Safety, reactogenicity and immunogenicity of an intranasal seasonal influenza vaccine adjuvanted with gram-positive matrix (GEM) particles (FluGEM) a randomized, double-blind, controlled, ascending dose study in healthy adults and elderly

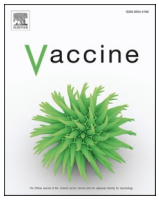
Plas, J.L. van der; Haijema, B.J.; Leenhouts, K.; Zoetweij, J.P.; Burggraaf, J.; Kamerling, I.M.C.

Vaccine 42 (2024) 125836

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Safety, reactogenicity and immunogenicity of an intranasal seasonal influenza vaccine adjuvanted with gram-positive matrix (GEM) particles (FluGEM): A randomized, double-blind, controlled, ascending dose study in healthy adults and elderly

Johan L. van der Plas [a,b,*], Bert-Jan Haijema [c,d], Kees Leenhouts [c,e], J. Paul Zoeteweij [f], Jacobus Burggraaf [a,g], Ingrid M.C. Kamerling [a,b]

[a] Centre for Human Drug Research, Leiden, the Netherlands
[b] Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands
[c] Mucosis B.V., Groningen, the Netherlands
[d] 3D-PharmXchange, Tilburg, the Netherlands
[e] Allero Therapeutics B.V., Rotterdam, the Netherlands
[f] Viroclinics Biosciences B.V., Rotterdam, the Netherlands
[g] Leiden Academic Centre for Drug Research, Leiden, the Netherlands

ARTICLE INFO

Keywords:
Mucosa
Vaccination
Influenza
Bacteria-like
Adjuvant
Intranasal

ABSTRACT

Background: Intranasal administration of respiratory vaccines offers many advantages such as eliciting both systemic and mucosal immunity at the point of viral entry. Immunogenicity of intranasal vaccination can be improved through the use of adjuvants. Bacteria-like particles derived from Lactococcus lactis have the potential to serve as a vaccine adjuvant. This clinical study investigated the safety, reactogenicity and immunogenicity of intranasal seasonal influenza vaccine adjuvanted with gram-positive matrix particles (FluGEM®).

Methods: This was a first-in-human, randomized, double-blind, controlled, dose-escalation study performed at the Centre for Human Drug Research (CHDR), the Netherlands. Participants aged 18–49 were randomized in a 3:1 ratio to receive FluGem® in ascending doses (two-dose regimens) together with a standard trivalent inactivated influenza vaccine or unadjuvanted TIV only. Primary outcomes were safety and tolerability. Secondary outcomes were serum hemagglutination inhibition (HI) antibody titers and mucosal IgA. The most immunogenic dose was used in an additional elderly cohort (>65 years).

Results: Ninty participants were included. Intranasal FluGem® was safe and well tolerated. The majority of adverse events were mild (97.4 %) with (un)solicited adverse events comparable across all dose levels and control groups. All groups showed geometric mean increases ≥ 2.5-fold. Seroconversion (≥40 % participants) was achieved at both day 21 (single-dose) and 42 (two-dose) for the 1.25 mg dose and on day 42 (two-dose only) for the 2.5 mg dose. Highest geometric mean IgA increases were observed in the 1.25 mg group on day 21. Immunogenicity was less pronounced in elderly.

Conclusions: Intranasal vaccination of FluGEM® was safe and tolerable in healthy adult volunteers aged 18–49 years and 65 and older. Highest immunogenicity was observed for 1.25 mg and 2.5 mg doses (compared to 5 mg) suggesting a potential non-linear dose–response relationship. More research is needed to further investigate the capabilities of bacteria-like peptides as adjuvants.

1. Introduction

It has been estimated that seasonal influenza causes roughly between 290 000 and 650 000 deaths annually worldwide. [1] Risk of serious influenza-related complications and mortality are highest in children younger than 5 years of age and adults above 65 years. [2,3] While there have been vaccines available against influenza for many decades there are still challenges to overcome. Eliciting effective and lasting vaccine-

* Corresponding author at: Centre for Human Drug Research, Zernikedreef 8, 2333 CL, Leiden, The Netherlands.
E-mail address: JvdPlas@chdr.nl (J.L. van der Plas).

https://doi.org/10.1016/j.vaccine.2024.03.063
Received 19 November 2023; Received in revised form 19 March 2024; Accepted 24 March 2024
Available online 20 May 2024

induced immune responses in high-risk groups may be complicated; in young children caused by immature immune systems and in elderly people by immunosenescence. [4–6].

Immunogenicity of currently available vaccines can potentially be improved through the use of adjuvants. Most currently licensed seasonal vaccines consist of trivalent or quadrivalent inactivated influenza viruses and are commonly intramuscularly administered to elicit a systemic immune response. Since the outbreak of the coronavirus disease 2019 (COVID-19) there has been a renewed interest in intranasal vaccines for respiratory viruses as immunity at the mucosa might better prevent transmission since the infection may be halted at the point of entry. [7] Especially for influenza, due to the constant antigenic drift and pandemic threat associated with antigenic shift, there is an urgent need for vaccination strategies that improve cross-protection against heterologous strains. Mucosal IgA elicited from intranasal immunization has the potential to provide cross-protection against heterologous and drifted strains. [8,9] In addition, intranasal vaccination has the advantage of patient-friendly needle-free administration with greater capacity for mass immunization compared to the traditional intramuscular route. [10].

Currently, only live-attenuated vaccines are licensed for intranasal administration (Fluenz Tetra/Flumist Quadrivalent, MedImmune, Gaithersburg, United States), however, its use is limited to 2–18 year olds due to higher incidence of hospital admission and wheezing in children < 2 years and lower efficacy in adults compared to injected influenza vaccines. [11] Adjuvants can help to boost immunogenicity of intranasal vaccination approaches. [12] Enterotoxin proteins, including *Escherichia coli* heat-labile toxin and derivatives, have previously shown to be potent and efficacious mucosal adjuvants but their use has been associated with facial nerve paralysis. [13,14].

Alternative safe and potent mucosal vaccine adjuvants are thus highly needed and Gram-positive Enhancer Matrix (GEM) may be a candidate. GEM adjuvant is composed of non-living bacterium-like particles (BLPs) derived from the lactic acid bacterium (LAB) *Lactococcus lactis*, a food-grade non-pathogenic Gram-positive bacterium that does not produce endotoxins and does not colonize the human mucosal cavities. GEM are BLPs that consist of a peptidoglycan outer surface. [15,16] Peptidoglycan is known to have immunostimulant properties and is presumed to play an important role in the observed adjuvant properties of GEM. [17,18] Studies in mice showed that intranasal administration of GEM mixed with influenza virus antigen (FluGEM) was safe and elicited hemagglutination inhibition (HI) titers equivalent to intramuscular vaccination after one booster dose. [19] Moreover, intranasal FluGEM administration yielded a strong mucosal IgA response, was fully protective in homologous and heterologous influenza virus challenge models in mice, with better protection rates compared to non-adjuvanted influenza vaccination. [19].

Here we present the results from a first-in-human clinical trial that had the objective to assess the safety, tolerability, reactogenicity and immunogenicity of intranasal vaccination with FluGEM in healthy adults and elderly (aged 65 and older).

2. Material and methods

2.1. Study design

This was a first-in-human, randomized, double-blind, controlled, dose-escalation study performed at the Centre for Human Drug Research (CHDR), Leiden, the Netherlands. Participants were randomized in a 3:1 ratio to receive nasally FluGEM (GEM-adjuvant with trivalent inactivated influenza vaccine [TIV]: B, A/H1N1 and A/H3N2 strains) or TIV only (control group). Randomization codes were generated by a statistician in SAS V9.1.3 for Windows (SAS Institute, Cary, NC, USA). Both study staff and subjects were blinded for the treatment allocation.

A staggered-enrollment strategy was used for the dose-escalation part of the study (age 18–49 years). In every dose level 4 subjects (3 active: 1 control) were vaccinated and followed-up until at least 7 days post-vaccination after which a preliminary safety assessment was made and predefined halting rules (supplementary appendix) were checked prior to the enrollment of an additional 16 (12 active: 4 control) participants. An independent safety monitoring committee (SMC) was to be consulted if one of the halting criteria was met. In addition, the SMC decided upon the selection of the intranasal doses to be tested in a subpopulation of elderly subjects aged 65 years or older.

The study was approved by the Central Committee on Research Involving Human Subjects (CCMO), The Netherlands (EudraCT 2010–024346-30). All study-related procedures were performed in accordance with the Declaration of Helsinki and the Dutch Act regarding Medical Research Involving Human Subjects. All subjects provided informed consent in writing prior to study activities.

2.2. Participants

All participants underwent a full medical screening prior to enrollment. Male and female volunteers were included if they were 18–49 years of age (part 1), or 65 years or older (part 2) and overtly healthy according to the medical screening procedure. Pregnant women were excluded from participation. Participants were excluded if they received an influenza vaccine that same year, had HI titers $> 1:10$ against two or more vaccine strains (B, H1N1 or H3N2) or suffered from moderate or severe illness 72 h prior to the planned nasal FluGEM or TIV vaccination (fever $\geq 38\,^{\circ}C$ or determined by the investigator). A full list of eligibility criteria is provided as supplementary material.

2.3. Vaccine formulations and nasal administration

FluGEM was administered intranasally and consisted of 1.25 mg, 2.5 mg and 5.0 mg doses of the GEM-adjuvant in conjunction with a standard TIV antigen dose (VAXIGRIP, 15 μg of A/California/7/2009 [H1N1], 15 μg of A/Perth/16/2009 [H3N2] and 15 μg of B/Brisbane/60/2008). FluGEM was administered as a two-dose regimen with a 21-day interval between first and second dose. The control group received nasally the TIV antigen only, diluted in phosphate buffered saline solution, in the same dose regimen as the FluGEM groups. FluGEM and control vaccine formulations were indistinguishable. Study treatments were administered by a trained physician using a disposable pipette to instill droplets of the vaccine, 0.125 ml in each nostril (0.250 in total), while the subject remained in supine position. Subjects were instructed to hold their breath during vaccine administration and pronounce a hard 'G' sound to prevent the vaccine of distributing to the lower airways.

2.4. Safety and tolerability assessment

The primary objective of the study was to assess safety and tolerability of intranasal doses of FluGEM. Routine laboratory safety assessments (blood biochemistry, hematology and urinalysis) were performed at screening, day 21 and day 42. Vital signs were measured prior to vaccination administration, 30 and 60 min after vaccination. Subjects remained in the clinical unit for at least 60 min for monitoring of any untoward medical event and were subsequently discharged if they had no adverse events, events were resolved or per discretion of the study physician. During the first 7 days post-vaccination subjects recorded the occurrence of any solicited local or systemic solicited adverse events (supplementary appendix) and measured body temperatures daily on a diary card. Unsolicited adverse events were monitored up to day 42 and afterwards subjects were monitored for serious adverse events only (until day 210).

2.5. Immunogenicity assessments

2.5.1. Systemic immunity: Hemagglutination inhibition assays

Sampling times throughout the study are depicted in Fig. 1. Presence

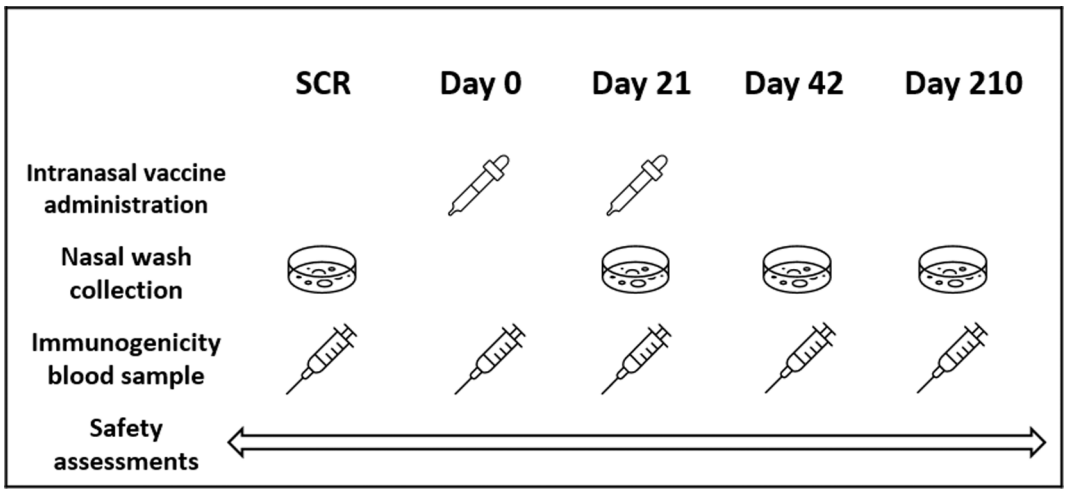


Fig. 1. Simplified study schedule SCR = screening. Blood samples for the assessment of hemagglutinin inhibition (HI) titers were collected at screening (up to 7 days prior to study start) or baseline (day 0) prior to receiving the first vaccination and on day 21 prior to the second vaccination. Finally on day 210 ± 15 final blood sample were collected for assessment of persistence of antibodies. Nasal washes for IgA determination were collected at screening, day 21 (prior to the second vaccination), day 42 and day 210 ± 15. Safety was assessed throughout the whole follow-up period until day 210. Solicited adverse events were recorded for 7 days following each vaccination.

of antibodies against hemagglutinin (of each of the respective vaccine components) were assessed in sera by a HI assay performed by Viroclinics Biosciences B.V., the Netherlands. In short, serial two-fold dilutions of serum samples (pre-treated to remove non-specific anti-HI activity) and quality control sera were incubated with the hemagglutinin virus suspension (previously titrated to adjust the dilution to 4 hemagglutination Units / 25 µl). After 30 min incubation at 37 ± 1 °C, 25 µl of 1 % (v/v) turkey erythrocytes were added in each plate, and further incubated for 1 h at 4 ± 1 °C. Duplicate plates were scored independently by two technicians. The serum titer was defined as the highest dilution that showed complete inhibition.

2.5.2. Mucosal immunity: IgA in nasal fluid

Nasal fluid was collected by gently instilling 4 ml of sterile solutions of phosphate buffered saline (PBS), at a temperature of 37 °C, into each nostril. Subjects were instructed to keep the solution in the nose for at least 20 s (with their neck extended approximately 45°) after which the nasal fluid was collected on a Petri dish. Nasal fluid material was subsequently transferred to conical polystyrene tubes and centrifuged for 10 min (360 g) at 4 °C. Nasal IgA concentrations were measured by enzyme-linked immunosorbent assay (ELISA) at Texcell, France. Six three-fold dilutions of the first 1:75 dilution of the nasal washes (or control samples) were added to empty wells that were previously incubated for 18 h at 5 ± 3 °C to coat with influenza antigens matching the vaccine strain hemagglutinins (50 µl of a 1 µg/ml solution). After 1 h incubation at 37 ± 2 °C, 50 µl of anti-human IgA peroxidase substrate was added and incubation was continued for 95 min at 37 ± 2 °C. A peroxidase substrate solution (50 µl) was added and the reaction was stopped after 20 min at 37 ± 2 °C by adding H_2SO_4. Optical density at 450 nm (OD_{450}) was measured and IgA values were read against the standard curve of the ELISA.

2.5.3. Exploratory: Serum IgG and subclass determination

As exploratory endpoint antigen-specific serum concentrations of total IgG and IgG subclasses were determined (age group 18 – 49 only). Serum IgG, and IgG1 and IgG3 subtypes were measured by ELISA at Texcell, France. Six three-fold dilutions of the first 1:50 dilution of the serum (or control samples) were added to wells coated with influenza antigens matching the TIV haemagglutinins as described above. After 1 h at 37 ± 2 °C, 50 µl of anti-human IgG (or IgG1 or IgG3) peroxidase substrate was added and incubated for 95 min at 37 ± 2 °C. A peroxidase substrate solution (50 µl) was added and the reaction was stopped after 20 min at 37 ± 2 °C by adding H_2SO_4. The OD_{450} was measured and IgG, IgG1 and IgG3 values were read against a standard curve of the ELISA.

2.6. Statistical analyses

This phase I study utilized group sizes that were conventional for early phase trials but was not powered to test a pre-defined hypothesis. Descriptive statistics were used for safety data. For immunogenicity parameters, geometric mean titers (GMT) and associated 95 % confidence intervals (CI), standard deviation and/or coefficient of variation and ratio's (GMR) were calculated. The following correlates of seroprotection were determined: 1) the proportion of subjects in each group exhibiting seroconversion on days 21 and/or 42, defined as either a four-fold rise in post-vaccination antibody HI titers compared to baseline HI, or a postvaccination titer ≥ 1:40 in subjects with baseline titer < 1:10; 2) the proportion of subjects in each group exhibiting seroprotection defined as HI ≥ 1:40; GMT increase defined as GMT ratio compared to baseline (GMR ≥ 2.5). [20] SAS for windows V9.1.3 (SAS Institute, Inc., Cary, NC, USA) was used for data analysis.

3. Results

3.1. Study population and baseline characteristics

The study was executed from 2011 till 2012. Sixty eligible subjects were included for the initial 18 – 49 years age group (part 1). All subjects completed the two-dose regimen of FluGEM or TIV, except for 3 individuals. Two subjects in the 1.25 mg group did not receive the second dose and a third subject was lost in the follow-up. Another subject in the 1.25 mg group had an intermittent severe AE (food allergy, also see 3.2) 6 days after the 1st administration and was withdrawn per protocol. In the 2.5 mg group a subject did not receive a second dose due to recurrent epistaxis. Baseline characteristics (Table 1) were overall similar for the dose groups. Subsequently, 30 elderly subjects were included to receive a selected dose of 1.25 mg FluGEM (n = 15) or TIV only as control group (n = 15). In the elderly group there was a slight predominance of female subjects in the control group (53.3 %) compared to the FluGEM group (33.3 %). All elderly subjects received 2 intranasal doses of FluGEM or control treatment.

Table 1
Baseline characteristics.

Characteristics	Age group 18–49				Age group 65 years and older	
	Control group n = 15	1.25 mg FluGEM n = 15	2.5 mg FluGEM n = 15	5.0 mg FluGEM n = 15	Control group n = 15	1.25 mg FluGEM n = 15
Age – years						
Mean (SD)	30.0 (10.4)	27.5 (9.0)	28.5 (8.3)	25.6 (7.9)	71.1 (3.3)	69.9 (4.1)
Range	18—47	20—47	19—46	20—46	67–78	65–78
Sex – n (%)						
Female	9 (60.0)	10 (66.7)	10 (66.7)	9 (60.0)	8 (53.3)	5(33.3)
BMI, kg/m^2, mean (SD)·	24.1 (2.9)	23.4 (2.2)	23.8 (3.3)	22.9 (2.64)	25.1 (2.6)	26.7 (2.5)
Race or ethnic group – n (%)						
Asian	0	1 (6.7)	0	0	0	0
Black	1 (6.7)	1 (6.7)	1 (6.7)	0	0	0
Hispanic	0	1 (6.7)	0	0	0	0
White	13 (86.7)	11 (73.3)	13 (86.7)	14 (93.3)	15 (100)	15 (100)
Mixed	1 (6.7)	1 (6.7)	0	1 (6.7)	0	0
Other	0	0	1 (6.7)	0	0	0

3.2. Safety and tolerability evaluation

Age group 18 – 49 years

All doses of FluGEM were well tolerated and there were no signs of increased reactogenicity following the second dose of FluGEM. The percentage of subjects reporting ≥ 1 treatment emergent adverse events (either solicited or unsolicited) was comparable across all FluGEM groups (86.7 – 100 %) and this was similar in the control group (93.3 %). The vast majority of adverse events were mild (97.4 %), there were two cases of moderate influenza-like illness in the 1.25 mg group that were self-limiting, two subjects had severe adverse advents: one subject had a concussion following an unrelated traumatic injury, another subject developed an anaphylactic reaction shortly after eating Thai food, 6 days after the first vaccination (1.25 mg group). Per protocol halting rules, the subject did not receive a second vaccination due to an intermittent severe adverse event and was withdrawn. The most frequent unsolicited adverse events were respiratory complaints, most frequently being throat irritation (22 % in active groups; 27 % in the control group). If respiratory complaints were reported by subjects they were mostly reported within 48 h following vaccination, however, timing of these adverse events were comparable for both active and control group (55 % to 65 % of respiratory adverse events were reported < 48 h in the active group versus 60 % in the control group. Epistaxis was reported with a low incidence (6.7 % – 13.3 %) in both control, 1.25 mg and 2.5 mg groups, but not in the highest 5.0 mg dose group, suggesting no apparent dose-related effect. There were no serious adverse events (SAE) observed.

Solicited adverse events (occurring within 7 days following vaccination) did not increase with increasing doses of FluGEM (Table 2). The frequency of solicited adverse events following FluGEM administration was comparable to the control group, with headache being reported most frequently (67 %) in both the control and the 2.5 mg group. There were no signs of dose-limiting toxicities or neurotoxicity. No findings of clinical concern in blood chemistry, hematology and urinalysis assessments were observed.

Age group 65 and older.

The frequency of treatment emergent adverse events in the elderly group was comparable to that of the 18—49 age group (86.7 %) as was the nature of the adverse events. Two unrelated SAEs occurred in the FluGEM (1.25 mg) dose group: one subject (age: 67) had a myocardial infarction (8 days following the 2nd vaccination) and needed percutaneous coronary intervention with stent placement due to atherosclerosis; another subject (age: 67) developed sinus node dysfunction (3 months after the 2nd vaccination) for which pacemaker insertion was needed. All other adverse events reported in the elderly group were mild, except for a single case of moderate gastro-enteritis that was unrelated to the vaccination.

Solicited local and systemic adverse events in the FluGEM group were similar to that in the control group by nature and frequency. Notably, sneezing was reported more often in the elderly age group (60 % and 73.3 % in the control and FluGEM 1.25 mg group, respectively) compared to the 18–49 years cohorts. Overall, FluGEM was well

Table 2
Solicited local and systemic adverse events.

Adverse events, n (%)	Age group 18–49				Age group 65 years and older	
	Control group n = 15	1.25 mg FluGEM n = 15	2.5 mg FluGEM n = 15	5.0 mg FluGEM n = 15	Control group n = 15	1.25 mg FluGEM n = 15
Nasal discomfort	1 (6.7)	1 (6.7)	1 (6.7)	2 (13.3)	2 (13.3)	1 (6.7)
Sneezing	2 (13.3)			2 (13.3)	9 (60.0)	11 (73.3)
Nasal congestion	4 (26.7)	4 (26.7)	5 (33.3)	4 (26.7)	3 (20.0)	2 (13.3)
Runny nose	6 (40.0)	2 (13.3)	3 (20.0)	5 (33.3)	6 (40.0)	4 (26.7)
Loss of smell					1 (6.7)	
Red eyes					1 (6.7)	
Lacrimation			1 (6.7)		1 (6.7)	2 (13.3)
Facial swelling						
Nasal pain						
Headache	10 (66.7)	7 (46.7)	10 (66.7)	7 (46.7)	4 (26.7)	5 (33.3)
Malaise		1 (6.7)	1 (6.7)		3 (20.0)	2 (13.3)
Myalgia	1 (6.7)	2 (13.3)	2 (13.3)	1 (6.7)	3 (20.0)	3 (20.0)
Chills		1 (6.7)	1 (6.7)		1 (6.7)	
Nausea			3 (20.0)		3 (20.0)	1 (6.7)
Vomiting		1 (6.7)			1 (6.7)	1 (6.7)

tolerated in participants aged 65 and older.

3.3. Immunogenicity

3.3.1. Systemic antibody response (18 – 49 years)

Baseline (day of start treatment administration) HI titers against influenza B virus, H1N1 and H3N2 showed a broad distribution across treatment groups (sup. Table S1) in the 18 – 49 years age group. Seronegativity (HI titer < 1:40) at baseline varied per strain, with percentages comparable across dose groups (range: 80 – 93 % [B], 53 – 67 % [H1N1], and 60 – 73 % [H3N2]). FluGEM doses of 1.25 mg and 2.5 mg showed a more rapid increase and higher magnitude of HI titers for the B strain and H1N1 strain compared to the control group (Table 3).

All treatment groups had GMT fold increases ≥ 2.5 post-vaccination for all three influenza strains (Table 3). For the B strain GMRs were highest following vaccination with 1.25 mg and 2.5 mg FluGEM (approximately 2 times higher than the GMRs of the control). For the H1N1 strain the increase in GMRs following FluGEM vaccination on day 21 and 42 were comparable to the control group. However, GMRs were markedly higher at day 210 in the 1.25 mg and 2.5 mg FluGEM dose groups for both the B strain and A/H1N1 strain (GMR of 7.7 and 5.7 [1.25 mg] and 8.8 and 4.9 [2.5 mg] versus 4.8 and 3.9 [control group] for the B and A/H1N1 strain, respectively). All formulations, including the non-adjuvanted control group, showed very strong HI responses to the H3N2 strain. FluGEM adjuvanted doses did not elicite higher GMRs compared to the non-adjuvanted control group for the A/H3N2 strain on the investigated time points.

In the study population as a whole, seroconversion in ≥ 40 % of subjects was achieved at both day 21 (single dose) and day 42 (two-dose) in the 1.25 mg for all tested strains and at day 42 (two-dose) only for the 2.5 mg dose (Table 4). The non-adjuvanted control group fulfilled this criterion for the H3N2 strain only. Seroconversion rates were higher for seronegative subjects. In the seronegative subpopulation both the 1.25 mg and 2.5 mg (but not the 5 mg group) and the unadjuvanted control group had seroconversion rates ≥ 40 % for all strains (sup. Table S2).

Seroprotection was highest for the influenza A strains (H1N1 and H3N2) with all treatment groups reaching protection rates of ≥ 70 % (Table 5). The highest seroprotection rate for the B strain was achieved with the 1.25 mg FluGEM dose level with a seroprotection rate of 73 % on day 21 and 64.3 on day 42. Seroprotection rates were consistently lower for subjects seronegative at baseline (sup. Table S3).

3.3.2. Nasal IgA response (18 – 49 years)

In the control group, 2.5 mg and 5 mg FluGEM group, the majority of subjects had pre-vaccination IgA titers below the detection limit (>73 % of subjects per group). However, in the 1.25 mg dose group only six subjects had IgA below this limit (Table 6). All groups showed increased nasal IgA levels following vaccination. Highest IgA GMRs were observed in the 1.25 mg FluGEM dose group on day 21 (ratio: 1.8). This effect was more pronounced in subjects with non-detectable IgA levels at baseline (GMR: 3.7 and 3.2 on day 21 and 42, respectively). Such effect of low pre-existing IgA levels on GMR was not observed in the other dose groups. At day 210, IgA returned to baseline levels in all treatment groups.

3.3.3. Total IgG, IgG1 and IgG3 subclasses (18 – 49 years)

Total influenza virus specific HA IgG in serum and IgG1 and IgG3 subclasses are listed in Table 7. Total IgG, IgG1 and IgG3 increased in all treatment groups following the first vaccination, FluGEM-adjuvanted dose groups showed a relatively faster peak titer following the first vaccination, effects on total IgG following a second vaccination were less pronounced in the FluGEM adjuvanted groups. All treatment groups showed a IgG1 dominant response following vaccination, with highest IgG1/IgG3 ratio's being observed in the 1.25 mg dose group (data not shown).

3.3.4. Immunogenicity: Age group 65 and older

Following interim-analysis of safety and immunogenicity data of the previous dose levels in 18 – 49 year olds, the 1.25 mg dose was selected by the SMC to be assessed in elderly subjects, as this dose showed overall the best immunogenicity profile. In general, systemic immunogenicity to both the non-adjuvanted inactivated trivalent vaccine (control group) and 1.25 mg of FluGEM was less pronounced than in the 18 – 49 year groups (sup. Table S4). Seroconversion and protection rates were similar for both control and 1.25 mg FluGEM (sup. Tables S5 and S6). Criteria for 40 % seroconversion and 70 % seroprotection were not fulfilled in both dose groups. At baseline there was a difference with more subjects (93 %) having IgA titers below the lower limit of detection in the control group compared to the 1.25 mg FluGEM group (64 %). No apparent treatment effects on nasal IgA GMT's and ratios were observed in subjects ≥ 65 years (sup. Table S7).

4. Discussion

In this study FluGEM – trivalent inactivated influenza vaccine

Table 3
Hemaglutinin inhibiton geometric mean titers and ratio to baseline titers in subjects with data available at all time points.

FluGEM dose	Study Day	N	GMT B strain	GMR B strain	N	GMT A/H1N1 strain	GMR A/H1N1 strain	N	GMT A/H3N2 strain	GMR A/H3N2 strain
TIV only (control)	0	13	10.5	1.0	11	10.4	1.0	15	13.2	1.0
	21	13	29.9	2.8	11	49.5	4.8	15	197.2	14.9
	42	13	32.4	3.1	11	53.8	5.2	15	243.7	18.5
	210	13	50.6	4.8	11	40.8	3.9	15	179.5	13.6
1.25 mg FluGEM	0	10	8.5	1.0	11	23.2	1.0	12	23.1	1.0
	21	10	48.1	5.7	11	118.0	5.1	12	261.9	11.3
	42	10	47.8	5.6	11	109.9	4.7	12	287.0	12.4
	210	10	63.9	7.6	11	131.7	5.7	12	244.3	10.6
2.5 mg FluGEM	0	14	8.6	1.0	12	15.9	1.0	14	18.3	1.0
	21	14	54.0	6.3	12	78.7	5.0	14	152.5	8.3
	42	14	56.9	6.6	12	88.7	5.6	14	179.8	9.8
	210	14	75.9	8.8	12	78.0	4.9	14	141.3	7.7
5 mg FluGEM	0	13	7.5	1.0	12	15.5	1.0	14	17.5	1.0
	21	13	28.7	3.8	12	59.0	3.8	14	177.3	10.1
	42	13	30.3	4.0	12	82.3	5.3	14	228.5	13.0
	210	13	53.7	7.2	12	83.0	5.4	14	187.2	10.7

GMR = geometric mean titer ratio to baseline; GMT = geometric mean titer; HI = hemagglutination inhibition; TIV = trivalent inactivated influenza vaccine.

Table 4
Seroconversion rates, 18 – 49 years.

FluGEM dose	B strain				A/H1N1 strain				A/H3N2 strain			
	Day 21		Day 42		Day 21		Day 42		Day 21		Day 42	
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
TIV only (control)	5/13	38.5	5/13	38.5	5/14	35.7	5/14	35.7	12/15	80.0	13/15	86.7
1.25 mg FluGEM	5/12	41.7	6/12	50.0	6/13	46.1	6/13	46.1	7/13	53.8	7/13	53.8
2.5 mg FluGEM	8/15	53.3	6/14	42.8	5/15	33.3	6/14	42.8	8/15	53.3	8/14	57.1
5 mg FluGEM	4/13	30.8	5/14	35.7	4/14	28.6	5/14	35.7	9/14	64.3	10/14	71.4

TIV = trivalent inactivated influenza vaccine.

Table 5
Seroprotection rates, 18 – 49 years.

FluGEM dose	B strain				A/H1N1 strain				A/H3N2 strain			
	Day 21		Day 42		Day 21		Day 42		Day 21		Day 42	
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
TIV only (control)	8/13	61.5	8/13	61.5	10/14	71.4	10/14	71.4	14/15	93.3	15/15	100
1.25 mg FluGEM	7/12	58.3	7/12	58.3	10/13	76.9	10/13	76.9	13/13	100	13/13	100
2.5 mg FluGEM	11/15	73.3	9/14	64.3	12/15	80.0	13/14	92.8	13/15	86.7	12/14	85.7
5 mg FluGEM	5/13	38.5	6/14	42.8	10/14	71.4	10/14	71.4	12/14	85.7	13/14	92.8

TIV = trivalent inactivated influenza vaccine.

Table 6
Nasal IgA titers from subjects with data available at all time points.

		All subjects			Subjects with baseline IgA titers below detection level		
FluGEM dose	Study Day	N	GMT	GMR	N	GMT	GMR
TIV only (control)	0	14	57.7	1.0	12	50.0	1.0
	21	14	78.3	1.4	12	76.3	1.5
	42	14	86.1	1.5	12	83.5	1.7
	210	14	53.7	0.9	12	50.0	1.0
1.25 mg FluGEM	0	13	77.3	1.0	6	50.0	1.0
	21	13	145.7	1.9	6	186.9	3.7
	42	13	140.4	1.8	6	161.6	3.2
	210	13	61.9	0.8	6	67.2	1.3
2.5 mg FluGEM	0	14	57.0	1.0	11	50.0	1.0
	21	14	61.5	1.1	11	53.9	1.1
	42	14	105.1	1.8	11	75.0	1.5
	210	14	51.6	0.9	11	50.0	1.0
5 mg FluGEM	0	15	50.0	1.0	14	50.0	1.0
	21	15	79.4	1.6	14	79.4	1.6
	42	15	85.0	1.7	14	85.0	1.7
	210	15	55.0	1.1	14	55.0	1.1

GMR = geometric mean titer ratio to baseline; GMT = geometric mean titer; TIV = trivalent inactivated influenza vaccine.

Table 7
Influenza-specific IgG, IgG1 and IgG3 subclasses from subjects with data available at all time points.

		GMT					
FluGEM dose	Study Day	N	Total IgG	N	IgG1	N	IgG3
TIV only (control)	0	15	15,349	15	2,858.5	15	80.7
	21	15	37,853	15	7,261.3	15	203.5
	42	15	53,358	14	7,582.0	14	168.2
	210	15	39,820	14	7,129.4	15	122.8
1.25 mg FluGEM	0	15	19,031	15	4,269.2	15	62.4
	21	14	57,754	13	7,136.4	14	125.7
	42	13	60,839	11	8,983.7	12	181.6
	210	13	51,463	11	7,450.1	12	126.5
2.5 mg FluGEM	0	15	15,613	15	2,612.7	15	68.6
	21	14	52,834	15	8,401.7	15	362.6
	42	14	45,451	13	8,022.5	14	339.2
	210	14	40,591	14	7,306.5	14	160.0
5 mg FluGEM	0	15	22,729	15	2,860.5	15	124.5
	21	15	59,815	15	6,574.2	15	287.9
	42	15	55,098	15	6,174.4	14	213.0
	210	15	52,730	15	5,819.7	15	164.9

TIV = trivalent inactivated influenza vaccine.

adjuvanted with GEM particles − was administered for the first time in humans via the intranasal route. We found that all explored intranasal doses (up to 5 mg in the 18–49 years group and 1.25 mg in elderly) were well tolerated. The frequency and intensity of adverse events following FluGEM vaccination were comparable to that of the unadjuvanted TIV.

In the 18–49 years age group, favorable effects of FluGEM on the humoral systemic immune response were observed compared to unadjuvanted TIV, particularly the 1.25 mg and 2.5 mg dose levels yielded the highest HI titers overall against multiple influenza strains. Several parameters for serological protection were used for the evaluation of vaccine immunogenicity. Geometric mean titer (GMT) increases ≥ 2.5-fold were observed in all treatment groups (including the control group). However, in the 1.25 mg FluGEM group seroconversion (≥40 %) for all strains was achieved after only a single dose. Seroconversion against all strains was also achieved in the 2.5 mg FluGEM group, but after two doses. Seroprotection (≥70 %) for all strains was only observed for the 2.5 mg dose (day 21 only). Historically, one out of three of these criteria should be met for annual vaccination with seasonal inactivated vaccines. [20] FluGEM doses performed better for both seroconversion and seroprotection criteria compared to the unadjuvanted intranasal trivalent vaccine (control), signifying the potential adjuvant function of FluGEM for these endpoints. In addition, FluGEM appears to elicit a more persistent systemic humoral response for both B and H1N1 strains (but not for H3N2) when HI GMT ratios at day 210 are compared to the control group.

Adjuvant effects of FluGEM on mucosal IgA were most pronounced in subjects in the 1.25 mg dose group with no pre-existing nasal IgA titers, with peak IgA titers following the first vaccination (GMT ratio: 3.7). Although there is no well-established correlate of protection for mucosal IgA, especially against currently circulating influenza strains, the

magnitude of IgA responses (fold-increases in IgA) in the present study were in a similar-to-higher range than IgA levels that were considered protective in previous human challenge studies with influenza virus. [21,22] Eliciting sufficient IgA responses is essential for a mucosal influenza vaccine candidate, as mucosal IgA can neutralize virus at the mucosal interface before viral entry and clear the virus from respiratory epithelial cells, preventing downstream adverse host immune responses and possibly direct transmission. [8,23] These effects are not readily expected from intramuscular vaccines that do not elicit mucosal IgA responses. At day 210 we found that IgA levels declined to baseline levels, further characterization of mucosal immune response would be needed to determine the timing and frequency of revaccination.

We found that in the 18–49 year population FluGEM intranasal doses of 1.25 mg and 2.5 mg were more immunogenic than the highest dose level of 5 mg. In a first-in-human study of SynGEM – an intranasal vaccine candidate based on the same bacterium-like particle platform with recombinant respiratory syncytial virus (RSV) F-protein as primary antigen attached to the BLP – higher serum IgG titers were achieved following boosting of the low dose group compared to a high-dose regimen. [24] However, for other endpoints the higher dose appeared more immunogenic. It is assumed that the mode of action of GEM is through toll-like receptor (TLR) – 2 signaling. [25] While TLR2 activation most often shows dose-dependent downstream effects, non-linear 'bell-shaped' dose–response relationships have been described for some TLR2 agonists. [26] The exact immunological basis for the dose–response relationship of FluGEM remains unknown and to be explored in future studies.

Intranasal administration of FluGEM was also well tolerated in elderly subjects (≥65 years). The explored 1.25 mg dose did not have as pronounced immune effects in elderly compared to the 18 – 49 years population. Mucosal immunization of the elderly population remains a well-known challenge for mucosal vaccine candidates, which is likely due to immunosenescence. Animal data suggest that nasal IgA responses to immunization are likely to be diminished by age-related decline of the immune system. [27,28] Even following controlled human infection challenge with a respiratory virus, nasal IgA production can be defective in the elderly population. [29] In addition to immunosenescence, anatomical age-related changes to the nose such as mucosal atrophy and increased size of the nasal cavity may also challenge the adequate delivery of intranasal vaccines. [30] Further dose-exploration in the elderly, optimization vaccine formulation for instance using mucoadhesive agents to enhance the vaccine residence time and increased uptake of active compounds, or delivery systems could be explored to improve immunogenicity in this specific population. [31].

The study's eligibility criteria allowed only for inclusion of volunteers with relatively low HI serum titers against the influenza virus strains that were present in the trivalent vaccine. High levels of pre-existing antibodies may negatively impact the magnitude of the fold-increase in serum antibody titers following vaccination. [32] In this study, pre-existing levels of mucosal antibodies were not implemented as eligibility criteria. However, pre-existing mucosal immunity could, analogous to serum responses, possibly impair the magnitude of the mucosal vaccine response. In subjects without pre-existing IgA titers, the 1.25 mg dose of FluGEM had a markedly higher magnitude of fold increase. Although limited by the sample size, these results suggest a potential for the GEM-adjuvanted intranasal vaccine platform to induce mucosal immunity against drifted strains or novel pathogens for which there is no pre-existing immunity.

Some limitations of the study should be noted. The influenza virus specific IgA assay used in this study were not standardized for total IgA or total protein measurements, mucosal IgA and protein concentration can vary between individuals. The study population had a highly immunogenic response to the H3N2 strain, illustrated by already high HI titers following vaccination with the plain trivalent vaccine. Interestingly, influenza A/H3N2 strains did not circulate on large scale during the preceding annual flu epidemic in the Netherlands, nor in the year before. [33] Adjuvant effects of FluGEM on the H3N2 strain in this study could be blunted by the already pre-existing strong immunogenic response to the primary vaccine antigen. While the group sizes in this study were conventional for phase I vaccine trials, the study was not powered to test pre-defined hypothesis on immunogenic endpoints, larger immunogenicity trials are needed for formal statistical interference on the observed adjuvant effects of FluGEM. Since the completion of this study, newer editions of vaccine guidelines have suggested a more elaborate assessment of immunogenicity including functional antibodies determined by virus neutralization assays and cellular immunity for influenza vaccine. [34] Future studies will need to be performed to further investigate the immunogenicity of FluGEM.

In conclusion, this study described the first-in-human administration of FluGEM, all explored intranasal doses were safe and well-tolerated with highest immunogenicity observed for the 1.25 mg and 2.5 mg doses in subjects between 18–49 years of age. Further research is warranted to assess immunogenicity of intranasal FluGEM in next phase clinical trials and in targeted subpopulations.

CRediT authorship contribution statement

Johan L. van der Plas: Writing – review & editing, Writing – original draft. **Bert-Jan Haijema:** Writing – review & editing, Project administration. **Kees Leenhouts:** Writing – review & editing, Project administration, Methodology, Conceptualization. **J. Paul Zoeteweij:** Writing – review & editing, Validation, Project administration, Methodology. **Jacobus Burggraaf:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Ingrid M.C. Kamerling:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

Kees Leenhouts and Bert-Jan Haijema were former employees of Mucosis BV. The study was sponsored by Mucosis BV. For the other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.03.063.

References

[1] Iuliano AD, Roguski KM, Chang HH, et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. Lancet 2018;391:1285–300.
[2] Molinari NA, Ortega-Sanchez IR, Messonnier ML, et al. The annual impact of seasonal influenza in the US: measuring disease burden and costs. Vaccine 2007;25:5086–96.
[3] Ruf BR, Knuf M. The burden of seasonal and pandemic influenza in infants and children. Eur J Pediatr 2014;173:265–76.
[4] Haq K, McElhaney JE. Immunosenescence: Influenza vaccination and the elderly. Curr Opin Immunol 2014;29:38–42.
[5] Targonski PV, Jacobson RM, Poland GA. Immunosenescence: role and measurement in influenza vaccine response among the elderly. Vaccine 2007;25:3066–9.
[6] Alexander-Miller MA. Challenges for the Newborn Following Influenza Virus Infection and Prospects for an Effective Vaccine. Front Immunol 2020;11:568651.
[7] Rubin R. Trying to Block SARS-CoV-2 Transmission With Intranasal Vaccines. JAMA 2021;326:1661–3.
[8] van Riet E, Ainai A, Suzuki T, Hasegawa H. Mucosal IgA responses in influenza virus infections; thoughts for vaccine design. Vaccine 2012;30:5893–900.
[9] Tamura S, Funato H, Hirabayashi Y, et al. Cross-protection against influenza A virus infection by passively transferred respiratory tract IgA antibodies to different hemagglutinin molecules. Eur J Immunol 1991;21:1337–44.
[10] Skwarczynski M, Toth I. Non-invasive mucosal vaccine delivery: advantages, challenges and the future. Expert Opin Drug Deliv 2020;17:435–7.

[11] Summary of Product Characteristics FluenzTetra nasal spray suspension. AstraZeneca AB. (Accessed 17 Oct 2022, at https://www.ema.europa.eu/en/documents/product-information/fluenz-tetra-epar-product-information_en.pdf.).
[12] Rhee JH, Lee SE, Kim SY. Mucosal vaccine adjuvants update. Clin Exp Vaccine Res 2012;1:50–63.
[13] Mutsch M, Zhou W, Rhodes P, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004;350:896–903.
[14] Lewis DJ, Huo Z, Barnett S, et al. Transient facial nerve paralysis (Bell's palsy) following intranasal delivery of a genetically detoxified mutant of Escherichia coli heat labile toxin. PLoS One 2009;4:e6999.
[15] Bosma T, Kanninga R, Neef J, et al. Novel surface display system for proteins on non-genetically modified gram-positive bacteria. Appl Environ Microbiol 2006;72:880–9.
[16] van Roosmalen ML, Kanninga R, El Khattabi M, et al. Mucosal vaccine delivery of antigens tightly bound to an adjuvant particle made from food-grade bacteria. Methods 2006;38:144–9.
[17] Dziarski R, Gupta D. Staphylococcus aureus peptidoglycan is a toll-like receptor 2 activator: a reevaluation. Infect Immun 2005;73:5212–6.
[18] Kusumoto S, Fukase K, Shiba T. Key structures of bacterial peptidoglycan and lipopolysaccharide triggering the innate immune system of higher animals: Chemical synthesis and functional studies". Proc Jpn Acad Ser B Phys Biol Sci 2010;86:322–37.
[19] Saluja V, Amorij JP, van Roosmalen ML, et al. Intranasal delivery of influenza subunit vaccine formulated with GEM particles as an adjuvant. AAPS J 2010;12:109–16.
[20] Committee for Human Medicinal Products 1997;CPMP/BWP/214/96.
[21] Belshe RB, Gruber WC, Mendelman PM, et al. Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. J Infect Dis 2000;181:1133–7.
[22] Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J Clin Microbiol 1986;24:157–60.
[23] Seibert CW, Rahmat S, Krause JC, et al. Recombinant IgA is sufficient to prevent influenza virus transmission in guinea pigs. J Virol 2013;87:7793–804.
[24] Ascough S, Vlachantoni I, Kalyan M, et al. Local and Systemic Immunity Against RSV Induced by a Novel Intranasal Vaccine: A Randomised, Double- Blind, Placebo-Controlled Trial. Am J Respir Crit Care Med 2019.
[25] Ramirez K, Ditamo Y, Rodriguez L, et al. Neonatal mucosal immunization with a non-living, non-genetically modified Lactococcus lactis vaccine carrier induces systemic and local Th1-type immunity and protects against lethal bacterial infection. Mucosal Immunol 2010;3:159–71.
[26] Young SH, Wolfarth MG, Roberts JR, Kashon ML, Antonini JM. Adjuvant effect of zymosan after pulmonary treatment in a mouse ovalbumin allergy model. Exp Lung Res 2013;39:48–57.
[27] Fukuiwa T, Sekine S, Kobayashi R, et al. A combination of Flt3 ligand cDNA and CpG ODN as nasal adjuvant elicits NALT dendritic cells for prolonged mucosal immunity. Vaccine 2008;26:4849–59.
[28] Hagiwara Y, McGhee JR, Fujihashi K, et al. Protective mucosal immunity in aging is associated with functional CD4+ T cells in nasopharyngeal-associated lymphoreticular tissue. J Immunol 2003;170:1754–62.
[29] Ascough S, Dayananda P, Kalyan M, et al. Divergent age-related humoral correlates of protection against respiratory syncytial virus infection in older and young adults: a pilot, controlled, human infection challenge model. Lancet Healthy Longev 2022;3:e405–16.
[30] Ganjaei KG, Soler ZM, Mappus ED, et al. Radiologic changes in the aging nasal cavity. Rhinology 2019;57:117–24.
[31] Yusuf H, Kett V. Current prospects and future challenges for nasal vaccine delivery. Hum Vaccin Immunother 2017;13:34–45.
[32] Ellebedy AH, Nachbagauer R, Jackson KJL, et al. Adjuvanted H5N1 influenza vaccine enhances both cross-reactive memory B cell and strain-specific naive B cell responses in humans. Proc Natl Acad Sci U S A 2020;117:17957–64.
[33] de Jong JC, Donker GA, Meijer A, van der Hoek W, Rimmelzwaan GF, Osterhaus ADME. Het influenzaseizoen 2010/2011 in Nederland: het nieuwe A (H1N1)-virus van 2009 blijft actief. Nederlands Tijdschrift voor Medische Microbiologie 2011, 2011,;19(4):21–7.
[34] Wijnans L, Voordouw B. A review of the changes to the licensing of influenza vaccines in Europe. Influenza Other Respir Viruses 2016;10:2–8.