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Safety and immunogenicity studies in animal models support clinical development of a bivalent norovirus-like particle vaccine produced in plants

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ABSTRACT

Noroviruses (NoV) are the leading cause of epidemic acute gastroenteritis in humans worldwide. A safe and effective vaccine that prevents NoV infection or minimizes NoV disease burden is needed, especially for children and the elderly who are particularly susceptible to NoV disease. A plant-based expression system (magnICON®) was used to manufacture two different virus-like particle (VLP) immunogens derived from human NoV genogroups I and II, genotype 4 (GI.4 and GII.4), which were subsequently blended 1:1 (w/w) into a bivalent vaccine composition (rNV-2v). Here, we report on the safety and immunogenicity of rNV-2v from one pilot and two GLP-compliant toxicity studies in New Zealand White rabbits administered the vaccine subcutaneously (SC) or intramuscularly (IM). Strong genogroup-specific immune responses were induced by vaccination without adjuvant at various doses (200 to 400 μg VLP/administration) and administration schedules (Days 1 and 7; or Days 1, 15 and 29). The results showed sporadic local irritation at the injection site, which resolved over time, and was non-adverse and consistent with expected reactogenicity. There were no signs of systemic toxicity related to vaccine administration relative to vehicle-treated controls with respect to clinical chemistry, haematology, organ weights, macroscopic examinations, or histopathology. In a 3-administration regimen (n + 1 the clinical regimen), the NOAEL for rNV-2v via the SC or IM route was initially determined to be 200 μg. An improved GI.4 VLP variant mixed 1:1 (w/w) with the wild-type GII.4 VLP was subsequently evaluated via the IM route at a higher dose in the same 3-administration model, and the NOAEL was raised to 300 μg. Serology performed in samples of both toxicity studies showed significant and substantial anti-VLP-specific antibody titers for rNV-2v vaccines administered via the IM or SC route, as well as relevant NoV blocking antibody responses. These results support initiation of clinical development of the plant-made NoV vaccine.

1. Introduction

Noroviruses (NoV) are the leading cause of epidemic acute gastroenteritis and foodborne diarrheal diseases in humans worldwide [1,2]. These infections can occur in all age groups and commonly result in significant morbidity and mortality, particularly in the very old and very young. It is estimated that up to 200,000 children die from complications of NoV infection worldwide annually [3]. Infection is characterized by severe vomiting, diarrhea, and abdominal cramping for 28 – 60 h within 10 – 51 h of exposure [4]. The virus is transmitted by the fecal/oral route and virus particles exhibit high environmental stability on exposed surfaces [5]. Severe outbreaks

Abbreviations: NoV, norovirus; GI.4, NoV genogroup I, genotype 4 antigen; GII.4, NoV genogroup II, genotype 4 antigen; VLP, virus-like particle; rNV-2v, recombinant NoV bivalent vaccine; NOAEL, no observed adverse effect level; SC, subcutaneous; IM, intramuscular; HBGA, histo-blood group antigens; MTD, maximum tolerated dose; PEG, polyethylene glycol; PGM, pig gastric mucin; BCA, bicinchoninic acid assay; CGE, capillary gel electrophoresis; HPLC, high-performance liquid chromatography; LAL, *Limulus* amebocyte lysate assay; EMA, European Medicines Agency; FDA, Food and Drug Administration; MHLW, Ministry of Health, Labor and Welfare; USDA, US Department of Agriculture.

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typically occur in close-quartered environments such as hospitals, schools, day care centers, elder care facilities, and ships [6–8]. Therefore, widespread vaccination to minimize overall NoV disease burden would bring economic and clinical benefits to the entire population [9]. To date, there is no approved vaccine to prevent NoV gastroenteritis. Consequently, in 2016 the World Health Organization stated that the development of a NoV vaccine should be considered an absolute priority [10].

Noroviruses are members of the Caliciviridae family, have a single plus-sense strand of RNA and lack a surface envelope [2]. Fucosylated glycans belonging to histo-blood group antigens (HBGAs) present on intestinal epithelial cells are considered putative receptors for human NoV attachment and cell entry in a manner similar to rotavirus [11]. Because HBGAs in an individual are under specific genetic control, susceptibility to NoV infection varies [12,13]. NoV are divided into 10 genogroups and 48 genotypes [14]. Most human disease is caused by members of genogroups I and II (GI and GII), which are comprised of at least 9 and 27 distinct genotypes, respectively [14,15]. GI and GII strains are typically responsible for 10% and 90% of human NoV disease, respectively, with genotype GII.4 being responsible for >70% of outbreaks since the 1990s [16]. Consequently, GII.4 NoV strains have been of primary interest for vaccine development [16], although a multivalent GI + GII vaccine might be a preferred option for inducing broad and efficient protection.

The NoV virion is composed of 90 dimers of the major capsid protein VP1 [17]. Two conserved domains of VP flank a central variable domain that likely carries antigenic determinants that define strain specificity [18]. Recombinantly produced variants of VP1 have been exploited as immunogens in the development of NoV vaccines. The most advanced NoV vaccine candidates in clinical development consist of VP1 monomers expressed in animal cell culture systems, which spontaneously form virus-like particles (VLP). Purified VLPs made in such systems have been evaluated clinically when administered orally, intranasally and intramuscularly, usually in combination with adjuvants that improve immunogenicity (reviewed in [2,9,19]) For example, a mixed NoV GI.1 + GII.4 VLP vaccine adjuvanted with MPL and/or aluminum hydroxide has been clinically evaluated for safety and immunogenicity in various age groups, including healthy adults [20–23], as well as infants, toddlers and children [24]. These VLP vaccines proved to be generally well tolerated and immunogenic.

The NoV vaccine in the present study is a non-adjuvanted, recombinantly produced, bivalent VLP composition (i.e. mixed NoV GI.4 + GII.4 antigens) designated "rNV-2v". The product is manufactured using the magnICON® system (Icon Genetics, Halle, Germany), a rapid and cost-effective proprietary platform comprising *Agrobacterium*-mediated transient gene expression in non-transgenic host plants of the species *Nicotiana benthamiana*. This cGMP-compliant manufacturing technology, and variations thereof, have been widely applied to produce vaccines, enzymes, therapeutic antibodies and other biologics, as extensively reviewed [25–30]. In particular, multiple personalized plant-made vaccines manufactured with the magnICON® process have demonstrated clinical safety and antigen-relevant immunogenicity [31]. Here, we describe results of preclinical toxicity studies with the plant-made rNV-2v that helped establish safety and tolerability and serological analyses that helped define a target clinical dose and administration regimen. These studies were designed to support regulatory submissions for first-in-human clinical evaluation of rNV-2v.

2. Materials and methods

2.1. Production of NoV VLPs in Nicotiana benthamiana

GI.4 and GII.4 NoV VLPs were expressed in *Nicotiana benthamiana* plants using magnICON® vectors based on a tobacco mosaic virus (TMV) RNA replicon system, purified and characterized by ICON Genetics GmbH (Halle, Germany), as described [32–34]. Briefly, *N. benthamiana* plants were vacuum-infiltrated (80–100 mbar for 3–4 min) with diluted *Agrobacterium tumefaciens* cultures with TMV-based assembled magnICON® vectors carrying codon-optimized VP1 DNA (GI.4 or GII.4-2006a) cloned for expression and plant material was harvested 6–14 days post infiltration. Biomass was homogenized and clarified by single-use depth filtration. Norovirus VLP were sedimented and purified by PEG precipitation and filtration. VLP formation was confirmed by size exclusion HPLC (Agilent 1200); protein concentration by BCA assay; protein purity by reduced CGE (Agilent 2100); endotoxin by chromogenic LAL assay (Lonza QCL-1000) and residual host cell DNA by Quant-iT dsDNA High Sensitivity Kit (Thermo Fisher). High-order structures and morphology of the proteins were imaged by transmission electron microscopy (Carl Zeiss EM900). The doses, homogeneity and stability of the vaccine formulations and their matched vehicle controls were verified pre and post each toxicity study.

2.2. Ethics statements for research institutions

Three animal studies were conducted by two accredited contract research organizations (CRO). One pilot study (Study A) and one GLP regulation-compliant (21CFR58) toxicity study (Study B) in rabbits were conducted by SRI International (Menlo Park, CA, USA). All procedures for animal care and housing complied with the current requirements of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), the Guide for the Care and Use of Laboratory Animals (National Research Council), and the USDA's Animal Welfare Act and Animal Welfare regulations (July 2020). All in-life and termination protocols were as approved by SRI's Institutional Animal Care and Use Committee (IACUC). An additional toxicity study in rabbits (Study C) was conducted by Laboratory for Pharmacology and Toxicology (LPT; Hamburg, Germany) in compliance with current EC GLP regulations enacted in Germany and regulated by Directive 2004/10/EC of the European Parliament and the Council of 11 February 2004.

2.3. Toxicity studies

2.3.1. Model system and test article administration

Test article formulations were provided to the CROs ready to use by the manufacturer (Icon Genetics) without the need for dilution. Vaccine and vehicle storage was at 5 °C ± 3 °C, as prescribed by the manufacturer's Certificates of Analysis. The appropriate number of vials were brought to room temperature at least 30 min before usage, used within 8 h of removal from refrigerated temperature, and mixed by 5–8 roundtrip inversions before administration. Three toxicity studies (Studies A, B and C) were conducted in New Zealand White (NZW) rabbits (obtained from Covance, Denver, PA, USA in Studies A and B, and from Charles River Laboratories, Sulzfeld, Germany in Study C). Animals were examined upon arrival, quarantined, weighed, randomized and assigned to study groups based on approved protocols by each CRO's IACUC. Injections of test articles were performed using a 1 ml Luer-Lok™ syringe (Becton Dickinson BD#309628) with a 25-gauge 5/8-inch needle (Becton Dickinson BD#305122). Vaccine or vehicle control was administered into (IM) or above (SC) the foreleg triceps muscle. Administrations were conducted on Days 1 and 7 in Study A, and on Days 1, 15 and 29 in Studies B and C. Additional details of each study are described in Results.

Toxicological evaluations. Standard local and systemic toxicity evaluations to support Phase 1 human clinical research were conducted in each of the nonclinical studies, following regulatory agencies' (EMEA 1997; WHO 2005; FDA 2006; ICH 2009; ICH

2011) guidance for preventative vaccines for infectious diseases [35–39]. Observations at necropsy of the main group animals were compared to those of recovery group animals to assess reversibility of any reactions and their correlation to vaccination and dose. Local tolerance including erythema, eschar formation and edema were scored by the method of Draize (1944) [40]. Body weights were recorded upon animal delivery, at group allocation, on the day of commencement of treatment, and once weekly thereafter. Food and water consumption, behavioral changes, morbidity and mortality were recorded daily. Body temperature was recorded predose, 1 and 4 h prior to dosing, and 1, 4 and 24 h post dosing. Ophthalmological and auditory examinations were performed before first dose, one day after each dosing, and at the end of the dosing period. Blood samples were collected before each dose for hematological examination, coagulation tests, clinical chemistry, CRP determination, and immunogenicity evaluations. Euthanasia was via overdose of sodium pentobarbital by IV injection following subcutaneous administration of a sedative cocktail. Histology was performed on multiple tissue samples from all major organs.

2.3.2. Control of bias

While evaluating the responses of the animals and conducting the analyses, the technical staff was aware of the treatment history of each animal and sample. Based on the relatively objective endpoints examined, bias was not expected to influence the results of the study.

2.3.3. Statistical evaluations

In Study A and Study B, means and standard deviations were calculated for body weight, body temperature and clinical pathology data at each evaluation interval. Calculations were performed using Provantis® version 9.3.1.1, MS Excel 2010 or later software. Statistical evaluations consisted of one-way analysis of variance (ANOVA), followed by Dunnett's test (if the ANOVA was significant). Criteria for null hypothesis rejection was $p \leq 0.05$. All other numeric parameters were evaluated by Student's *t*-test. In Study C statistical analyses included Student's *t*-test ($p \leq 0.05$ and $p \leq 0.01$ for body weight, food consumption, hematology, coagulation. clinical chemistry, relative and absolute organ weights); Chi-square test ($p \leq 0.01$ for bone marrow analysis), and exact test of R.A. Fischer ($p \leq 0.05$ for histopathology).

2.4. Immune response assays

2.4.1. Serology

Serological evaluations were conducted by the Vaccine Research Center (VRC), Tampere University, Finland. Sera of individually collected whole blood samples obtained by the CROs were separated and stored at −20 °C and shipped frozen with temperature control and recording to VRC's research laboratory for analysis, together with requisite chain-of-custody documentation [35].

2.4.2. Humoral immune response

Titers of antigen-specific (NoV GI.4 and GII.4) IgG and IgA in sera obtained from the toxicity studies were quantified by enzyme linked immunosorbent assay (ELISA), as described [41,42].

2.4.3. Antibody NoV blocking potential

A pig gastric mucin (PGM)-based blocking assay [43,44] was used to determine the ability of rabbit immune sera from Studies B and C to block binding of NoV VLPs to the putative NoV receptors, human histo-blood group antigens (HBGA), as described [45].

2.4.4. Statistical evaluations

Results of serology were analyzed in Microsoft Excel® and the means and SEM of study groups were determined for descriptive evaluations. Selective comparisons were made using the Student's *t*-test and/or MedCalc Comparison of Means software v20, with rejection criteria at $p < 0.05$.

3. Results

3.1. Safety of rNV-2v as determined in toxicity studies in New Zealand White rabbits

The designs of the three animal studies to assess safety, tolerability and immune response to the vaccine are summarized in Table 1. The first or pilot study (Study A) was designed to assess acute toxicity and maximum tolerated dose (MTD) and was the first administration of rNV-2v to rabbits. The results helped inform the design, route of administration and dosing regimen in regulation-compliant Studies B and C. Summary results of each study follow.

3.1.1. Study A – Pilot (range-finding) toxicity study of rNV-2v in male rabbits

The objective of this pilot study was to assess the potential toxicity of rNV-2v (Lot DSDV092) after two administrations at doses of 200 or 400 μg total antigen per administration. Control solution (Lot DSDV097/2) consisted of sterile vehicle for injection. Vaccine or vehicle control was administered IM or SC to three young adult male rabbits per group. Group 1 and 2 rabbits received 0.5 ml of vehicle or 200 μg vaccine, respectively on Days 1 and 7 by IM injection to a single site. Group 3 and 4 rabbits received 400 μg vaccine on Days 1 and 7 by IM or SC injection, respectively, equally split across two injection sites.

General observations. The results showed no vaccine-related toxicological effects. All rabbits survived to their scheduled sacrifice on Day 10. No clinical observations were attributed to the vaccine, and three were no statistically significant body weight differences between vaccine-treated and vehicle-treated rabbits.

Local irritation. Pre-dose erythema was observed for each dose day in one to two rabbits from each group, including the vehicle control group, and was attributed to shaving of the dose site the day prior to injection. Slight edema at the dose site was observed in only one Group 3 (IM, high-dose) rabbit. Neither the severity nor the incidence of these events was dose limiting and the events were treatment- but not vaccine-related.

Clinical pathology evaluations. There were no toxicologically meaningful changes in any of the clinical pathology evaluations, including hematology, coagulation and clinical chemistry, with no differential findings between vaccine-treated and vehicle-treated animals on Days 3 and 10.

Necropsy observations and histopathology. There were no vaccine-related macroscopic findings. Following IM or SC injection, minimal inflammation was present in the subcutaneous tissue and minimum mononuclear cell infiltrates were present at the injection site in one to two animals of each vaccine group. These observations are consistent with responses to vaccination, were considered non-adverse and not toxicologically significant.

The MTD was not reached at the highest dose applied, and the NOAEL for either IM or SC administration was 400 μg per administration after two administrations. A summary of the findings from the pilot toxicity study are presented in Table 2.

3.1.2. Study B – Repeat-dose toxicity study of rNV-2v with recovery period in male and female rabbits

The objective of this GLP-compliant study was to assess the potential toxicity of rNV-2v (Lots DSDV112 and DSDV114) at a dose of 200 μg total antigen per administration, or vehicle (lot DSDV119), after three IM or SC administrations at 14-day intervals

Table 1
Toxicity studies with bivalent NoV vaccine rNV-2v in New Zealand White rabbits.

Study A: Pilot toxicity study

Group	Treatment[a]	Route	Dose Level (μg)[b]	Dose Conc. (μg/ml)	Volume (ml)[c]	No. Animals	Sacrifice Day 10
1	Vehicle	IM	0	0	0.5	3M	3M
2	rNV-2v	IM	200	400	0.5	3M	3M
3	rNV-2v	IM	400	400	0.5 x 2	3M	3M
4	rNV-2v	SC	400	400	0.5 x 2	3M	3M

[a]Animals were administered test articles twice, on Day 1 and Day 7.
[b]200 μg total mixed VLP per administration represented at the time the highest planned clinical dose.
[c]Rabbits were dosed with a constant volume regardless of body weight.

Study B: Repeat-dose toxicity study of Wild type GI.4 + Wild type GII.4 VLPs with recovery period (GLP)

Group	Treatment[a]	Route	Dose Level (μg)[b]	Dose Conc. (μg/ml)	Total No. Animals [c]	Main Sacrifice Day 32[c]	Recovery Sacrifice Day 50[c]
1	Vehicle	IM	0	0	10M/10F	5M/5F	5M/5F
2	rNV-2v	IM	200	400	10M/10F	5M/5F	5M/5F
3	Vehicle	SC	0	0	10M/10F	5M/5F	5M/5F
4	rNV-2v	SC	200	400	10M/10F	5M/5F	5M/5F

[a]In Study B recombinant bivalent NoV vaccine rNV-2v consisted of Wild type VLP1 (GI.4) and Wild type VLP2 (GII.4) mixed at a 1:1 ratio (w/w).
[b]Rabbits were administered 0.5 ml of vehicle or vaccine on Days 1, 15, and 29. Administration was to a single site to simulate the planned clinical administration.
[c]M, males; F, females.

Study C: Repeat-dose toxicity study of Mutant GI.4 + Wild type GII.4 VLPs with recovery period (GLP)

Group	Treatment[a]	Route	Dose Level (μg)[b]	Dose Conc. (μg/ml)	Total No. Animals[c]	Main Sacrifice Day 32[c]	Recovery Sacrifice Day 50[c]
1	Vehicle	IM	0	0	10M/10F	5M/5F	5M/5F
2	rNV-2v	IM	300	600	10M/10F	5M/5F	5M/5F

[a]In Study C recombinant bivalent NoV vaccine rNV-2v consisted of Mut VLP1 ($GI.4_{mut}$) and Wt VLP2 (GII.4) mixed at a 1:1 ratio (w/w).
[b]Rabbits were administered 0.5 ml of vehicle or vaccine on Days 1, 15, and 29. Administration was to a single site to simulate the planned clinical administration.
[c]M, males; F, females.

followed by a 3-week recovery period. The design of the study is shown in Table 1. Young adult male and female rabbits aged 7.5–9.5 months were administered test articles on Days 1, 15, and 29 as a 0.5 ml injection of either vehicle or a 400 μg/ml vaccine stock solution, regardless of body weight. Scheduled necropsies were performed on Days 32 (Main) and 50 (Recovery) to five rabbits/sex/group.

General observations. All animals survived to their scheduled sacrifice on Day 32 or Day 50. Moderate to slight swelling on Days 17–20 at or near the dose site (i.e., dose site or left scapular region) was observed in 2–3 female rabbits out of 10 each in Group 2 (IM vaccine), Group 3 (SC vehicle), and Group 4 (SC vaccine). This swelling began two days after the second SC administration in three vehicle control females and two vaccine-treated females and therefore is most likely a result of the SC injection itself and not the vaccine. No other clinical observations were considered to be related to administration of the rNV-rv2 vaccine by either the IM or SC route. There were no statistically significant differences in mean Group body weight throughout the study when the vaccine-treated animals in Group 2 (IM) and Group 4 (SC) were compared with their respective vehicle control group. Body temperature was not affected by vaccine administration by either the IM or SC route.

Local irritation. The mild erythema seen in the control Group 1 (IM vehicle) and Group 3 (SC vehicle) males was associated with shaving of the dose area. Erythema in females was generally of similar incidence and severity in control and treated rabbits after each injection. Dose site edema was infrequently seen in males and never seen in the females. All instances of local irritation resolved and were not considered adverse or dose limiting.

Ophthalmology. Ophthalmology examinations revealed no vehicle or vaccine related effects.

Clinical pathology evaluations. None of the hematology, clinical chemistry, or coagulation parameters that were evaluated on this study differed significantly between vaccine and control treatment groups in a toxicologically meaningful manner.

Necropsy observations and histopathology. There were no vaccine-related macroscopic findings. All macroscopic observations were considered incidental, did not correlate with microscopic findings, and were not considered toxicologically significant. No microscopic histology observations were considered test-article related or toxicologically significant and were either spontaneous or incidental and/or occurred with similar incidence across groups including the control groups. IM and SC administration of rNV-2v was associated with non-adverse localized inflammation at the injection site and non-adverse findings in the axillary (draining) lymph node. Findings in each of these tissues were partially reversible following a 3-week recovery period.

The NOAEL was 200 μg per administration after 3 administrations for either the IM or SC route. A summary of the findings from this repeat-dose toxicity study with recovery is included in Table 2.

3.1.3. Study C – Repeat-dose toxicity study of modified composition of rNV-2v with recovery period in male and female rabbits

The objective of this GLP-compliant study was to assess the potential toxicity of a new composition of rNV-2v (Lot DSDV142) at a dose of 300 μg total antigen (0.5 ml injection of 600 μg/ml dose concentration) per administration, or vehicle (lot DSDV146), after three IM administrations at 14-day intervals followed by a 3-week recovery period. The design of the study is shown in Table 1. This study aimed to complement Study B and followed generally the same experimental procedures; however, using (a) 50% higher dose of VLP total antigen (300 μg vs. 200 μg in Study B) and (b) Wild type GII.4 VLP as in Study B but admixed with Mutant GI.4 VLP ($GI.4_{mut}$) that instilled higher stability to the VLP particle during prolonged storage. Briefly, male and female rabbits 3–4 months of age were administered test articles IM on Days 1, 15, and 29. A 21-day recovery period after the last dose followed to assess reversibility of adverse effects. Scheduled necropsies were performed on Days 32 (Main) and 50 (Recovery) to five rabbits/sex/group.

Table 2
Toxicology tabulated summary.

Study number	Toxicity study		
	Study A	Study B	Study C
Parameter			
Testing facility	SRI International. Menlo Park, CA, USA	SRI International Menlo Park, CA, USA	LPT GmbH Hamburg, Germany
Toxicity study GLP compliance	Non-GLP	GLP	GLP
Species and sex of animals	NZW rabbit, M only	NZW rabbit, M+F	NZW rabbit, M+F
Number of animals per study group	3 M	5 M + 5 F	5 M + 5 F
Total number of animals on study	12	80	40
Route of administration	IM, SC	IM, SC	IM
rNV-2v vaccine composition evaluated	GI.4 + GII.4	GI.4 + GII.4	$GI.4_{mut}$ + GII.4
VLP GI.4:GII.4 antigen mix ratio (wt:wt)	1:1	1:1	1:1
Dose of total VLP antigen/ administration	200 μg SC 400 μg IM and SC	200 μg IM and SC	300 μg IM
Dosing schedule (day number)	D1, D7	D1, D15, D29	D1, D15, D29
Necropsy, main and recovery groups	D10	D32 Main; D50 Rec.	D32 Main; D50 Rec.
Results and observations			
Local tolerance	Minor reactogenicity	Minor reactogenicity	Minor reactogenicity
Systemic tolerance or toxicity			
Mortality	All animals survived	All animals survived	All animals survived
Behaviour; external appearance, faeces	No vaccine effects	No vaccine effects	No vaccine effects
Body weights and body weight gain	No vaccine effects	No vaccine effects	No vaccine effects
Food and water consumption	No vaccine effects	No vaccine effects	No vaccine effects
Body temperature changes post vaccine administration relative to vehicle	Transient minor increase	No vaccine effects	No vaccine effects
Haematology; coagulation time	No vaccine effects	No vaccine effects	No vaccine effects
Clinical chemistry	No vaccine effects	No vaccine effects	No vaccine effects
C-reactive protein	Not evaluated	Not evaluated	No vaccine effects
Ophthalmological and auditory exams	Not evaluated	No vaccine effects	No vaccine effects
Final examinations			
Macroscopic examination	No vaccine effects	No vaccine effects	No vaccine effects
Organ weights and weight ratios	Not evaluated	No vaccine effects	No vaccine effects
Bone marrow examination	Not evaluated	No vaccine effects	No vaccine effects
Histopathology	No vaccine effects	No vaccine effects	No vaccine effects
NOAEL for rNV-2v vaccine	400 μg IM or SC	200 μg IM or SC	300 μg IM

NZW, New Zealand White rabbit; M, male; F, female; IM, intramuscular; SC, subcutaneous; $GI.4_{mut}$, mutant version of GI.4 VLP.

General observations. All animals survived to the conclusion of the study. No test article-related influence was noted on the behavioral pattern, external appearance or the consistency of the faeces following vaccine administration. No significant differences were noted with respect to food consumption, body weight or body weight gain among the groups. Sporadic increases (males) and decreases (females) in body temperature were observed in some animals, but they were transient and not statistically significant.

Local irritation. No significant local intolerance reactions were noted during observations following IM administration of test articles on Days 1, 15 or 29.

Ophthalmology and auditory. No ophthalmological changes were noted on the *adnexa oculi* (i.e. lids, lacrimal apparatus), conjunctiva, cornea, anterior chamber, lens vitreous body and fundus (retina, optic disc). Likewise, the auditory examination did not reveal any test-article related changes.

Clinical pathology evaluations. Statistically significant differences in some biochemical parameters between the vaccine- and vehicle-treated groups were noted, but were sporadic in nature, occurred in a few animals including some in the control group, and were not considered to be vaccine-related or toxicologically meaningful.

C-Reactive protein. CRP increased significantly in only one male animal receiving vaccine; however, the value normalized upon subsequent evaluations and was considered sporadic and not toxicologically meaningful.

Bone marrow examination. During dissection fresh bone marrow was obtained from the *os femoris* (3 air-dried smears/animal followed by Pappenheim staining) of the first 5 animals/sex/group of Group 1 (vehicle) and Group 2 (IM vaccine). The myeloid-to-erythroid ratios were determined by cell differentiation (counting of 200 nuclei-containing cells). No test-article related influence was noted between vaccine and control groups with respect to bone marrow cellular profile.

Necropsy observations and histopathology. There were no significant test-article related macroscopic changes observed. Morphological changes considered to be spontaneous or incidental in nature were noted in only a few animals in both vaccine and control groups. There were no significant differences in organ weights between the groups. Statistically significant differences in relative organ weights between the vaccine group and the control group were few and not considered to be vaccine-related or toxicologically meaningful. Histopathological evaluation of rabbit organ tissues did not reveal any morphological lesions that were considered related to the test articles. Lymphohistiocytic inflammatory lesions in various organs were considered to be spontaneous organ changes and thus not test article-related. The minimal to mild inflammatory reactions with haemorrhages around the injection site were caused by the technical administration procedure. There was no difference between the groups.

The NOAEL for the IM route and schedule used in this study was 300 μg per administration after 3 administrations. A summary of the findings in this study are presented in Table 2.

3.2. Serology on samples from regulation-compliant toxicity Study B and Study C

3.2.1. Immune titers and VLP receptor binding blocking activity

Blood samples were collected and evaluated to determine the immune titers and the quality of the immune response induced by the original vaccine composition (Study B) and the improved, more stable version (Study C). The primary objective of serology was to determine the immunogenicity of the purified, plant produced norovirus GI.4 and GII.4 VLPs when administered via IM and SC routes in male and female rabbits after 3 vaccine administrations. In addition to quantifying NoV-specific IgG and IgA titers in rabbit sera, the ability of immune sera to block the binding of NoV VLPs to the putative NoV receptors, human histo-blood group antigens (HBGA), was assessed using a pig gastric mucin (PGM)-based homologous blocking assay, as described [43–45].

3.2.2. Immune responses in Study B

The humoral immune response to 200 μg per administration of Wild type GI.4 and Wild type GII.4 divalent VLP vaccine is summa-

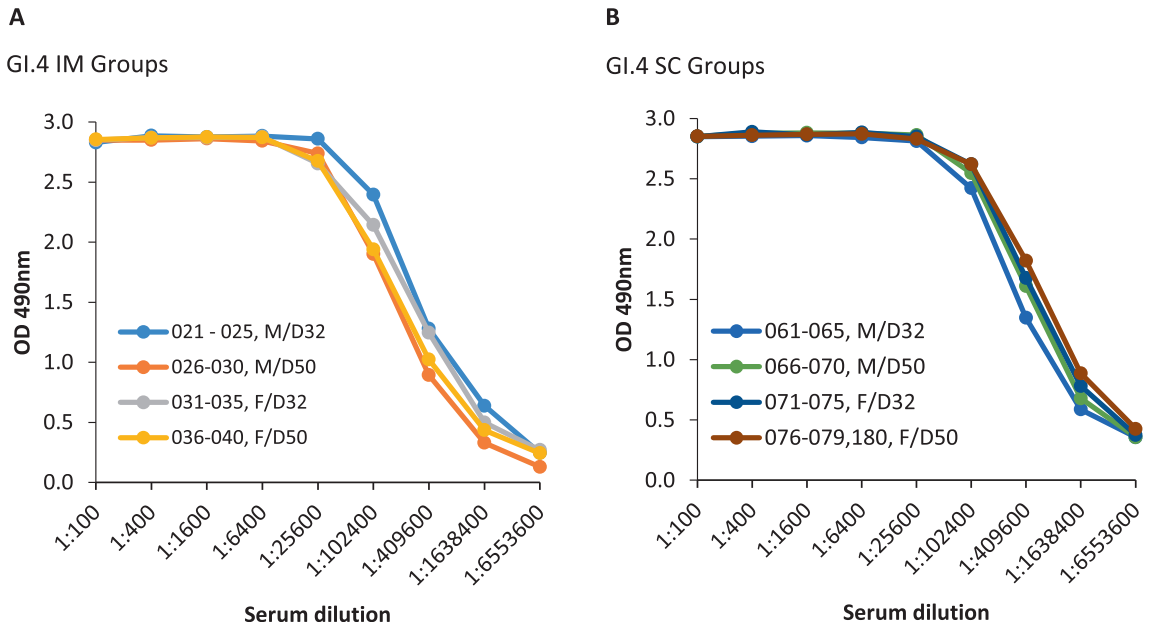

Fig. 1. Wild type GI.4-specific mean IgG titers of the IM and SC subgroups from Study B. Terminal sera of individual animals receiving 3 administrations of test article at 200 μg per administration via the intramuscular (A) or subcutaneous (B) administration route were analysed individually; shown are mean OD values of each subgroup. IM, intramuscular; SC, subcutaneous; M, male; F, female; D32, sera from Main group sacrifice on D32; D50, sera from Recovery group sacrifice on Day 50.

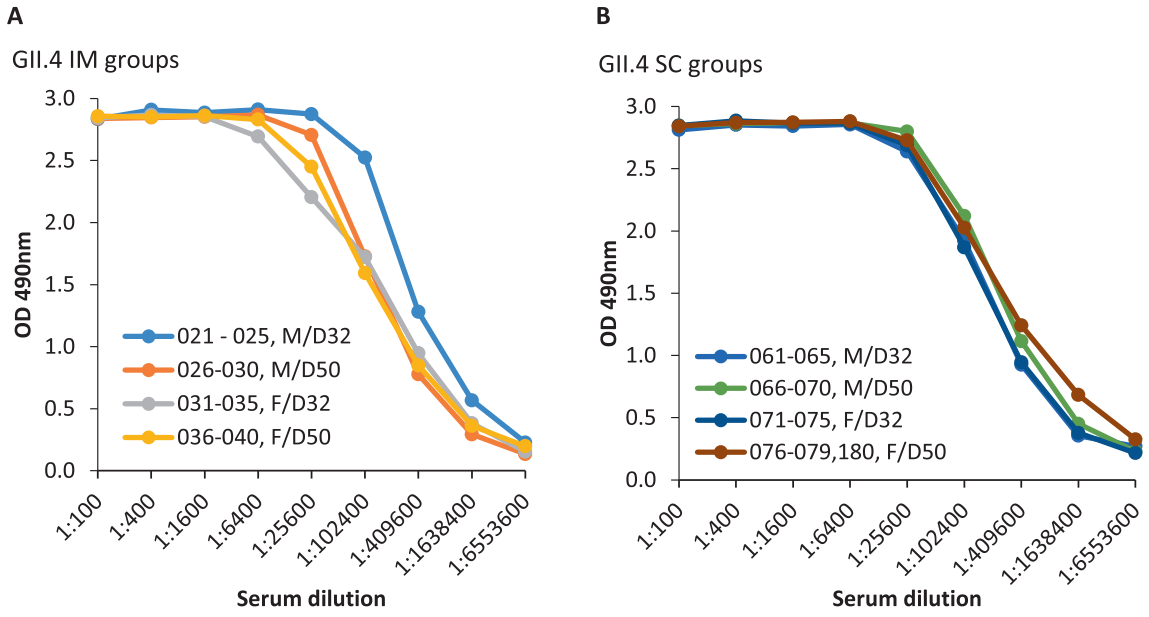

Fig. 2. Wild type GII.4-specific mean IgG titers of the IM and SC subgroups from Study B. Terminal sera of individual animals receiving 3 administrations of test article at 200 μg per administration via the intramuscular (A) or subcutaneous (B) administration route were analysed individually; shown are mean OD values of each subgroup. IM, intramuscular; SC, subcutaneous; M, male; F, female; D32, sera from Main group sacrifice on D32; D50, sera from Recovery group sacrifice on Day 50.

rized in Fig. 1 and Fig. 2. The results showed that the Wt rNV-2v composition is highly immunogenic in both male and female rabbits in the absence of a co-administered adjuvant. Although IgG titers to GI.4 and GII.4 VLPs were similar at Days 32 and 50 post immunization, higher GI.4-specific IgA titers were measured (Fig. 3). To the contrary, although very high blocking antibodies were observed to each VLP, a stronger response was observed to GII.4 (Fig. 4 and Fig. 5).

3.2.3. Immune responses in Study C

Serum IgG titers elicited by IM immunization with 300 μg per administration of non-adjuvanted mixed Mutant GI.4$_{mut}$ and Wt GII.4 VLPs were both high and at the same level at Days 32 (3 days after last dose) and 50 (21 days after last dose), as summarized in Fig. 6. Considerably higher GI.4-specific IgA levels than GII.4-specific IgA titers were detected, as shown in Fig. 7. High titers of

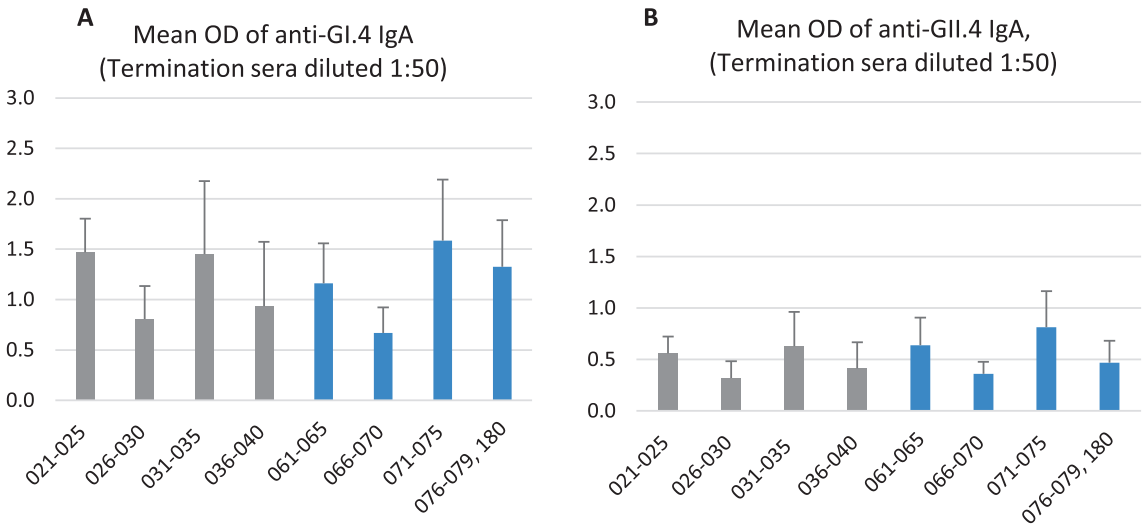

Fig. 3. Vaccine-specific IgA titers at termination of the IM and SC subgroups from Study B. Terminal sera of individual animals receiving test article via intramuscular (IM, grey bars, animals 021–040) or subcutaneous (SC, blue bars, animals 061–079 and 180) routes were analysed for Wild type GI.4-specific (A) and Wild type GII.4-specific (B) IgA at the dilution 1:50; shown are mean OD_{490} nm of each subgroup with standard errors of the mean. At D50, only the IgA response to GI.4 in females by SC route was greater than in males ($p < 0.05$). There were no other sex-related statistical differences between matched pairs via either route of administration. Animals 021–025 and 061–065, Main Study males D32; animals 026–30 and 066–070, Recovery Males D50; animals 031–035 and 071–075, Main Study females D32; animals 036–040 and 076–079, 180, Recovery females D50.

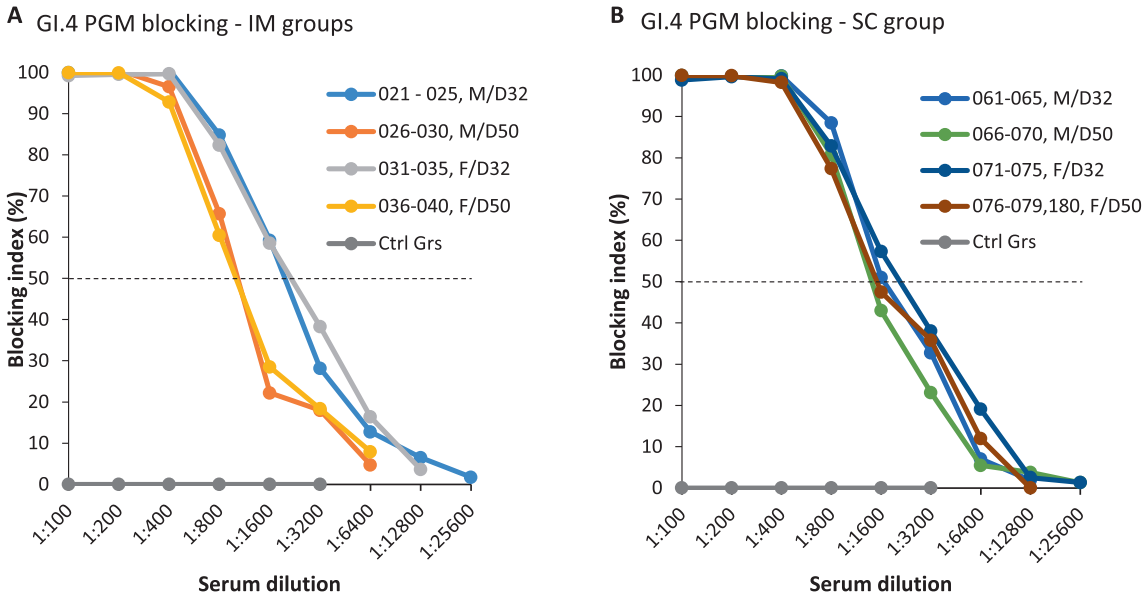

Fig. 4. GI.4-specific blocking IgG titers at termination of the IM and SC subgroups from Study B. Sera of rNV-2v-immunized rabbits collected at termination were pooled according to gender (M, males, F, females) and Termination Day 32 (Main sacrifice, D32) or Day 50 (Recovery period sacrifice, D50) for analysis of blocking of GI.4 VLP binding in PGM-based assay; blocking titers of rabbits immunized via intramuscular (A) or subcutaneous (B) route were titrated two-fold starting at 1:100; dashed line represents 50% blocking index. The blocking index (%) was calculated as 100% - [(OD wells with VLP-serum mix/OD maximum binding OD) × 100%]. Ctrl Grs, control groups.

blocking IgG antibodies were observed to both $GI.4_{mut}$ and Wt GII.4 VLPs, as shown in Fig. 8.

4. Discussion

The two rNV-2v VLP vaccine compositions evaluated in this program were manufactured in plants with magnICON® technology and showed high safety and tolerability in a rabbit model. These results echo previous preclinical and clinical results for other vaccines produced via the same system [31,46]. In the three studies described, the vaccines administered IM or SC at doses ranging from 200 to 400 μg per administration were very well tolerated and induced NoV genogroup/genotype-specific immune responses.

The lack of well-characterized NoV infection models *in vitro* and *in vivo* has limited the development of vaccines and therapeutics against human NoV (reviewed by Todd and Tripp, 2019 [47]). Consequently, there remains a need for nonclinical tools to aid in human NoV research and product development. Lacking other robust tools, immunogenicity against target NoV antigens has been the main endpoint for assessing induction of protection, including the ability of polyclonal sera to block the binding of NoV to its putative receptors (HBGAs).

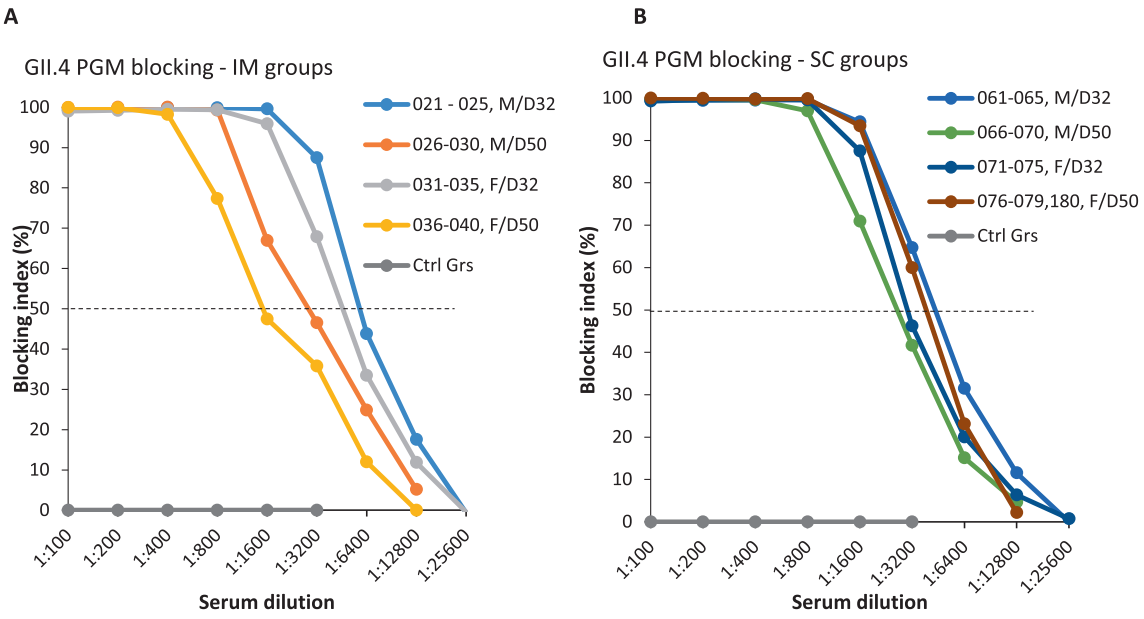

Fig. 5. GII.4-specific blocking IgG titers at termination of the IM and SC subgroups from Study B. Sera of immunized rabbits collected at termination were pooled according to gender (M, males, F, females) and Termination Day 32 (Main sacrifice, D32) or Day 50 (Recovery period sacrifice, D50) for analysis of blocking of GII.4 VLP binding in PGM-based assay, blocking titers of rabbits immunized via intramuscular (A) or subcutaneous (B) route were titrated two-fold starting at 1:100; dashed line represents 50% blocking index. Blocking index (%) was calculated as 100% - [(OD wells with VLP-serum mix/OD maximum binding OD) × 100%]. Ctrl Grs, control groups.

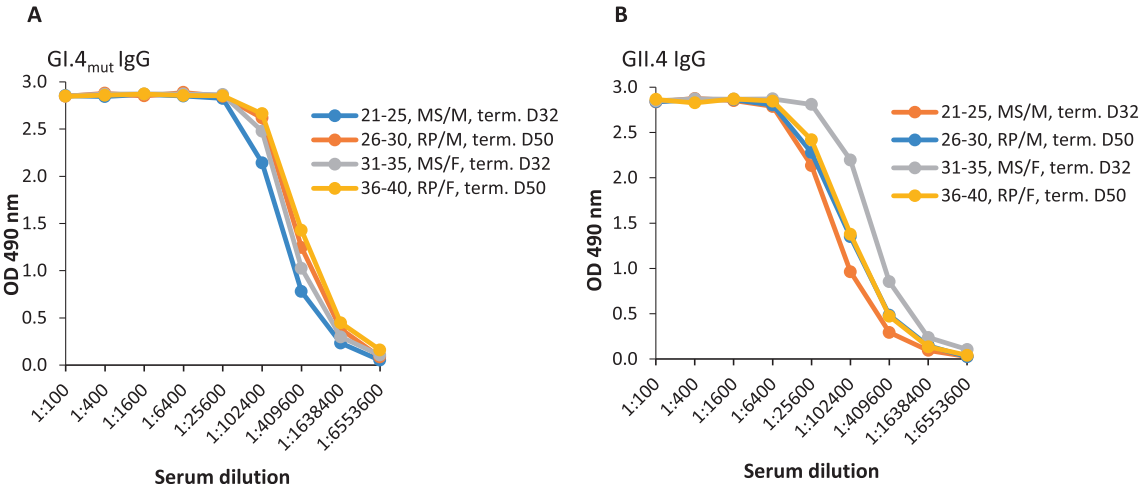

Fig. 6. Mutant GI.4$_{mut}$ + Wild type GII.4 VLP-specific mean IgG titers at termination of the IM groups from Study C. Antibodies in terminal sera of vaccinated animals were analyzed individually against mixed Mutant GI.4$_{mut}$ (A) and Wild type GII.4 (B) VLP's by ELISA; shown are mean OD values of each subgroup according to Main Study (MS, sera from Main sacrifice group, D32), or Recovery Period (RP, sera from Recovery Period group, D50) and gender (M, males; F, females).

Results of exploratory immunogenicity studies in mice with rNV-2v, not reported here and conducted prior to initiation of our safety program, helped define the quality of the immune response and guide the design and administration schedule in the rabbit studies. For example, murine studies helped characterize the antigen-specific IgG subtypes induced by Wild type rNV-2v vaccination as being balanced for both GI.4- and GII.4-specific Th2/Th1 responses, with a skew of the response to both antigens slightly to Th2-type (i.e. IgG1) with higher doses (data not shown). In mouse studies we also assessed the cellular immune response to the VLPs by ELISPOT assay, measuring the production of IFN-γ by exposed mouse splenocytes [42,48]. The cellular response was low (data not shown) relative to the humoral response; therefore, cellular response was not assessed in the rabbit studies. However, besides cross-reactive antibodies the T-cell-mediated immunity might provide the key for eliciting broad and protective immune responses after vaccination in humans [32]. Therefore, cell-mediated responses will be assessed in details in our Phase I clinical study.

Serology on samples from Studies B and C revealed that the norovirus GI.4 and GII.4 VLPs induced strong and specific immune responses in both male and female rabbits in the absence of co-administered adjuvants. IgG titers in Study B (Wild type GI.4 and GII.4 VLPs) and Study C (Mutant GI.4 $_{mut}$ and Wt GII.4 VLPs) were similar. In Study B, very high blocking antibodies were observed to each VLP but a relatively stronger response was observed to GII.4. In contrast, in Study C equally high titers of blocking IgG antibodies were observed to both GI.4 $_{mut}$ and GII.4 VLPs. These results

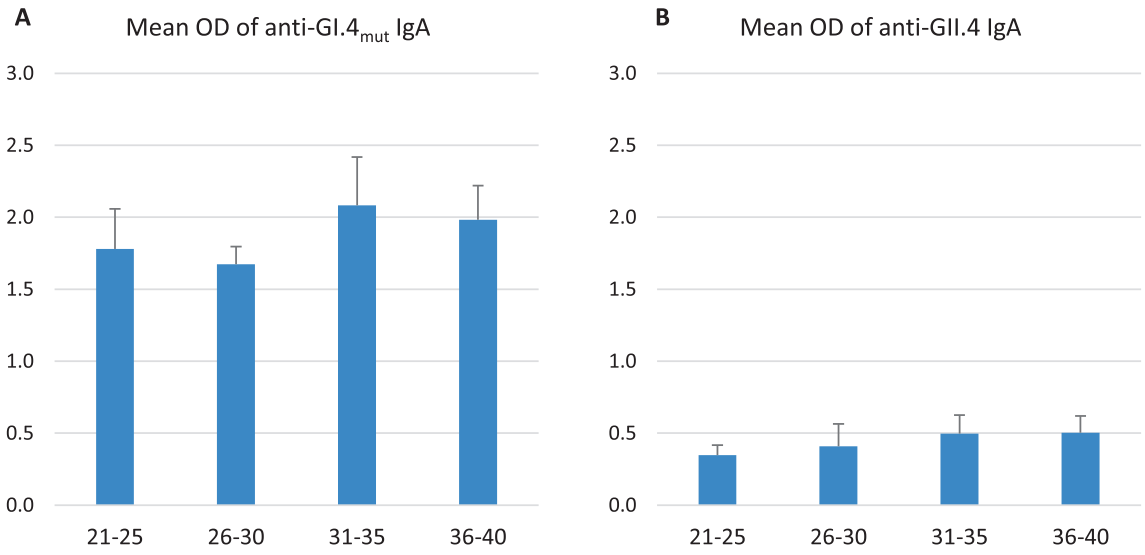

Fig. 7. Mutant GI.4$_{mut}$ + Wild type GII.4 VLP-specific mean IgA titers at termination of the IM groups from Study C. Terminal sera of individual animals that received bivalent VLP vaccine via the IM route in Study C were analyzed for Mutant GI.4$_{mut}$-specific (A) and Wild type GII.4-specific (B) IgA at the dilution 1:50; shown are mean OD_{490} nm of each subgroup with standard errors of the mean. There were no sex-related statistical differences between D32 or D50 matched pairs. Animals 21–25, Main Study males D32; animals 26–30, Recovery Period males, D50. Animals 31–35, Main Study females, Day 32; animals 36–40, Recovery Period females, Day 50.

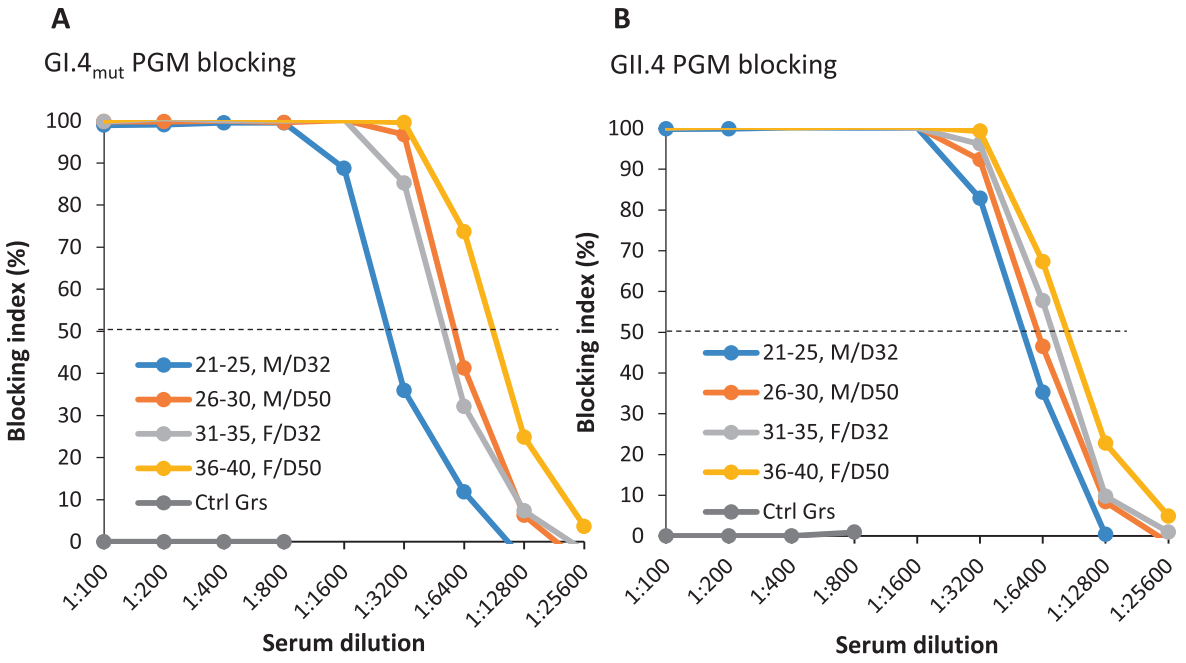

Fig. 8. Mutant GI.4$_{mut}$- and Wild type GII.4-specific VLP blocking IgG titers at termination of the IM subgroups from Study C. Sera of norovirus VLP-immunized rabbits collected at termination were pooled according to gender, males (M) or females (F) and Termination Day 32 (D32, Main Study) or Day 50 (D50, Recovery Period) for analysis of blocking of Mutant GI.4$_{mut}$ (A) or Wild type GII.4 (B) VLP binding in PGM-based assay. Blocking titers of rabbits immunized via the IM route were titrated two-fold starting at 1:100; dashed line represents 50% blocking index. The blocking index (%) was calculated as 100% - [(OD wells with VLP-serum mix/OD maximum binding OD) × 100%]. Ctrl Grs, control groups.

suggest that perhaps the mutation introduced to the GI.4$_{mut}$ VLP that confers higher stability to the particle may also confer a potential for inducing higher blocking titers than the previous Wild type composition.

The doses selected for the animal studies were based on the anticipated clinical dose range of 50–300 μg per administration given as two administrations 28 days apart (prime/boost). These doses and administration schedule were derived from results of clinical studies with baculovirus-produced NoV VLP vaccines [20,21,23], which suggested that these dose ranges and scheduling were adequate to induce the desired immune response. In our studies blocking antibodies to GI.4 and GII.4 in males and females were significantly ($p < 0.0001$) and uniformly higher in Study C (300 μg/dose) than in Study B (200 μg/dose) via the IM route, suggesting a dose–response effect and justifying the higher dose and IM route for clinical evaluation. Although the inclusion of adjuvants might enable the administration of lower doses of VLPs, adjuvants add complexity to formulations and could increase the

incidence and/or severity of adverse events [21,49]. Although IM and SC administration proved equally effective routes for immunization, we selected IM administration of the improved composition of rNV-2v (Study C) at 300 μg/dose for initial clinical evaluation because IM is a more accepted and reproducible route for vaccine delivery, especially in children and the elderly.

5. Conclusions

The results of the nonclinical studies summarized herein showed excellent immunogenicity and safety *in vivo* and mechanistic efficacy *in vitro* for the plant-made rNV-2v norovirus preventative vaccine. A maximum tolerated dose (MTD) was not reached, and the NOAEL was determined to be 200 μg/administration of the original VLP composition via the SC and IM routes, and 300 μg per administration of the improved VLP composition via the IM route, both evaluated using an n + 1 vaccine administration protocol [38]. Both vaccine compositions induced strong humoral immune responses in the absence of adjuvant as well as high titers of blocking antibodies, the latter considered a surrogate indicator of viral infection-blocking potential to putative NoV HBGA receptors. Taken together, these results support first use of the rNV-2v vaccine in a Phase I clinical study.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: DT was retained as a consultant by the funding source company to assist in the design and management of toxicity studies, analysis of results and development of the manuscript. MM, KT and VB were engaged by the funding source company to conduct immunological studies. AD, FT, FJ and VK are employees of a wholly owned subsidiary of the funding source company.

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Role of funding source

Icon Genetics manufactured and provided test articles for the studies, participated in the design of immunogenicity studies and managed operational aspects of the program, including monitoring data collection, statistical analyses, and writing of the report.

Contributors

All authors attest that they meet the ICMJE criteria for authorship. DT and FT contributed to the conceptual design of the toxicity studies and/or data acquisition and interpretation, including statistical analyses. VB, MM and KT contributed to the conceptual design and performance of the serological evaluations for immunogenicity, including data acquisition, interpretation and statistical analyses. AD, FT and FJ were responsible for manufacturing vaccine and control vehicle test articles for these studies. DT developed the initial draft of the manuscript and integrated the final version. All authors contributed to critical revision of this report, approved the final version, and are accountable for its accuracy and integrity. VK was overall program supervisor and provided management and coordination for the studies reported here.

Data Availability Statement

The dataset for toxicity studies is voluminous and contains proprietary information; consequently, only the results of safety analyses are included here. All other data generated or analysed during this study are included in this published article.

References

[1] Bresee J, Widdowson M-A, Monroe S, Glass R. Foodborne viral gastroenteritis: challenges and opportunities. Clin Infect Dis 2002;35(6):748–53. https://doi.org/10.1086/342386.
[2] Debbink K, Lindesmith LC, Baric RS. The state of norovirus vaccines. Clin Infect Dis 2014;58(12):1746–52. https://doi.org/10.1093/cid/ciu120.
[3] Patel MM, Hall AJ, Vinjé J, Parashar UD. Noroviruses: a comprehensive review. J Clin Virol 2009;44(1):1–8. https://doi.org/10.1016/j.jcv.2008.10.009.
[4] Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. N Engl J Med 2009;361(18):1776–85.
[5] Weber DJ, Rutala WA, Miller MB, Huslage K, Sickbert-Bennett E. Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, Clostridium difficile, and Acinetobacter species. Am J Infect Control 2010;38(5):S25–33. https://doi.org/10.1016/j.ajic.2010.04.196.
[6] Bert F, Scaioli G, Gualano MR, Passi S, Specchia ML, Cadeddu C, et al. Norovirus outbreaks on commercial cruise ships: a systematic review and new targets for the public health agenda. Food Environ Virol 2014;6(2):67–74. https://doi.org/10.1007/s12560-014-9145-5.
[7] Fankem SL, Boone SA, Gaither M, Gerba CP. Outbreak of norovirus illness in a college summer camp: impact of cleaning on occurrence of norovirus on fomites. J Environ Health 2014;76:20–6.
[8] Mitchell C, Meredith P, Richardson M, Greengross P, Smith GB. Reducing the number and impact of outbreaks of nosocomial viral gastroenteritis: time-series analysis of a multidimensional quality improvement initiative. BMJ Quality Safety 2016;25(6):466–74. https://doi.org/10.1136/bmjqs-2015-004134.
[9] Esposito S, Principi N. Norovirus vaccine: priorities for future research and development. Front Immunol 2020;11. .
[10] Giersing BK, Vekemans J, Nava S, Kaslow DC, Moorthy V. Report from the World Health Organization's third Product Development for Vaccines Advisory Committee (PDVAC) meeting, Geneva, 8–10th June 2016. Vaccine 2019;37(50):7315–27.
[11] Tan M, Jiang X. Histo-blood group antigens: a common niche for norovirus and rotavirus. Expert Rev Mol Med 2014;16. https://doi.org/10.1017/erm.2014.2.
[12] Clausen H, Hakornori S-I, ABH and Related Histo-Blood Group Antigens. Immunochemical differences in carrier isotypes and their distribution. 1989;56.
[13] Marionneau S, Cailleau-Thomas A, Rocher J, Le Moullac-Vaidye B, Ruvoën N, Clément M, et al. ABH and Lewis histo-blood group antigens, a model for the meaning of oligosaccharide diversity in the face of a changing world. Biochimie 2001;83(7):565–73. https://doi.org/10.1016/S0300-9084(01)01321-9.
[14] Chhabra P, de Graaf M, Parra GI, Chan M-W, Green K, Martella V, et al. Updated classification of norovirus genogroups and genotypes. J Gen Virol 2019;100(10):1393–406. https://doi.org/10.1099/jgv.0.001318.
[15] Kroneman A, Vega E, Vennema H, Vinjé J, White PA, Hansman G, et al. Proposal for a unified norovirus nomenclature and genotyping. Arch Virol 2013;158(10):2059–68. https://doi.org/10.1007/s00705-013-1708-5.
[16] Vega E, Barclay L, Gregoricus N, Shirley SH, Lee D, Vinje J. Genotypic and epidemiologic trends of norovirus outbreaks in the united states, 2009 to 2013. J Clin Microbiol 2014;52(1):147–55. https://doi.org/10.1128/JCM.02680-13.
[17] Chan MCW, Shan Kwan H, Chan PKS. Structure and genotypes of noroviruses. The Norovirus, Elsevier; 2017. doi: 10.1016/B978-0-12-804177-2.00004-X.
[18] Hardy ME. Norovirus protein structure and function. FEMS Microbiol Lett 2005;253:1–8. https://doi.org/10.1016/j.femsle.2005.08.031.
[19] Riddle MS, Walker RI. Status of vaccine research and development for norovirus. Vaccine 2016;34(26):2895–9. https://doi.org/10.1016/j.vaccine.2016.03.077.
[20] Treanor JJ, Atmar RL, Frey SE, Gormley R, Chen WH, Ferreira J, et al. A novel intramuscular bivalent norovirus viruslike particle vaccine candidate-reactogenicity, safety, and immunogenicity in a phase 1 trial in healthy adults. J Infect Dis 2014;210(11):1763–71. https://doi.org/10.1093/infdis/jiu337.

[21] Atmar RL, Baehner F, Cramer JP, Song E, Borkowski A, Mendelman PM. Rapid responses to 2 virus-like particle norovirus vaccine candidate formulations in healthy adults: a randomized controlled trial. J Infect Dis 2016;214(6):845–53.
[22] Leroux-Roels G, Cramer JP, Mendelman PM, Sherwood J, Clemens R, Aerssens A, et al. Safety and immunogenicity of different formulations of norovirus vaccine candidate in healthy adults: a randomized, controlled, double-blind clinical trial. J Infect Dis 2018;217(4):597–607. https://doi.org/10.1093/infdis/jix572.
[23] Sherwood J, Mendelman PM, Lloyd E, Liu M, Boslego J, Borkowski A, et al. Efficacy of an intramuscular bivalent norovirus GI.1/GII.4 virus-like particle vaccine candidate in healthy US adults. Vaccine 2020;38(41):6442–9. https://doi.org/10.1016/j.vaccine.2020.07.069.
[24] Masuda T, Lefevre I, Mendelman P, Sherwood J, Bizjajeva S, Borkowski A. Immunogenicity of takeda's bivalent virus-like particle (VLP) norovirus vaccine (NoV) candidate in children from 6 months up to 4 years of age. Open Forum Infectious Diseases 2018;5. https://doi.org/10.1093/ofid/ofy210.1929.
[25] Loh HS, Green BJ, Yusibov V. Using transgenic plants and modified plant viruses for the development of treatments for human diseases. Curr Opin Virol 2017;26:81–9. https://doi.org/10.1016/j.coviro.2017.07.019.
[26] Tusé D, Nandi S, McDonald KA, Buyel JF. The emergency response capacity of plant-based biopharmaceutical manufacturing-what it is and what it could be. Front Plant Sci 2020;11. .
[27] Dubey KK, Luke GA, Knox C, Kumar P, Pletschke BI, Singh PK, et al. Vaccine and antibody production in plants: developments and computational tools. Briefings Funct Genomics 2018;17(5):295–307. https://doi.org/10.1093/bfgp/ely020.
[28] Rosales-Mendoza S, Márquez-Escobar VA, González-Ortega O, Nieto-Gómez R, Arévalo-Villalobos JI. What does plant-based vaccine technology offer to the fight against COVID-19? Vaccines 2020;8. doi: 10.3390/vaccines8020183.
[29] Takeyama N, Kiyono H, Yuki Y. Plant-based vaccines for animals and humans: recent advances in technology and clinical trials. Ther Ad Vaccines 2015;3(5-6):139–54. https://doi.org/10.1177/2051013615613272.
[30] Rybicki EP. Plant-produced vaccines: promise and reality. Drug Discov Today 2009;14(1-2):16–24.
[31] Tusé D, Ku N, Bendandi M, Becerra C, Collins R, Langford N, et al. Clinical safety and immunogenicity of tumor-targeted, plant-made Id-KLH conjugate vaccines for follicular lymphoma. Biomed Res Int 2015;2015:1–15. https://doi.org/10.1155/2015/648143.
[32] Malm M, Diessner A, Tamminen K, Liebscher M, Vesikari T, Blazevic V. Rotavirus VP6 as an adjuvant for bivalent norovirus vaccine produced in Nicotiana benthamiana. Pharmaceutics 2019;11(5):229. https://doi.org/10.3390/pharmaceutics11050229.
[33] Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y. Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 2005;23(6):718–23. https://doi.org/10.1038/nbt1094.
[34] Klimyuk V, Pogue G, Herz S, Butler J, Haydon H. Production of recombinant antigens and antibodies in Nicotiana benthamiana using 'Magnifection' Technology: GMP-compliant facilities for small- and large-scale manufacturing. 2012. doi: 10.1007/82_2012_212.
[35] EMEA CPMP/SWP465/95. Note for guidance on preclinical pharmacological and toxicological testing of vaccines; 1997.
[36] World Health Organization. WHO guidelines on nonclinical evaluation of vaccines; 2005.
[37] FDA. Guidance for Industry: Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications; 2006.
[38] International Conference on Harmonization. ICH M3(R2) guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals; 2009.
[39] International Conference on Harmonization. ICH guideline S6(R1) – preclinical safety evaluation of biotechnology-derived pharmaceuticals; 2011.
[40] Draize J, Woodard G, Calvery H. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Therap 1944;82:377–90.
[41] Blazevic V, Lappalainen S, Nurminen K, Huhti L, Vesikari T. Norovirus VLPs and rotavirus VP6 protein as combined vaccine for childhood gastroenteritis. Vaccine 2011;29(45):8126–33. https://doi.org/10.1016/j.vaccine.2011.08.026.
[42] Tamminen K, Huhti L, Koho T, Lappalainen S, Hytönen VP, Vesikari T, et al. A comparison of immunogenicity of norovirus GII-4 virus-like particles and P-particles. Immunology 2012;135(1):89–99. https://doi.org/10.1111/j.1365-2567.2011.03516.x.
[43] Malm M, Heinimäki S, Vesikari T, Blazevic V. Rotavirus capsid VP6 tubular and spherical nanostructures act as local adjuvants when co-delivered with norovirus VLPs. Clin Exp Immunol 2017;189(3):331–41. https://doi.org/10.1111/cei.12977.
[44] Lindesmith LC, Debbink K, Swanstrom J, Vinje J, Costantini V, Baric RS, et al. Monoclonal antibody-based antigenic mapping of norovirus GII.4-2002. J Virol 2012;86(2):873–83.
[45] Uusi-Kerttula H, Tamminen K, Malm M, Vesikari T, Blazevic V. Comparison of human saliva and synthetic histo-blood group antigens usage as ligands in norovirus-like particle binding and blocking assays. Microbes Infect 2014;16(6):472–80. https://doi.org/10.1016/j.micinf.2014.02.010.
[46] Tusé D. Safety of plant-made pharmaceuticals: product development and regulatory considerations based on case studies of two autologous human cancer vaccines. Human Vaccines 2011;7(3):322–30. https://doi.org/10.4161/hv.7.3.14213.
[47] Todd K, Tripp R. Human norovirus: experimental models of infection. Viruses 2019;11(2):151. https://doi.org/10.3390/v11020151.
[48] Malm M, Tamminen K, Vesikari T, Blazevic V. Type-specific and cross-reactive antibodies and T cell responses in norovirus VLP immunized mice are targeted both to conserved and variable domains of capsid VP1 protein. Mol Immunol 2016;78:27–37. https://doi.org/10.1016/j.molimm.2016.08.009.
[49] Levie K, Gjorup I, Skinhøj P, Stoffel M. A 2-dose regimen of a recombinant hepatitis B vaccine with the immune stimulant AS04 compared with the standard 3-dose regimen of engerix-B in healthy young adults. Scand J Infect Dis 2002;34(8):610–4. https://doi.org/10.1080/00365540110080881.