and 12–35 month-old cohorts. Subjects in the 6–11 months age group were randomized in a 1:1 ratio to receive vaccine containing either 7.5 µg antigen with a full dose of MF59 (7.5-Full MF59) or 3.75 µg antigen with half a dose of MF59 (3.75-Half MF59). Subjects in the 12–35 months age group were randomized in a 2:2:1 ratio to receive 3.75-Half MF59 vaccine, 7.5-Full MF59 vaccine, and a non-adjuvanted formulation containing 15 µg antigen (15-No MF59), respectively. Subjects were randomly assigned to study groups using a system of sealed envelopes. The identity of the study vaccine was not revealed to the subject or their parent/legal guardian. All subjects received two doses of vaccine given three weeks apart. A booster dose of MF59-adjuvanted, seasonal, trivalent influenza vaccine (MF59-TIV) was administered one year after primary immunization. In addition, subjects who had never previously received seasonal vaccine were given a second dose of MF59-TIV three weeks later to complete the seasonal immunization course according to WHO recommendations.¹⁰ Therefore, subjects received one dose on Day 1, a second dose on Day 22 (primary doses), a third dose on Day 366 (booster), and, where applicable, a fourth dose on Day 387 (**Figure 1**). Vaccines were administered either in the deltoid muscle of the non-dominant arm (subjects ≥24 months-old) or in the anterolateral thigh muscle (subjects <24 months-old). Blood samples (~5 mL per sample) were collected for immunogenicity analyses on Day 1 (baseline, pre-vaccination), Day 22 (three weeks after first primary dose, before the second primary dose), Day 43 (three weeks after second primary dose), Day 366 (one year after primary immunization, before the booster), and Day 387 (three weeks after booster vaccination). The immunogenicity of the second seasonal immunization was not investigated. Safety data were collected up to 18 months after study entry.

Vaccines

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The investigational, egg-derived, monovalent, MF59-adjuvanted, pandemic vaccine, Focetria[®] (Novartis Vaccines and Diagnostics), contained haemagglutinin and neuraminidase surface antigens derived from the A/H1N1/California/7/2009 influenza strain. The vaccine seed A/H1N1 virus was

1 prepared from the reassortant virus, NYMC X-179A (New York Medical College, New York,
2 USA), generated from the A/California/7/2009 strain, as recommended by the World Health
3 Organization.¹¹ A full/standard dose of MF59, as used in the commercial seasonal influenza
4 vaccine, Flud[®] (Novartis Vaccines and Diagnostics), contains 9.75 mg squalene, 1.18 mg
5 polysorbate 80, 1.18 mg sorbitan trioleate, 0.66 mg sodium citrate dehydrate, and 0.04 mg citric
6 acid monohydrate. The booster dose of the seasonal, MF59-adjuvanted, trivalent influenza vaccine
7 (MF59-TIV), Flud, contained a standard dose of MF59, and 15 µg of each of the World Health
8 Organization reference strains recommended for the 2010/11 influenza season: A/California/7/2009
9 (H1N1); A/Perth/16/2009 (H3N2); and B/Brisbane/60/2008. Thus, the A/H1N1 antigen strain
10 contained in both priming and booster vaccines were identical. Vaccines were supplied in pre-filled
11 syringes. Single doses of 7.5-Full MF59 and 15-No MF59 vaccines were administered in a volume
12 of 0.5 mL. A single dose of the 3.75-Half MF59 formulation was administered in a volume of 0.25
13 mL. Single doses of seasonal vaccine were administered in volumes of 0.5 mL or 0.25 mL for
14 subjects aged ≥36 months and <36 months at the time of booster administration, respectively.
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32 *Immunogenicity assessments*

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37 Blood samples were obtained by venipuncture and centrifuged at 1500 g for 10 minutes; sera were
38 stored at a temperature of -18°C or below and shipped to the Novartis Vaccines Clinical Serology
39 Laboratory in Marburg, Germany, where antibody responses were assessed by haemagglutination
40 (HI) and microneutralization (MN) assays. The HI assay was based on the method of Stephenson
41 and colleagues;¹² HI titre was expressed as the reciprocal of the highest dilution at which
42 haemagglutination was totally inhibited. MN assays were performed according to a method
43 previously described by Nicholson and colleagues, serial dilutions of serum started at 1:20; the
44 reciprocals of two-fold dilutions that achieved ≥ 50% neutralization of viral growth were considered
45 a positive result.¹³ Titers were reported in terms of Geometric Mean Titers (GMT). Geometric Mean
46 Ration (GMR) were calculated as GMT Day 22/GMT Day 1, GMT Day 43/GMT Day 1, GMT Day
47 366/GMT Day 1 and GMT Day 387/GMT Day 366. In addition, HI GMT titers greater or equal
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than 1: 40 were assessed. Seroconversion, as assessed by HI assay, was defined as a negative pre-vaccination antibody titre of < 1:10 to a positive post-vaccination titre of \geq 1:40, or a minimum four-fold increase where pre-vaccination titres were \geq 1:10. HI titres below the detection limit of 1:10 were arbitrarily assigned to half that limit (1:5) for the purpose of analysis. The HI titer of 1:40, which has been determined as an immunologic correlate corresponding to a 50% reduction in the risk of contracting influenza, is based on studies in adults and may not be generalizable to children. Indeed, a recent study in a large pediatric population indicated that the conventional HI titer of 1:40 was only associated with 22% protection.¹⁴ Therefore, percentages of subjects with HI titers \geq 1:330 were also calculated, as this cut-off titer has been shown to predict an 80% clinical protective level in young children. Homologous HI and MN assays were performed using the vaccine antigen strain A/California/7/2009 (H1N1).

Safety assessment

Subjects were observed for 30 minutes after receipt of each vaccine dose to monitor for immediate adverse reactions. The frequency and severity of solicited local and systemic reactions occurring within one week of each vaccination were recorded on diary cards and summarized according to the Brighton collaboration case definition.¹⁵ Solicited local reactions were ecchymosis, erythema, induration, swelling, (graded as mild, moderate, severe based on measurement in mm) and tenderness. Solicited systemic reactions were sleepiness, diarrhoea, vomiting, irritability, altered eating habits, shivering, unusual crying, and fever (\geq 38°C). A high, grade 3 fever was defined as an axillary body temperature of \geq 40°C. Solicited systemic reactions were collected as present/not present. In addition, use of analgesic/antipyretic was collected as measure of reactogenicity. All unsolicited adverse events (AE) were recorded for three weeks after each vaccination. The onset of new chronic diseases, serious adverse events (SAEs), and AEs leading to withdrawal were recorded throughout the entire study period (Day 1–546). A physician rated AEs as mild, moderate, or severe if resulting in no limitation of, some limitation of, or inability to perform normal daily activities, respectively.

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3 *Statistical analyses*
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5 Sample sizes were chosen to meet or exceed the minimum requirements of the European guidelines
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7 for influenza vaccine clinical trials. No formal statistical hypothesis was tested, immunogenicity
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9 endpoints being based on HI licensure criteria established by the EU Committee for Medicinal
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11 Products for Human Use (CHMP).¹⁶ There are currently no pre-defined CHMP criteria for children,
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13 therefore, the following adult licensure criteria applied: the number of subjects achieving
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15 seroconversion or significant increase (defined as $HI \geq 1:40$ for subjects with a pre-vaccination HI
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17 titer $< 1:10$; a minimum 4-fold increase HI titer for subjects with a pre-vaccination HI titer $\geq 1:10$)
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19 should be $> 40\%$; geometric mean ratios (GMRs) should be > 2.5 ; and the proportion of subjects
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21 achieving an HI titre $\geq 1:40$ should be $> 70\%$. Percentages of subjects with HI titers $\geq 1:330$ were
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23 also calculated.¹⁴ Immunogenicity data reflecting the above endpoints as well as the corresponding
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25 two-sided 95% confidence intervals (CIs) were calculated for each vaccine group and age cohort
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27 using two-way analysis of variance (ANOVA) with factors for vaccine group and center. Safety
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29 data were evaluated descriptively and expressed as the proportion and number of subjects with AEs
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31 in each group. Immunogenicity analyses were run on the per-protocol (PP) set, which consisted of
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33 subjects who received all the relevant doses of vaccine correctly, provided evaluable serum sample
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35 at the relevant time points, and had no major protocol violations. Safety was analyzed for all
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37 subjects exposed to at least one study vaccination and provided safety data. All statistical analyses
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39 were performed by employees of Novartis Vaccines and Diagnostics using SAS 9.1[®] software.
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Results

1 After the removal of one study site, 330 subjects were included in the two age cohorts (6-11 months
2 and 12-35 months) covered in this paper. In this study, 64-78% across groups completed the study
3 protocol (**Figure 1**). An average of 93% and 72% of subjects across study groups completed the
4 primary immunization schedule (Day 43) and received the booster dose (Day 366), respectively. A
5 second dose of seasonal vaccine at Day 387 was administered to about 47% of subjects across
6 vaccine groups in both age cohorts.
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8 Demographic and other baseline population characteristics are shown in **Table 1**. The majority of
9 children were of Hispanic ethnic origin. Mean age of the subjects was 8.5 months and 24.2 months
10 in cohorts 6-11 months and 12-35 months, respectively
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Immunogenicity

15 Immunogenicity analyses were performed on the PP data set, which included 68-76% of subjects
16 across groups after the primary vaccination schedule (Day 43), 49-53% of subjects for persistence
17 analyses (Day 366), and 32-55% of subjects for post-booster analyses (Day 387).
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24 HI assay GMTs were low at baseline, with only 18–26% of subjects displaying HI titres of $\geq 1:40$
25 (**Table 2**). In the 6–11 months cohort, 7.5-Full MF59 vaccine induced greater GMTs than the 3.75-
26 Half MF59 formulation three weeks after both first and second doses (**Figure 2A**). One dose of
27 either MF59-adjuvanted vaccine was sufficient to meet the CHMP criterion for GMR (**Table 2**). In
28 the 12–35 months cohort, the 7.5-Full MF59 vaccine induced greater GMTs than the 3.75-Half
29 MF59 formulation after first and second doses (**Figure 2B**). GMTs were greater in response to both
30 MF59-adjuvanted vaccines compared with the 15 μg non-adjuvanted formulation (**Figure 2B**). All
31 three vaccine formulations met the licensure criterion for GMRs as early as 3 weeks after the first
32 vaccination (**Table 2**). Subjects in the 6–11 months cohort demonstrated high HI seroconversion
33 rates for both formulations after the first and second vaccinations (91–98%); the same was true in
34 subjects in the 12–35 months cohort who received adjuvanted formulations (97–100%; **Table 2**).
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1 Seroconversion rates were lower in subjects who received the 15-No MF59 formulation (52% on
2 Day 22 and 74% on Day 43) than those who received adjuvanted formulations. For both age
3 cohorts, seroconversion rates for all vaccines were above the CHMP licensure criterion of > 40%
4 after both the first and second vaccinations. Rates of subjects with HI titers \geq 1:40 were high after
5 first and second doses in response to both adjuvanted formulations in both age cohorts, ranging
6 from 91% to 100% (**Figure 3A and B**), exceeding the CHMP criterion of > 70%. Rates of subjects
7 with HI titers \geq 1:40 in the 15-No MF59 vaccination group of the 12–35 months cohort failed to
8 meet licensure criterion after the first dose (52%); a second dose was required for this criterion to be
9 achieved (81%) (**Figure 3B**). Analyses by MN assay supported HI data (**Figure 2C and D, Table**
10 **3**).

11 Long-term antibody persistence was demonstrated in subjects receiving MF59-adjuvanted vaccines;
12 95–100% of subjects across age groups achieved HI titers \geq 1:40 one year after immunization,
13 which is considerably higher than in those receiving the non-adjuvanted formulation (62%) (**Figure**
14 **3A and B**). Antibody titres were significantly higher after booster vaccination compared with
15 GMTs after priming doses (**Figure 2**). The licensure criteria for GMR and seroconversion were met
16 by all priming groups following booster vaccination in both age cohorts (**Table 2**) and all subjects
17 achieved HI titers \geq 1:40 in all vaccine groups in both age cohorts, regardless of priming
18 formulations.. Analyses by MN assay supported HI data (**Figure 2C and D, Table 3**).

19 Percentages of subjects with HI titers \geq 1:330 are reported in **Table 4**. In the 6–11 months cohort,
20 35% (3.75-Half MF59), 39% (7.5-Full MF59) and 82% (3.75-Half MF59), 100% (7.5-Full MF59)
21 of subjects achieved HI titers \geq 1:330 after the first and second primary doses, respectively, and all
22 subjects in both vaccine groups had HI titers \geq 1:330 after the booster vaccination.

23 In children 12-35 months of age receiving adjuvanted formulations, percentages of subjects
24 achieving HI titers \geq 1:330 were higher compared with subjects receiving the nonadjuvanted
25 formulation. HI titers \geq 1:330 were achieved in 37% (3.75-Half MF59), 47% (7.5 Full MF59), 23%
26 (15-No MF59), and 85% (3.75-Half MF59), 91% (7.5-Full MF59), 39% (15-No MF59) after the
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1 first and second primary doses, respectively and in 100% (both adjuvanted formulations) and 91%
2 (15-No MF59) of subjects after the booster dose.

3 *Safety and tolerability*

4 All subjects were exposed to at least one study vaccination and contributed to the safety analyses.

5 In the 6–11 months cohort, the percentage of subjects with solicited local reactions were similar

6 between first and second primary doses, and between the two adjuvanted formulations (**Table 5**).

7 The most frequently reported local reactions after primary immunizations were erythema (15–25%

8 of subjects) and tenderness (14–25%). There were no cases of severe local reactions after primary

9 vaccinations. Local reactions were generally more frequent after the booster dose than the primary

10 doses. Tenderness was the most frequently reported local reaction after the booster (34–39%) and

11 second (23–30%) seasonal dose, followed by erythema (28–31% and 22–30%, respectively). Severe

12 reactions were reported in up to 8% and 6% across groups after booster dose and second seasonal

13 dose, respectively, with tenderness as the most frequently reported severe local reaction. The

14 incidence of solicited systemic reactions was generally lower after the second primary vaccination

15 compared with the first primary vaccination in the 6–11 months cohort, no substantial differences

16 were observed between the vaccine groups. The incidence of systemic reactions after the booster

17 was similar to that after the second primary vaccination. The incidence of local and systemic

18 reactions tended to be lower after the second seasonal vaccination compared to the booster (**Table**

19 **5**).

20 In the 12–35 months cohort (**Table 6**), solicited local reactions were less frequent in the non-

21 adjuvanted formulation compared with the adjuvanted formulations and occurred at a similar rate

22 after the first and second primary doses.

23 Tenderness was the most frequently reported local reaction after the primary immunizations (22–

24 35%), increasing in frequency after the booster (34–48%), but decreasing in frequency after the

25 second seasonal dose (15–29%). Other local reactions occurred at a similar rate after the primary

26 and booster vaccinations, but tended to occur at a lower rate after the second seasonal vaccination.

27 After primary immunizations, severe tenderness was reported in 1–3% of subjects across adjuvanted

groups, and severe induration and erythema occurred in 1% of subjects receiving 7.5-full MF59.

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3 Following booster and second seasonal dose, severe tenderness was reported in up to 5% of subjects
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5 across adjuvanted formulations. Systemic reactions tended to be similar between the vaccine
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7 formulations after the primary immunizations in the 12–35 months cohort.

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9 There were three reports of fever $\geq 40^{\circ}\text{C}$: one in the 6-11 months cohort (3.75-Half MF59) and two
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11 in the 12-35 months cohort (n=1: 3.75-Half MF59, n=1: 7.5-Full MF59), all following the first
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13 vaccination. Cases occurred between 3-5 days postvaccination and all resolved within 1-2 days
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15 without sequelae.

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20 For spontaneously reported AEs, no differences in overall frequency of AEs were observed between
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22 the two adjuvanted formulations and between adjuvanted and nonadjuvanted vaccine groups.

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25 Between study Days 1 and 43, AEs were spontaneously reported for 51–60% of subjects across age
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27 and vaccine groups; 5–16% of these cases were considered at least possibly related to vaccination.

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30 In the 6-11 months cohort, the most commonly reported AEs were nasopharyngitis (23–31%),
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32 followed by diarrhea (12-13%); in the 12-35 months cohort, the most commonly reported AEs were
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34 nasopharyngitis (25-30%) and bronchitis (7-16%).

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37 Selected unsolicited AEs from Day 1-Day 546 are presented in **Table 7**. Between Days 1-366, there
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39 were 25 subjects with SAEs and 8 subjects with new onset chronic diseases. None of these SAEs
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41 and new onset of chronic diseases was considered as related to study vaccination. There were 3 AEs
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43 leading to premature study withdrawal, one AE was considered at least possibly related to study
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45 vaccination (6-11 months cohort, 3.75-Half MF59 group; subject experienced mild allergic
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47 dermatitis on day 2, the AE resolved in 6 days).

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51 After the booster vaccination, the most frequently reported unsolicited AEs were nasopharyngitis
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53 (12–13%), and diarrhoea (2–6%) in the 6–11 months cohort, and nasopharyngitis (6–9%) and
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55 conjunctivitis (0–5%) in the 12-15 months cohort. Nasopharyngitis was also the most frequently
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57 reported AE after the second dose of the seasonal MF59 vaccine in both the 6–11 months (15%)
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59 and 12–35 months (12%) cohorts.
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One 6–11 month-old subject in the 3.75-Half MF59 group experienced one SAE, and one 12–35 month-old subject in the 3.75-Half MF59 group experienced two SAEs between booster administration and the end of the study (Day 366–546). No SAEs were related to either the booster or the second seasonal vaccination. There was one new onset of chronic disease in 6-11 month (asthmatic crisis) and 12-35 month (allergic rhinitis) age cohorts, and one AE leading to premature study withdrawal in a 6-11 month-old subject (food allergy), none of which related to study vaccination between booster administration and the end of the study.

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Discussion

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3 All the investigational vaccines used in this study were shown to be highly immunogenic and well
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5 tolerated in children from 6 to 35 months of age. Both MF59-adjuvanted formulations induced
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7 higher antibody responses compared with the non-adjuvanted vaccine. A single dose of either the
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9 3.75-Half MF59 or 7.5-Full MF59 vaccines was sufficient to meet all three European licensure
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11 criteria for influenza vaccines; two doses of the non-adjuvanted vaccine were required to achieve
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13 similar results. The ability of MF59 to allow for reduced antigen content would potentially ensure a
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15 higher coverage during a pandemic outbreak, which is an important public health issue to consider
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17 in periods of heightened demand.
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22 The findings of this study are supported by similar trials. A cell culture-derived, MF59-adjuvanted
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24 A/H1N1 vaccine has been demonstrated to provide adequate levels of antibodies after a single dose
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26 in British adults ⁶ and Japanese adults and children.^{17, 18} Adults were found to require one 3.75-Half
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28 MF59 vaccine dose, while a single 7.5-Full MF59 dose was optimal for children in the Japanese
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30 study.¹⁸ A trial of A/H1N1 vaccine was conducted in Costa Rican children to compare an MF59-
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32 adjuvanted formulation containing 7.5 µg of A/California/7/2009 antigen with 15 µg and 30 µg
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34 non-adjuvanted vaccines.⁴ All three vaccines met the US Center for Biologics Evaluation and
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36 Research (CBER) and CHMP licensure criteria after one dose in subjects aged 9–17 years; only the
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38 MF59-adjuvanted formulation met these criteria after one dose in younger subjects of 3 to 8 years.
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40 Paediatric and adult clinical trials using an AS03[®]-adjuvanted (GlaxoSmithKline Biologicals,
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42 Wavre, Belgium) A/H1N1 vaccine have also demonstrated that a single dose containing 3.75 µg
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44 antigen is sufficient in combination with an oil-in-water adjuvant.^{19, 20}
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52 It has recently been demonstrated that the conventional titer of 1:40 correlate may not be
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54 appropriate in a pediatric population.¹⁴ A titer of 1:40 was only associated with 22% protection,
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56 whereas titers of 1:110 or 1:330 could predict a 50% or an 80% of clinical protective level.¹⁴ Our
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58 results demonstrate that in children receiving MF59 adjuvanted formulations, a high proportion
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60 reached the stringent HI cut-off titer \geq 1:330 postvaccination, i.e. 82-100% and 100% after primary
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1 and booster doses respectively, as compared with 39% in children receiving the non-adjuvanted
2 vaccine. The seasonal MF59 vaccine was able to boost the immune responses also in the group
3 primed with the nonadjuvanted formulation: indeed, 91% of subjects primed with the non-
4 adjuvanted vaccine reached the HI cut-off titer $\geq 1:330$. These findings give strong support for the
5 use of MF59 adjuvanted vaccines in the pediatric population.
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10 In the present study, significant antibody persistence was observed in response to the MF59-
11 adjuvanted vaccine formulations, with HI titers $\geq 1:40$ detected in 95–100% of subjects one year
12 after immunization. The ability of MF59 to promote long-term antibody persistence has also been
13 demonstrated in previous studies.^{21, 22} All subjects in the current study received two priming doses;
14 as such, further investigations are required to confirm that long-term antibody persistence and
15 response to booster vaccination is not negatively affected by a one-dose priming schedule. The
16 production of cross-reactive antibodies able to provide heterologous immunity was not analysed
17 during this study. However, the ability of MF59 to promote the production of cross-reactive
18 antibodies is well documented.^{21, 23-25}
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32 The overall safety and tolerability were as expected in this age group and were in line with previous
33 studies using MF59-adjuvanted vaccines in young children.^{18, 26-28} Solicited local reactions were
34 more common in adjuvanted formulations compared with nonadjuvanted vaccine, but there were no
35 MF59-related increase in the frequency of solicited systemic reactions or unsolicited adverse
36 events. This study demonstrated an acceptable safety profile for the adjuvanted vaccine over an 18-
37 month period; although subjects were repeatedly vaccinated with up to four doses of MF59-
38 adjuvanted vaccine, no vaccine-related SAEs were reported during the study period.
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49 In conclusion, a single dose of 3.75 μ g antigen together with half the standard dose of MF59 was
50 identified as optimal, provided adequate levels of immediate and long-term antibodies and an
51 acceptable safety profile in children 6-35 months of age. These data support the suitability of
52 Focetria for pandemic immunization in the paediatric population.
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Author contributions

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12 All authors participated in the conception, design and implementation of the trials. All authors were
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14 involved in the interpretation of analyzed data and the decision to submit for publication.
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Figure legends

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2 **Figure 1:** Design of clinical trial and subject disposition.
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7 **Figure 2:** GMTs by HI (A and B) and MN (C and D) assay at baseline (Day 1), three weeks after
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9 first (Day 22) and second (Day 43) priming doses, and pre- and post-booster vaccination in children
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11 aged 6–11 months- (A and C) and 12–35 months old (B and D).
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16 **Figure 3:** Percentages (95% CI) of subjects aged 6–11 months (A) and 12–35 months (B) achieving
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18 HI titer $\geq 1:40$ after primary and booster immunizations. Broken line represents the CHMP
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21 licensure criteria.
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Table 1: Study population demographics

	6–11 Months		12–35 Months		
	3.75-Half MF59 (n = 72)	7.5-Full MF59 (n = 72)	3.75-Half MF59 (n = 75)	7.5-Full MF59 (n = 74)	15-No MF59 (n = 37)
Age (months, SD)	8.8 ± 1.3	8.3 ± 1.4	24.0 ± 7.4	23.9 ± 6.4	25.1 ± 6.4
Male (%)	50	49	53	57	46
Asian (%)	3	0	5	5	5
Black (%)	3	0	1	3	3
Caucasian (%)	14	18	28	30	32
Hispanic (%)	76	79	56	54	54
Other (%)	4	3	9	8	5
Weight (kg, SD)	9 ± 1.2	8.9 ± 1.2	12.3 ± 2.3	12.6 ± 1.9	12.9 ± 2.1
Height (cm, SD)	71.7 ± 3.7	70.6 ± 3.6	86.0 ± 7.2	87.4 ± 5.8	88.5 ± 6.7
Body Mass Index (kg/m ² , SD)	17.5 ± 1.9	17.8 ± 2.0	16.7 ± 2.1	16.5 ± 1.6	16.6 ± 2.0
Previous influenza vaccination (%)	0	1	44	41	51

SD: standard deviation

Table 2: HI assay: immunogenicity analysis (95% CI) against A/H1N1/California/7/2009, at baseline (Day 1), three weeks after first (Day 22) and second (Day 43) primary doses, one year after vaccination (Day 366) and three weeks after booster vaccination (Day 387). **Bold:** CHMP criterion met

	6–11 Months		12–35 Months		
	3.75-Half MF59 (n prime = 55) (n MF59-TIV boost = 26)	7.5-Full MF59 (n prime = 49) (n MF59-TIV boost = 30)	3.75-Half MF59 (n prime = 60) (n MF59-TIV boost = 44)	7.5-Full MF59 (n prime = 58) (n MF59-TIV boost = 42)	15-No MF59 (n prime = 31) (n MF59-TIV boost = 23)
	Baseline values				
GMTs	12 (6.9–22)	15 (8.0–27)	7.7 (4.0–15)	10 (5.2–20)	12 (6.2–24)
% subjects HI titer ≥ 40	22 (12–35)	22 (12–37)	18 (10–30)	24 (14–37)	26 (12–45)
	GMRs				
Day 22 : 1 (1 st dose)	15 (9.8–22)	21 (14–33)	28 (17–44)	31 (19–51)	4.6 (2.8–7.5)
Day 43 : 1 (2 nd dose)	79 (44–142)	121 (66–225)	91 (51–162)	80 (44–146)	12 (6.4–21)
Day 366 : 1 (pre-booster)	23 (14–39) (n=38)	32 (19–55) (n=35)	39 (20–75) (n=46)	25 (13–48) (n=51)	3.4 (1.7–6.7) (n=26)
Day 387 : 366 (post-booster)	14 (8.4–24)	8.1 (5.0–13)	12 (5.8–24)	9.1 (4.5–19)	31 (15–64)
	% of subjects achieving seroconversion				
Day 22 (1 st dose)	91 (80–97)	96 (86–100)	97 (88–100)	100 (94–100)	52 (33–70)
Day 43 (2 nd dose)	96 (87–100)	98 (89–100)	100 (94–100)	100 (94–100)	74 (55–88)
Day 387 (post-booster) (from Day 366)	81 (61–93)	77 (58–90)	80 (65–90)	86 (71–95)	78 (56–93)

HI: haemagglutination inhibition; GMT: geometric mean titer; GMR: geometric mean ratio

Table 3: MN assay: immunogenicity analysis (95% CI) against A/H1N1/California/7/2009, at baseline (Day 1), three weeks after first (Day 22) and second (Day 43) primary doses, one year after vaccination (day 366) and three weeks after booster vaccination (Day 387)

	6–11 Months		12–35 Months		
	3.75-Half MF59 (n prime = 55) (n MF59-TIV boost = 26)	7.5-Full MF59 (n prime = 49) (n MF59-TIV boost = 30)	3.75-Half MF59 (n prime = 60) (n MF59-TIV boost = 44)	7.5-Full MF59 (n prime = 58) (n MF59-TIV boost = 42)	15-No MF59 (n prime = 31) (n MF59-TIV boost = 22)
Baseline values					
GMTs	12 (6.2–24)	14 (6.8–29)	7.7 (3.5–17)	10 (4.5–22)	11 (5.1–26)
% of subjects MN titer \geq 1:40	22 (12–35)	22 (12–37)	18 (10–30)	24 (14–37)	23 (10–41)
GMRs					
Day 22 : 1 (1 st dose)	28 (17–45)	36 (22–59)	36 (22–59)	36 (22–60)	4.7 (2.8–7.9)
Day 43 : 1 (2 nd dose)	144 (81–256)	208 (113–383)	226 (120–427)	202 (104–391)	34 (18–66)
Day 366 : 1 (pre-booster)	29 (15–54) (n=38)	32 (17–62) (n=35)	34 (17–66) (n=46)	21 (11–40) (n=51)	5.1 (2.5–10) (n=25)
Day 387 : 366 (post-booster)	12 (8.3–18)	9.2 (6.5–13)	18 (10–31)	14 (8.3–25)	21 (11–38)
% of subjects MN titer \geq 1:40					
Day 22 (1 st dose)	95 (85–99)	100 (93–100)	100 (94–100)	100 (94–100)	52 (33–70)
Day 43 (2 nd dose)	100 (94–100)	100 (93–100)	100 (94–100)	100 (94–100)	94 (79–99)
Day 387 (post-booster)	100 (87–100)	100 (88–100)	100 (92–100)	100 (92–100)	100 (85–100)

MN: microneutralization; GMT: geometric mean titer; GMR: geometric mean ratio

Table 4: Percentages of Subjects (95% CI) With HI Titer $\geq 1:330$ against the vaccine strain A/H1N1/California/7/2009 at baseline (Day 1), three weeks after first (Day 22) and second (Day 43) primary doses, one year after vaccination (Day 366), and three weeks after booster vaccination (Day 387)

	6–11 Months		12–35 Months		
	3.75-Half MF59 (n prime = 55) (n MF59-TIV boost = 26)	7.5-Full MF59 (n prime = 49) (n MF59-TIV boost = 30)	3.75-Half MF59 (n prime = 60) (n MF59-TIV boost = 44)	7.5-Full MF59 (n prime = 58) (n MF59-TIV boost = 42)	15-No MF59 (n prime = 31) (n MF59-TIV boost = 23)
	% of subjects with HI titers $\geq 1:330$				
Day 1 Baseline	4 (0-13)	8 (2-20)	2 (0.04–9)	0 (0-6)	6 (1-21)
Day 22 (1 st dose)	35 (22-49)	39 (25-54)	37 (25-50)	47 (33-60)	23 (10-41)
Day 43 (2 nd dose)	82 (69–91)	100 (93–100)	85 (73-93)	91 (81-97)	39 (22-58)
Day 366 (pre-booster)	42 (26–59) (n=38)	71 (54–85) (n=35)	50 (35–65) (n=46)	57 (42–71) (n=51)	27 (12–48) (n=26)
Day 387 (post-booster)	100 (87-100)	100 (88-100)	100 (92-100)	100 (92-100)	91 (72-99)

HI: haemagglutination inhibition

Table 5: 6–11 months cohort: percentages of subjects experiencing solicited local* and systemic reactions within one week of each vaccination. Percentages of subjects with reactions classified as severe are shown in brackets

	First Dose		Second Dose		MF59-TIV (Booster)		MF59-TIV (2 nd Seasonal Dose)	
	3.75-Half MF59 (n = 72)	7.5-Full MF59 (n = 73)	3.75-Half MF59 (n = 69)	7.5-Full MF59 (n = 68)	3.75-Half MF59 (n = 50)	7.5-Full MF59 (n = 49)	3.75-Half MF59 (n = 46)	7.5-Full MF59 (n = 47)
Ecchymosis*	4 (0)	3 (0)	4 (0)	3 (0)	8 (0)	8 (0)	0 (0)	2 (2)
Erythema*	21 (0)	25 (0)	19 (0)	15 (0)	28 (0)	31 (2)	22 (0)	30 (4)
Induration*	4 (0)	3 (0)	7 (0)	6 (0)	18 (0)	24 (2)	13 (0)	19 (2)
Swelling*	3 (0)	1 (0)	3 (0)	3 (0)	12 (0)	16 (2)	11 (0)	11 (2)
^a Tenderness*	15 (0)	25 (0)	14 (0)	16 (0)	34 (8)	39 (4)	30 (2)	23 (6)
Sleepiness	42	47	25	34	16	27	13	4
Diarrhoea	29	32	20	18	18	16	7	6
Vomiting	14	15	14	7	6	12	4	4
Irritability	31	27	17	31	28	35	13	11
Altered eating habits	25	27	14	18	20	27	4	6
Shivering	8	7	4	1	8	8	2	4
Crying	36	33	19	26	32	27	15	17
^b Fever ≥38°C	13 (1)	14 (0)	7 (0)	13 (0)	24 (0)	22 (0)	9 (0)	17 (0)
Use of analgesic/antipyretic	31	32	19	29	30	41	20	19

Solicited systemic reactions were collected as present/not present

^aTenderness was classified as severe if subjects cried on movement of limb. ^bFever was classified as severe if ≥ 40°C. Body temperatures were converted into axillary measured temperatures by subtracting 1.0°C from rectally, 0.5°C from orally and 0.2°C from tympanically measured body temperature

Table 6: 12–35 months cohort: percentages of subjects experiencing solicited local* and systemic reactions within one week of each vaccination. Percentages of subjects with reactions classified as severe are shown in brackets

	First Dose			Second Dose			MF59-TIV (Booster)			MF59-TIV (2 nd Seasonal Dose)		
	3.75- Half MF59	7.5- Full MF59	15- No MF59	3.75- Half MF59	7.5- Full MF59	15- No MF59	3.75- Half MF59	7.5- Full MF59	15- No MF59	3.75- Half MF59	7.5- Full MF59	15- No MF59
	(n = 75)	(n = 73)	(n = 37)	(n = 73)	(n = 72)	(n = 36)	(n = 52)	(n = 57)	(n = 29)	(n = 24)	(n = 27)	(n = 13)
Ecchymosis*	11 (0)	10 (0)	5 (0)	7 (0)	13 (0)	11 (0)	10 (0)	9 (0)	3 (0)	8 (0)	7 (0)	0 (0)
Erythema*	28 (0)	25 (0)	19 (0)	18 (0)	25 (1)	22 (0)	38 (0)	28 (0)	10 (0)	25 (0)	22 (0)	8 (0)
Induration*	5 (0)	11 (0)	5 (0)	11 (0)	10 (1)	3 (0)	31 (0)	18 (0)	7 (0)	25 (0)	7 (0)	0 (0)
Swelling*	4 (0)	3 (0)	3 (0)	5 (0)	3 (0)	3 (0)	23 (0)	12 (0)	7 (0)	17 (0)	4 (0)	0 (0)
^a Tenderness*	35 (3)	34 (3)	22 (0)	25 (1)	33 (1)	28 (0)	48 (2)	40 (5)	34 (0)	29 (4)	15 (4)	23 (0)
Sleepiness	39	29	30	23	18	33	13	9	14	8	4	8
Diarrhoea	24	21	24	14	17	14	8	9	7	17	7	8
Vomiting	9	5	11	10	10	11	4	11	3	4	0	0
Irritability	28	29	27	26	22	31	25	21	17	13	7	8
Altered eating habits	24	19	16	22	15	22	10	5	14	17	7	8
Shivering	11	10	11	10	3	8	4	7	7	0	0	8
Crying	31	29	24	18	19	22	21	18	17	13	15	8
^b Fever ≥ 38°C	9 (1)	15 (1)	8 (0)	11 (0)	14 (0)	0 (0)	12 (0)	19 (0)	7 (0)	8 (0)	0 (0)	0 (0)
Use of analgesic/antipyretic	15	19	22	21	21	17	25	25	14	4	4	0

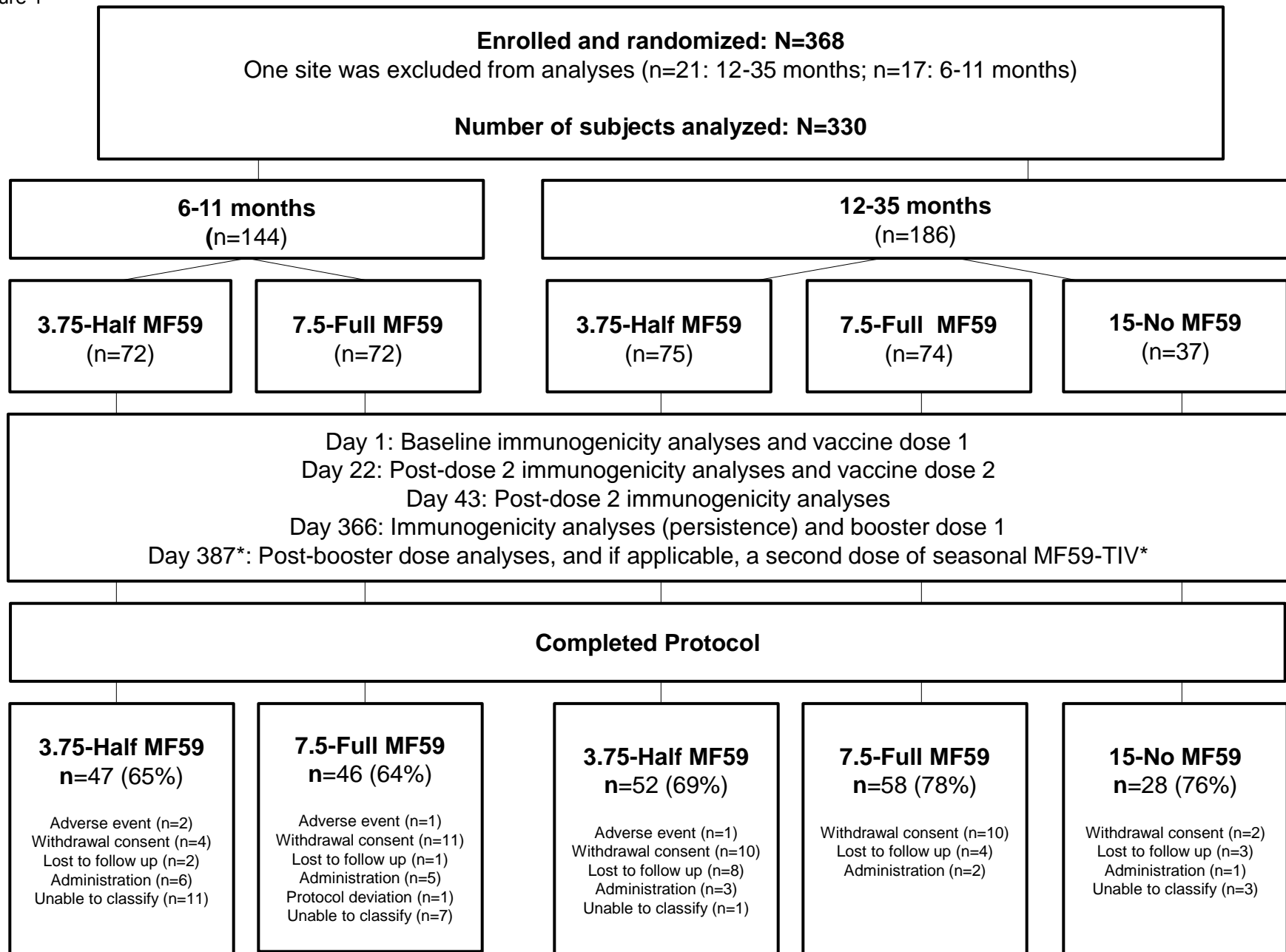
Solicited systemic reactions were collected as present/not present

^aTenderness was classified as severe if subjects cried on movement of limb. ^bFever was classified as severe if ≥ 40°C. Body temperatures were converted into axillary measured temperatures by subtracting 1.0°C from rectally, 0.5°C from orally and 0.2°C from tympanically measured body temperature

Table 7. Number and percentages of subjects with selected unsolicited adverse events, Days 1-546

	6–11 Months		12–35 Months		
	3.75-Half MF59 (n = 72)	7.5-Full MF59 (n = 72)	3.75-Half MF59 (n = 75)	7.5-Full MF59 (n = 74)	15-No MF59 (n = 37)
Day 1-366					
Serious AEs	8 (11%)	6 (8%)	6 (8%)	3 (4%)	2 (5%)
At least possibly related SAEs	0	0	0	0	0
AEs leading to discontinuation	2 (3%)	0	1 (1%)	0	0
At least possibly related AEs leading to discontinuation	1 (1%)	0	0	0	0
New onset of chronic diseases	5 (7%)	1 (1%)	0	1 (1%)	1 (3%)
At least possibly related new onset of chronic diseases	0	0	0	0	0
Day 366-546					
	3.75-Half MF59 (n = 68)	7.5-Full MF59 (n = 62)	3.75-Half MF59 (n = 63)	7.5-Full MF59 (n = 68)	15-No MF59 (n = 35)
Serious AEs	1 (1%)	0	1 (2%)	0	0
At least possibly related SAEs	0	0	0	0	0
AEs leading to discontinuation	0	1 (2%)	0	0	0
At least possibly related AEs leading to discontinuation	0	0	0	0	0
New onset of chronic diseases	1 (1%)	0	1 (2%)	0	0
At least possibly related new onset of chronic diseases	0	0	0	0	0

Figure 1



One German site was excluded from analyses due to noncompliance with the protocol requirements for safety reporting for a different study
 *subjects who had never previously received seasonal vaccine were given a second dose of seasonal MF59-TIV at Day 387 to complete the seasonal immunization course according to WHO recommendations; the immunogenicity of this second seasonal immunization was not investigated.

Figure 2

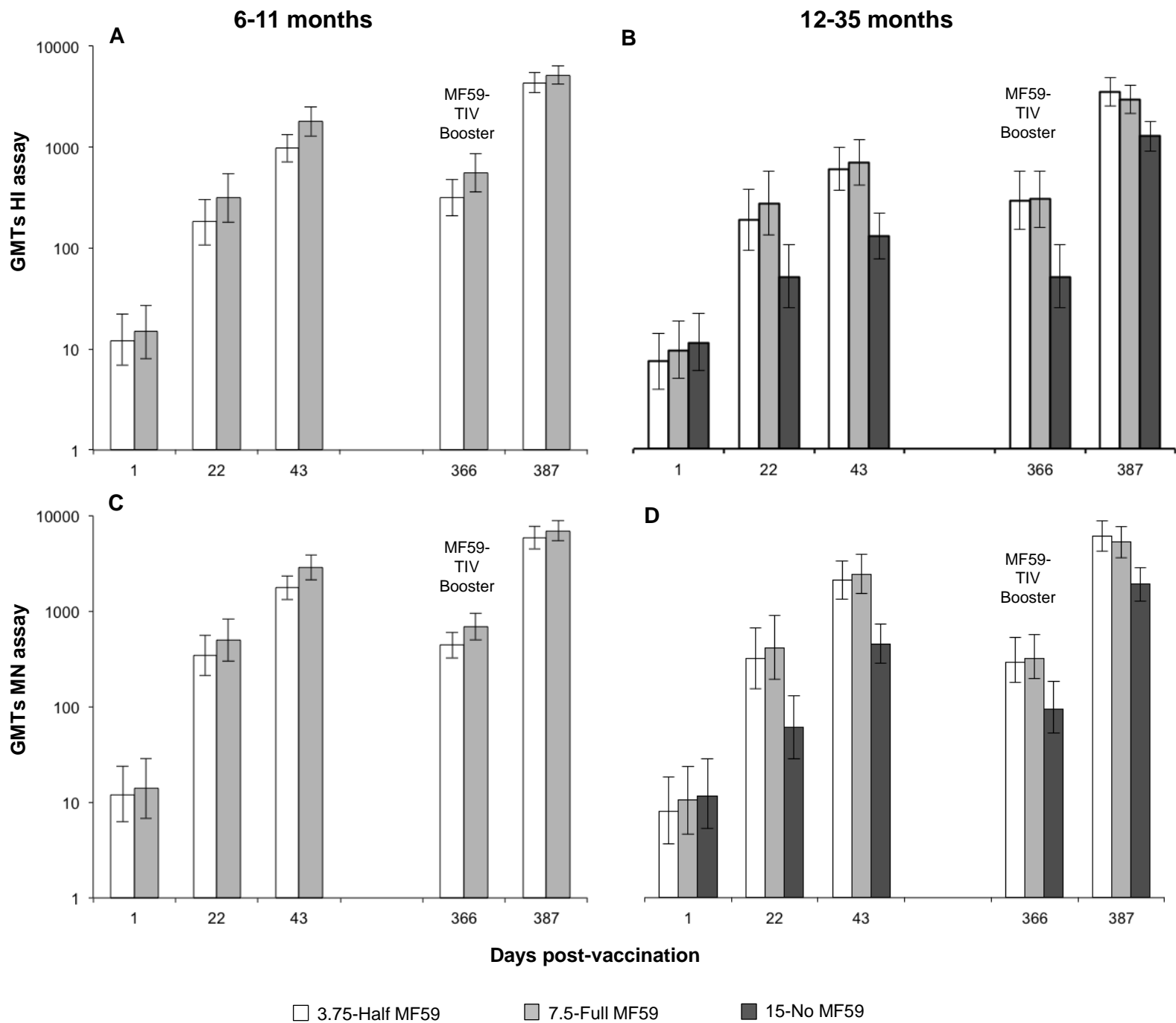


Figure 3

