PROJECT FINAL REPORT

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1. Final publishable summary report

1.1 An executive summary

The NASANVAC project was undertaken in response to the need to develop an effective user friendly, heat stable vaccine to control the highly pathogenic form of avian influenza virus (H5N1) which has the potential to cause another global influenza pandemic. A significant number of deliverables have been achieved, despite the challenges that arose during the project. Crucially, the GMP surface subunit antigen of H5N1 strain NIBRG-14 raised in eggs was promptly sourced from a new supplier (Novartis) and an MTA signed permitting it's use in preclinical studies after the first supplier (Solvay) could not supply the antigen subsequent to changes in corporate structure and management. A bridging study in the mouse confirmed that the Novartis antigen was sufficiently similar to the egg-based antigen originally sourced from Solvay and thus, would be suitable for use in preclinical and clinical studies.

Formulation development work has confirmed that liquid and powder formulations of H5N1 adjuvanted with the biopolymer chitosan are stable and suitable for clinical use . Serological assays (surrogate correlates of protection) in mouse and ferret sera were initially established to enable the evaluation of vaccine efficacy. Preclinical studies in mice and ferrets have confirmed that both chitosan glutamate (CSN) and trimethyl-chitosan (TM-CSN) as solutions are efficacious in augmenting serology responses. Mouse studies further indicated that it is possible to enhance the humoral and cellular responses obtained after administering two doses of an intranasal CSN-adjuvanted vaccine by first priming with a subcutaneous injection of antigen. CSN was also shown to be a safe mucosal adjuvant and significantly no pro-inflammatory response was observed in the hypothalamus of the brain (allaying some concerns over Bell's palsy-like adverse effects which have manifested in clinical practice after administration of some intranasal influenza vaccines). Based on collective findings, the consortium initially selected a CSN liquid formulation to proceed to a Phase I proof-of-principle clinical study, which could potentially be supported without the need for further preclinical toxicology. Following the decision by Novartis not to supply antigen for clinical use a definitive challenge study to establish efficacy was conducted in the ferret model using liquid vaccine formulations based on both CSN and TM-CSN. A significant aspect of this study was as a comparison of intratracheal and intranasal challenge, the first time such studies have been attempted.

In this project we planned to develop a prototype nasal H5N1vaccine, which could be delivered through a commercial nasal delivery applicator and which showed a positive immunogenicity response in humans; thereby forming the basis for further exploitation of the project results in terms of Phase II/III clinical trials. The specific objective of conducting a Phase I clinical trial was not met due to non-availability of suitable GMP antigen. Nevertheless, the excellent challenge study data obtained in the ferret should allow strategic decisions to be made over further development of an intranasal vaccine. The data clearly demonstrated protective immunity against lethal H5N1 A/Vietnam/1194/2004 virus infection following intranasal vaccination adjuvanted with both CSN and TM-CSN, though the latter appeared to be more efficacious in the recognised model of human influenza, the ferret. In view of the excellent safety profile of chitosan, these data are highly encouraging given that traditionally vaccine adjuvants tend to be relatively more toxic moieties. Thus, the risk : benefit ratio for a chitosan-based vaccine may be favourable when compared with that of more potent adjuvants. A H5N1 pandemic could have a major impact on public services, making self reliant treatment and management of the disease mandatory. The NASPANVAC project has clearly demonstrated

the potential to develop a nasal vaccine which could be self administered and also enable rapid mass vaccination with minimum aid from specialist healthcare providers.

1.2 A summary description of project context and objectives

Annual outbreaks of influenza A and B, and pandemics of influenza A, are responsible for substantial mortality and morbidity, particularly in high-risk groups including the elderly and those with chronic underlying medical conditions. Vaccines are the most effective means of defence against influenza to prevent infection and control its spread. Despite the availability of effective parenteral vaccines, influenza still incurs considerable medical and socio-economic costs. Barriers limiting vaccine uptake include the intramuscular route of injection, and the perception of vaccine ineffectiveness. Furthermore the capacity to manufacture to meet a global demand is limited.

Delivery of an influenza vaccine by the nasal route, targeted to the mucosal site of virus entry and principal location of its replication, offers potential advantages over parenteral delivery of the vaccine. While conventional intramuscular influenza vaccines are effective at inducing serum IgG neutralizing antibodies, they are poor at stimulating mucosal IgA. Mucosal IgA exhibits both heterosubtypic cross-reactivity to influenza virus strains and potent immunological memory, properties that offer potential wider protection against variants of influenza that have drifted antigenically from the vaccine strain.

Immunity to influenza infection in man is multifactorial, and the precise contribution of innate immunity, serum IgG to haemagglutinin (HA) and neuraminidase (NA), local secretory IgA and Th1- and Th2-type immune responses has been difficult to ascertain. Live virus vaccines and inactivated vaccine induce different arms of the immune response, with sufficient antibody in either serum or nasal secretions being capable of conferring resistance.

Stimulation of both local and systemic immune responses following influenza vaccination may enhance vaccine efficacy, particularly among the elderly, who exhibit age-related reductions in immunity to vaccination. In addition, induction of mucosal responses could be important if stockpiled vaccine, prepared in advance of a pandemic, differs significantly antigenically from the emergent pandemic strain or subtype. The simpler intranasal route of administration also offers the possibility for self-administration and could reduce the healthcare costs of delivery and increase vaccine uptake.

This project is being undertaken in response to the need to develop an effective user friendly, heat stable vaccine to control the highly pathogenic form of avian influenza virus (H5N1) which has the potential to cause another global influenza pandemic.

The principal challenges to pandemic vaccine deployment are (a) avian haemagglutinin (H5 and H7) is poorly immunogenic compared to H1 and H3 haemagglutinin; (b) there is limited manufacturing capacity for subunit vaccines; (c) H5 and other avian influenza strains are continuously evolving limiting the usefulness of pandemic vaccine stockpiling; and (d) dose-sparing strategies for the antigen component of the vaccine are not optimised.

The overall objective of this programme was to develop a nasal avian influenza vaccine using ChiSys® (chitosan), which is a chitosan-based vaccine delivery technology of Archimedes Development Ltd. The most effective way of controlling a pandemic flu would be via the nasal route as this route has the advantage of allowing the generation of both systemic and mucosal immune responses. Mucosal antibodies may facilitate control of the virus at its point of entry. In comparison, the currently used injectable vaccine cannot induce mucosal immune

responses. The nose is an excellent route for vaccination as the nasal cavity has a large surface area available for absorption, with a highly vascularized underlying epithelium as well as its own lymphoid tissue. Intranasal vaccination has several advantages over parenteral delivery:

- combats the pathogen both at its point of entry and also systemically
- avoids the need for injection and for purchase and safe disposal of syringes
- eliminate the risk of HIV transmission through re-use of syringes or accidental contact with body fluids for example through needle-stick injuries, especially in the developing world
- have greater public compliance
- well-suited to rapid mass global vaccination programmes

The scientific objectives of the project were:

- To develop an intranasal influenza vaccine that induces both systemic and mucosal immunity
- To develop an influenza vaccine that induces a substantial level of cross-immunity against a drifted (diverse) strain of H5
- To develop an influenza vaccine that is effective at low dose, to meet the global demands from limited vaccine stockpiles
- To develop an influenza vaccine that has thermal stability, so as to avoid refrigeration for storage and transportation
- To employ a user friendly cost effective vaccine applicator
- To evaluate, adjuvant combinations, dose, schedule, efficacy and toxicity in pre-clinical studies.
- To evaluate efficacy and safety profile in humans (proof-of-principle)
- To disseminate the findings to scientific community and industry

The project was structured into the following interconnectd parts:

1. Supply of antigen, formulation development and selection of a device.
2. Carry out pre-clinical studies in mouse and ferret models to establish the optimum antigen dose, schedule and formulation; vaccine efficacy was to be evaluated using antibody and T cell assays and challenge tests.
3. To carry out toxicity and proof-of-principle Phase I clinical studies using GMP antigen.
4. Coordination of the project, data management of all the results from the work-packages and dissemination of results.

Each of the above parts had further subparts which corresponded to individual work packages and are discussed in the next section.

1.3 **A description of the main S&T results/foregrounds**

1.3.1 *Supply of antigen and formulation development*

1.3.1.1 Supply of antigen

Bulk antigen (inactivated purified surface subunit antigen of H5N1 strain NIBRG-14, propagated in chicken eggs), Batch 1 as supplied by Solvay Pharmaceuticals in July 2008 was concentrated to a level necessary for use in the programme (primarily to facilitate the conduct of immunology studies in the mouse) using Membrane-based Tangential Flow Filtration (TFF) equipment and was found to retain its potency. The concentrated antigen (SU) was

used for developing solution and powder formulations utilising chitosan as potential adjuvant. A further supply of the bulk antigen (inactivated purified surface subunit antigen of H5N1 strain NIBRG-14 (SU-c) propagated in cell culture), Batch 2 was received in January 2010.

Due to changes in corporate structure and management, Solvay declined to supply subunit antigen of GMP H5N1 (A/Vietnam/1194/2004 (NIBRG-14)) propagated in eggs or cell culture, for continuing the work. Hence the consortium looked for another supplier who could supply a similar antigen. Participant 3 opened the communications with Novartis Vaccines and Diagnostics Srl and Participant 1 proceeded with subsequent communications and negotiations. Under an MTA agreement, Novartis initially supplied relevant GMP subunit antigen (NIBRG-14) for preclinical work with indications for supply of antigen for a clinical study at a later stage, as it would require more extensive legal paper work. However, on 23 Oct 2011, Novartis declined to supply the GMP subunit antigen for clinical work. As a result the clinical study could not be carried out, hence the attention was focused on a challenge study in ferrets to obtain robust protection efficacy data.

The various antigen sources used in this project are summarised in Table 1.

Table 1. Summary of antigen sources used during NASPANVAC project

Antigen used	Test facility	Outline details
Subunit NIBRG-14, Non-GMP, egg-based, *Concentrated antigen* [Solvay]	ADL	In vitro: formulation development and stability testing
	UIB	In vivo: mouse immunogenicity – preliminary screening study (Mouse Study 1)
	UIB	In vivo: mouse, immunogenicity – dose response study (Mouse Study 2)
	TCD	In vivo: mouse, immunogenicity – Comparison of CSN and TM-CSN (Mouse Study 3)
	TCD	In vivo: mouse, immunogenicity – Effect of H3 or H5 priming on nasal boost with H5 (Mouse Study 4)
	TCD	In vivo: mouse, immunogenicity – Comparative study examining mucosal versus parenteral priming (Mouse Study 5)
	UIB	In vivo: statistically powered mouse study CSN vs TM-CSN; + c-di-GMP (Mouse Study 6)
	TCD	In vivo: mouse bridging study (Mouse Study 7)
	ADL	In vitro: investigation of effect of freeze-drying on antigen potency
Subunit NIBRG-14, Non-GMP, egg-based, *Bulk antigen* [Solvay]	ADL	In vitro: formulation development and stability testing
	RV	In vivo; ferret immunogenicity – Preliminary study (Ferret Study 1)
Subunit NIBRG-14, Non-GMP, cell-based, *Bulk antigen* [Solvay]	ADL	In vitro: formulation development and stability testing
	RV	In vivo; ferret immunogenicity – Second study (Ferret Study 2)
Whole NIBRG-14 virus	UIB	In vivo: mouse, immunogenicity – dose response study (Mouse Study 2)
Sub-unit NIBRG-14, GMP, egg-based *Bulk antigen* [Novartis]	ADL	In vitro: formulation development and stability testing
	TCD	In vivo: Mouse bridging study (Mouse Study 7)
	RV/Viroclinics	In vivo: Ferret challenge study (Ferret Study 3)

1.3.1.2 *Formulation development and selection of device*

a) Solutions

Various grades of chitosan, chitosan glutamate (CSN) which is the proprietary nasal delivery system of participant 1 (Archimedes), carboxy-methyl chitosan (CM-CSN), trimethyl-chitosan (TM-CSN) and (for the first time in any investigative study) diquaternary piperazine chitosan (DP-CSN) were assessed to determine the most efficacious adjuvant. Of all the chitosans CM-CSN was found to be most compatible with the antigen and the solution formulation was stable for at least 6 months when stored at 2-8ºC in terms of potency measure by participant 4 using Single Radial Immunodiffusion (SRID) assay. Of the chitosan stock solutions (without antigen), CM-CSN, TM-CSN and DP-CSN were stable at all storage conditions (2-8ºC or 25ºC) up to 12 months. With increasing temperature and with time the viscosity of CSN solution decreased while that of CM-CSN at higher concentration (20 mg/ml) increased and solution ultimately formed a gel.

A commercially available Pfeiffer metered dose nasal spray pump with paediatric nozzle attached to a 5 ml amber bottle was found to be most suitable as a delivery device for solution formualtios after assessment for delivery in terms of fill volume, the number of priming sprays required and delivered dose weight.

A clinical study was planned to evaluate two nasal formulations containing H5N1 antigen either with or without chitosan glutamate (CSN). In both cases, it was proposed to supply each formulation to the study site in two separate containers to be mixed just prior to use; one container to hold antigen solution and the other to hold either chitosan glutamate solution or saline diluent (for use in preparing chitosan-free formulation). A study was carried out to evaluate stability of the antigen solution when stored at 2-8°C in glass vials and in plastic syringes over a period of 12 weeks in order to select a suitable package for clinical use. Results of this study clearly indicated that the antigen was stable in terms of appearance, potency and integrity using SRID assay in both glass vial and BD Plastipak syringe; therefore either package would be suitable for the clinical supplies. Further investigations were carried out to study the effect of pH on antigen potency and integrity which were evaluated by SRID assay. The results showed that the antigen lost its potency significantly when pH $\leq$ 6; however when pH > 6 (e.g., 6.2 and 7) the antigen was stable for at least 4 hours, ample time to enable administration.

b) Powders

In order to facilitate the development of a thermally stable vaccine, powder formulations were developed. The effect of various excipients (mannitol, sucrose and trehalose) and freezing methods on the potency of freeze dried antigen was studied. Based on the handling properties of the freeze dried solids and final powder formulations, mannitol was identified as a necessary bulking agent for use in the freeze drying process. Prototype powder formulations were prepared satisfactorily. The antigen content of the final powder formulations could not be measured using the SRID assay by participant 4 due to interference with the assay when the powder was solubilised Investigations were carried out to enable potency measurement of the antigen in powder formulations containing chitosan using the single radial immunodiffusion (SRID). The antigen was found to be soluble in both glycerine and dimethyl sulfoxide (DMSO) but failed to be detected by the SRID method in powder extracts. Solid phase extraction of the antigen from chitosan formulations followed by high pressure liquid chromatography (HPLC) resulted in only 70% recovery possibly due to interaction between the antigen and chitosan. The effect of fast and slow freezing, excipients and buffer on antigen potency was also examined. The results confirmed that fast freezing in liquid

nitrogen was a suitable process, while mannitol and phosphate buffered saline (PBS) were appropriate excipient and buffer respectively, for preparing the powder formulations (prior to blending with chitosan).

The delivery characteristics of the CSN powders were assessed using the commercially available Pfeiffer unit dose nasal powder devices. When loaded with 10.0-10.5 mg of powder approximately 98% of the dose was delivered when the device was fired. The mean particle size was approximately 37 µm, indicating suitability of this device for intranasal dosing to humans.

1.3.2 *Manufacture of vaccine formulations*

Solution vaccine formulations were manufactured for three mouse studies performed by participant 7 and four mouse studies by participant 5. Both solution and powder formulations were manufactured for two immunogenicity studies in ferret performed by participant 4. Solution formulations were also manufactured for the challenge study in ferret subcontracted to Viroclinics by participant 4.

Due to unavailability of the GMP subunit antigen, toxicology and clinical studies could not be carried out. Hence the formulations were not manufactured for these studies for the 12 and 9 month stability studies to support the toxicology and the clinical studies respectively.

1.3.3 *In-Vitro studies*

To induce an effective mucosal immune response, a successful vaccine should be directed toward effectively triggering the innate immune response. Innate immunity is critical for orchestrating the adaptive immune response through the activation of antigen-presenting cells (APC) such as the dendritic cell (DC). Initial systemic responses are governed by the innate immune system, which produces pro-inflammatory responses (cytokines and chemokines) to the initial insult of an infectious agent. Furthermore, this initial rapid pro-inflammatory response is considered to be the critical trigger provided by traditional immunological adjuvants. Thus, many vaccine formulations strategies include innate immune triggers to provide this adjuvant signal.

In vitro studies were performed to examine the ability of various chitosan solutions to activate/potentiate an innate immune response. The results showed that both CSN and TM-CSN solutions gave positive results; with TM-CSN being more efficient in it's ability to enhance TLR-induced cytokine production skewing the T cell response towards a mixed Th1/ Th17 phenotype. Further studies to evaluate the effects of chitosan on TLR-induced cytokine production by plasmacytoid dendritic cells (pDC) showed that TM-CSN enhanced CpG induced IP-10 which is a Th1 cell chemokine, whereas CSN induced LPS induced IL-12p40 and IL-1β which are Th17 polarising cytokines. CSN had significant suppressive effect on both LPS and CpG induced LP27 which is known to stimulate the production of IFN-α and has been shown to exhibit both pro- and anti-inflammatory properties. Further studies would be required to investigate this effect.

The chitosan formulations that exhibited the best ability to potentiate the innate immune response *in vitro* supported the identification of formulations with the best chance of succeeding *in vivo*.

1.3.4 *In vivo Mouse studies*

Several *in vivo* studies were carried out in mice to evaluate various chitosans, antigen dose response, efficacy of the adjuvanted antigen, effect of previous immunity, establish vaccination regime and examine the effect of nasally administered chitosan on local brain cytokine production.

1.3.4.1 *Evaluation of different chitosans and dose response*

Of the three chitosans (CSN, carboxymethyl chitosan and diquaternary piperazine chhitosan) tested, CSN produced the highest overall immune response (IgG, nasal wash IgA and serum radial haemolysis (SRH)). Furthermore CSN enhanced SRH titres after one dose as compared to the antigen alone. A dose response study in mice showed that CSN augmented the highest serum IgG and IgA, nasal wash IgA, seroprotection (SRH titres) shown in Figures 1 and 2 respectively below and the quality of the T cell responses. The SU (7.5µg HA) formulated with CSN induced good nasal wash IgA and serum IgG responses after the second immunisation and low frequencies of double cytokine producing $CD4^+$ cells (not shown).

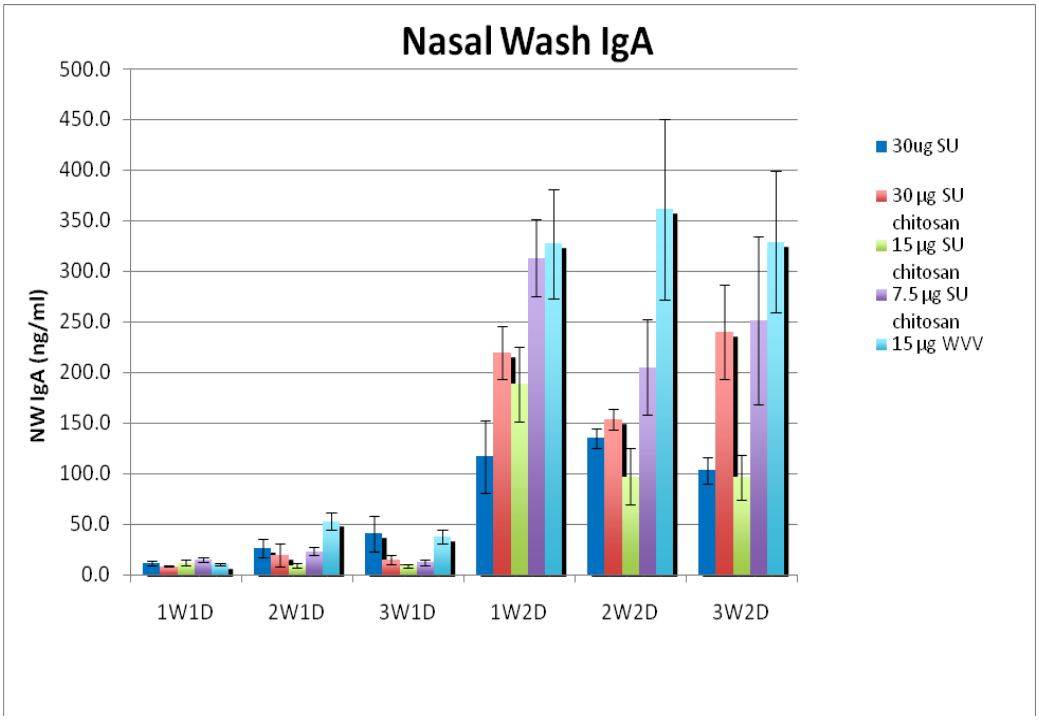


Figure 1. The local nasal antibody response

1W1D = 1 week after dose 1, 2W1D = 2 weeks after dose 1, 3W1D = 3 weeks after dose 1
1W2D = 1 week after dose 2, 2W2D = 2 weeks after dose 2, 3W2D = 3 weeks after dose 2

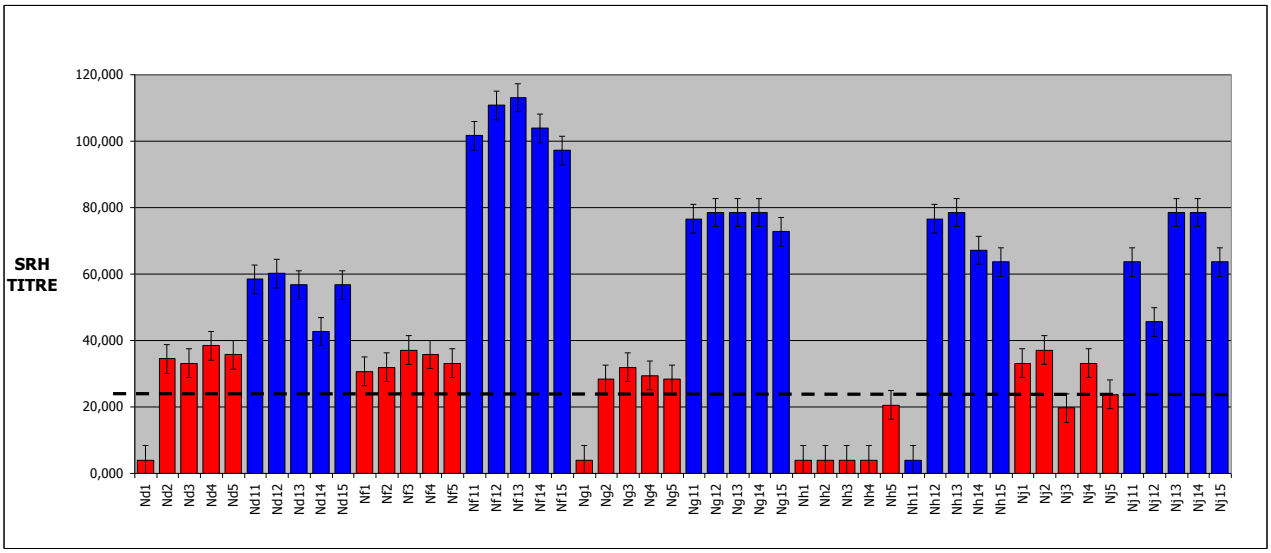


Figure 2. The serological SRH antibody response
red = 3weeks after dose 1, blue = 3weeks after dose 2
----- seroprotection level

1.3.4.2 *Evaluation of vaccine regime*

To discover the most efficacious vaccination regime, parenteral priming with either alum or cytosine-phosphate-guanine dinucleotide (CpG) followed by nasal vaccination with the antigen formulated with CSN and TM-CSN was explored. CSN and TM-CSN enhanced HA-specific IgG1 in sera of alum and CpG primed mice, whereas TM-CSN strongly enhanced HA-specific IgG2a in sera of CpG primed mice (Figures 3A and 3B). Priming with antigen in the presence of alum polarised the antigen specific T cell response towards a Th2 phenotype with high concentrations of antigen-specific IL-4 and IL-5 detected in both the spleen and lymph nodes of immunised mice. In contrast, priming with antigen in the presence of CpG followed by intranasal boosting with antigen and TM-CSN induced a more mixed Th1/Th2 profile in the spleen of immunised mice.

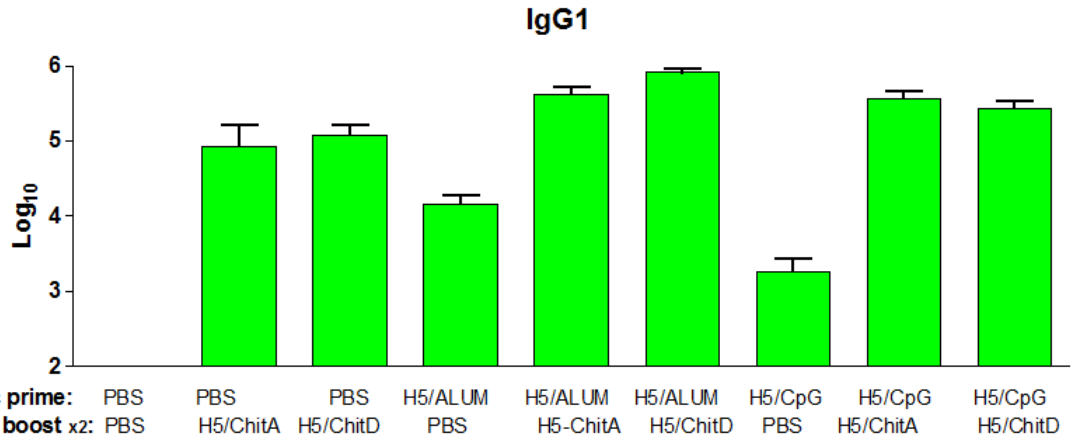


Figure 3A. IgG1 antibody responses to CSN and TM-CSN adjuvanted vaccine in sera of either CpG or ALUM primed mice.
Antibody titres are expressed as the $\log_{10}$ values of reciprocal end point titres.

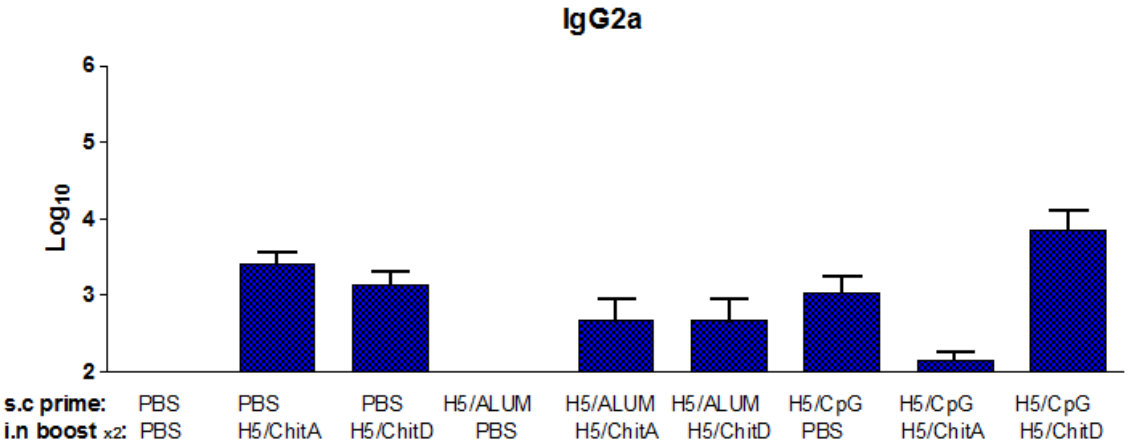


Figure 3B. IgG2a antibody responses to CSN and TM-CSN adjuvanted vaccine in sera of either CpG or ALUM primed mice.
Antibody titres are expressed as the $\log_{10}$ values of reciprocal end point titres.

1.3.4.3 *Effect of previous H3 or H5 immunity*

As the general human population is already exposed to H3 influenza viruses the effect of previous H3 immunity on nasal immunization with H5N1was investigated. The results showed that two nasal doses of H5/CSN administered intranasally following H3 or H5 priming in the presence of CpG considerably enhanced the antibody responses (Figures 4A and 4B). This indicated that subcutaneous prime / nasal boost strategy could provide protection against H5 viral infection.

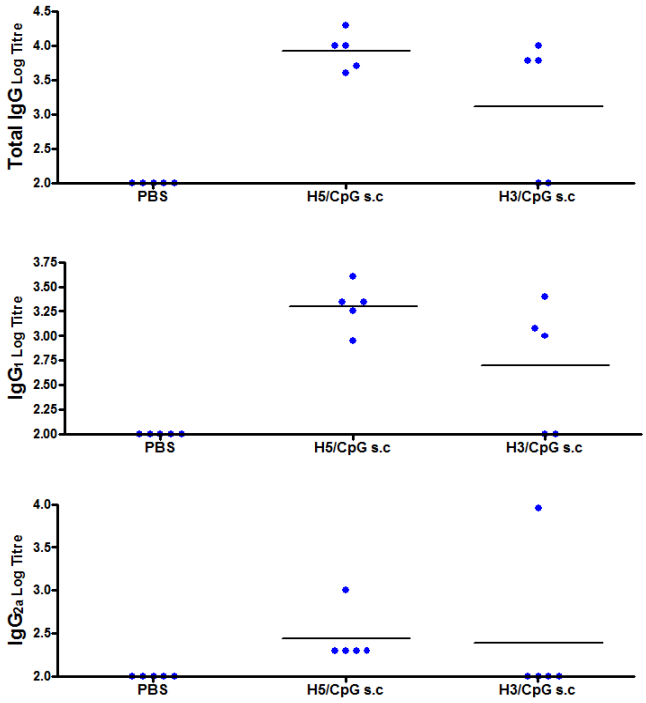


Figure 4A. Humoral immune responses in H3 or H5 primed mice in the presence of CpG. Antibody titres are expressed as the $\log_{10}$ values of reciprocal end point titres.

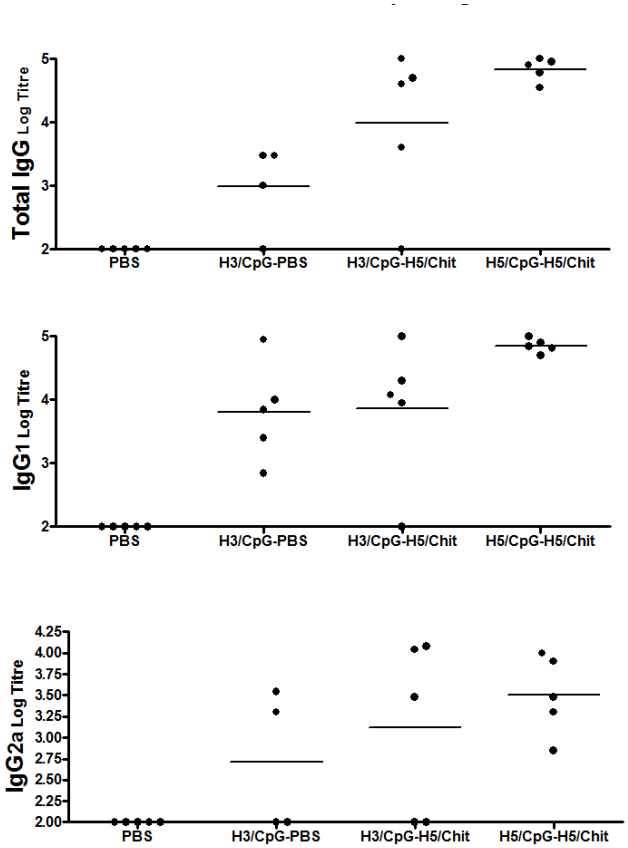


Figure 4B. Humoral immune responses in H3 or H5 primed mice followed by two nasal boosts with H5 in the presence of CSN.
Antibody titres are expressed as the $\log_{10}$ values of reciprocal end point titres.

Further prime / boost studies confirmed that two doses of nasal chitosan adjuvanted H5 vaccine could produce both humoral and cellular immune responses. These responses could be further enhanced by priming the mice subcutaneously with CpG adjuvanted H5 vaccine followed by two doses of chitosan adjuvanted H5 vaccine. A comparative study examining mucosal versus parenteral priming showed that CSN elicits a strong IFN-γ response when administered intranasally and also induces a cytokine IL-22 which is known to play an important role in the regulation of immunity and inflammation at barrier surfaces (Figure 5).

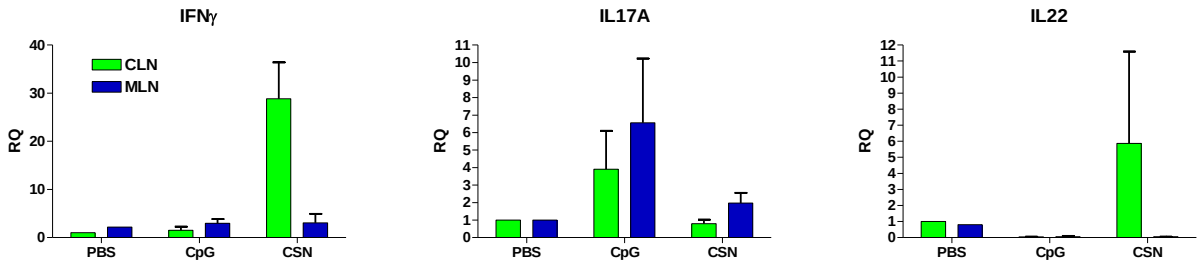


Figure 5. Cytokine response of cervical lymph nodes (CLN) and mesenteric lymph nodes (MLN) following intranasal or subcutaneous vaccination with PBS, CpG or CSN.
IFN-g, IL-17A and IL-22 mRNA levels measured by real time PCR. (Green: CLN, Blue: MLN)

1.3.4.4 *Effect of nasally administered CSN on local brain cytokine production*

When vaccinating via the nasal route in the presence of an adjuvant its important to investigate its effect on the local lymphatic tissues as well the brain (due to its close proximity to the nasal cavities). The nasal associated lymphoid tissue (NALT) is a mucosal lymphoid organ which is thought to play a pivotal role in directing the development of B and T cells that can protect the respiratory tract. This study aimed to investigate the effectiveness of CSN used in the vaccine formulations at activating immunity in the NALT. CSN activated the production of IL-1β, IL-6 (important for Th17 responses) and IL-12p35, a critical polarising cytokine for Th1 development. In the NALT CSN also induced the production of IL-22 which is known to play an important role in the regulation of immunity and inflammation at barrier surfaces. Early studies have indicated that IL-22 does not appear to play a substantial direct role in immunity to viral pathogens. However, the importance of IL-22 in vaccine induced protective immunity to influenza would require further investigation.

On evaluating the cytokine production in the hypothalamus region of the brain the results showed that CSN did not augment the production of pro-inflammatory cytokines, IL-1β and IL-6 after intranasal immunisation; thereby supporting the claim that chitosan is a safe adjuvant to use intranasally (Figure 6).

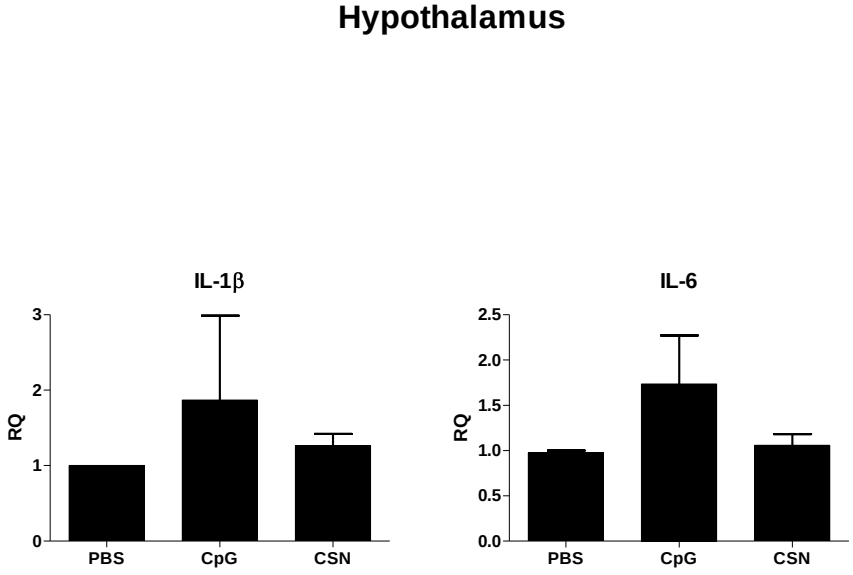


Figure 6. Cytokine response of the hypothalamus region of the brain following intranasal administration with PBS, CpG or CSN.

Cytokine mRNA expression was evaluated by real-time PCR normalized to 18 S rRNA and relative to NALT tissue from control (PBS) mice.

1.3.4.5 *Intranasal studies evaluating C-di-GMP adjuvanted antigen with or without CSN*

A novel experimental adjuvant c-di-GMP was found to enhance systemic and local antibody responses in terms of serum IgG1, IgG2a, SRH, haemagglutinin inhibition (HI) and virus neutralisation (VN) antibody titres. VN titres against the homologous strains in all the adjuvanted groups were significantly higher than in the non-adjuvanted group. The evaluation of cross clade reactive antibodies against the clade 2.1 virus A/Indonesia/5/05 (IDON) showed no neutralisation of this virus in the antigen alone groups, whilst some animals in the

adjuvanted groups were seropositive (Figure7). However no reactive antibodies against A/Indonesia/05/2005 were observed.

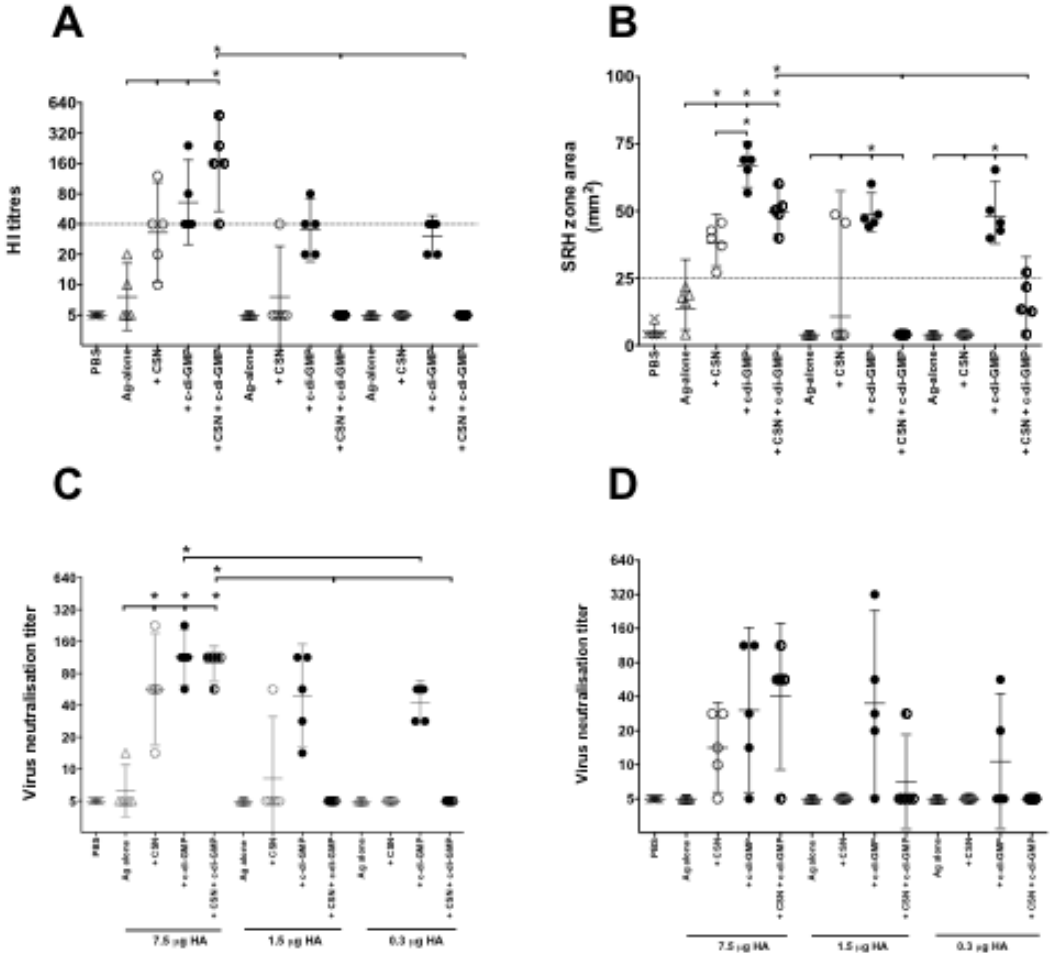


Figure 7. The serological antibody response

A) serum haemagglutination inhibition (HI) titres, **B)** single radial haemolysis (SRH) zone areas (mm^2) and **C)** virus neutralisation titres measured at three weeks after the second vaccination against the homologous strain. **D)** cross-clade neutralising antibody responses against A/Indonesia/05/2005. The lines represent the geometric mean titre (GMT) ±95% CI, and each symbol represents one animal. The limit of detection of the HI assay was 10 and negative titres were assigned an arbitrary value of 5. The dotted lines represent the protective HI titre (40) and SRH (25 mm^2) zone areas. *significant difference ($p<0.05$).

A clear dose response was observed for all vaccine groups, with adjuvants augmenting the highest local nasal wash IgA response at an antigen dose of 7.5µg after the second administration; whereas no local IgA was found in the control animals that had been dosed with PBS. At two and three weeks after the second dose no influenza specific serum IgG or IgA were detected in the control group, whereas the c-di-GMP or combination c-di-GMP+CSN groups had the highest humoral responses closely followed by the CSN group. Both CSN and c-di-GMP effectively enhanced splenocyte proliferation as compared to antigen alone. The evaluation of T-helper cell profile showed that c-di-GMP adjuvanted vaccine produced a Th1/Th17 biased response, whilst the CSN adjuvanted vaccine group had a cytokine profile indicating a more Th2 skewed response.

Overall this study showed that both CSN and c-di-GMP boosted functional antibody responses. The c-di-GMP adjuvant provided significant dose-sparing in terms of seroprotection, whereas the adjuvant combination did not. However, the adjuvant combination skewed the immune responses towards a more balanced Th profile than either of the adjuvants alone. This study highlights the importance of assessing the humoral and cellular immune responses following immunisation to allow direct evaluation novel mucosal influenza vaccines for finding the optimal vaccine formulation.

1.3.4.6 *Comparison of CSN and TM-CSN adjuvanted influenza (H5N1) vaccine*

Since conclusive immunological results were not obtained from two ferret studies (sections 1.3.5.1 and 1.3.5.2), a statistically powered study was performed to compare the efficacy of CSN and TM-CSN as mucosal adjuvants. Both CSN and TM-CSN augmented the antibody response, with SRH titres above the protective threshold of 25mm^2 and no significant difference was observed in HI titres to the two chitosans but CSN resulted in significantly higher SRH titres than TM-CSN. The homologous neutralising antibody response was significantly higher in the CSN group than all the other groups (Figure 8). In terms of the heterologous antibody response cross-reactive neutralising antibody response to A/Indonesia/1/2005 strain was observed in majority of the CSN immunized animals (7 of 10) and none in the other groups.

Low concentrations of local nasal wash IgA were detected after the first dose with the highest response detected in the two chitosan groups (Figure 9A). The second dose significantly augmented the local IgA particularly in the chitosan groups, with the CSN group at two weeks and the TM-CSN group at two and three weeks post vaccination (Figures 9 B and C).

The splenocyte proliferation assay showed a significant adjuvant effect of CSN, and TM-CSN suggesting an enhancement of the cellular immune response. Of the two chitosan adjuvants CSN appeared to be more effective than TM-CSN. In terms of the cytokine response the antigen alone and chitosan adjuvants elicited Th1 (IL2, IFN-γ), Th2 (IL4, IL5 and IL10) and Th17 response (Figure 10). A significantly high IL-17 response was observed in two chitosan groups.

Overall this study confirmed that both CSN and TM-CSN boosted functional antibody as well as local IgA responses with CSN appearing to be marginally more efficacious than TM-CSN. Similar observations were made in terms of T cell proliferation. However TM-CSN showed a significantly higher IL-23 response than CSN.

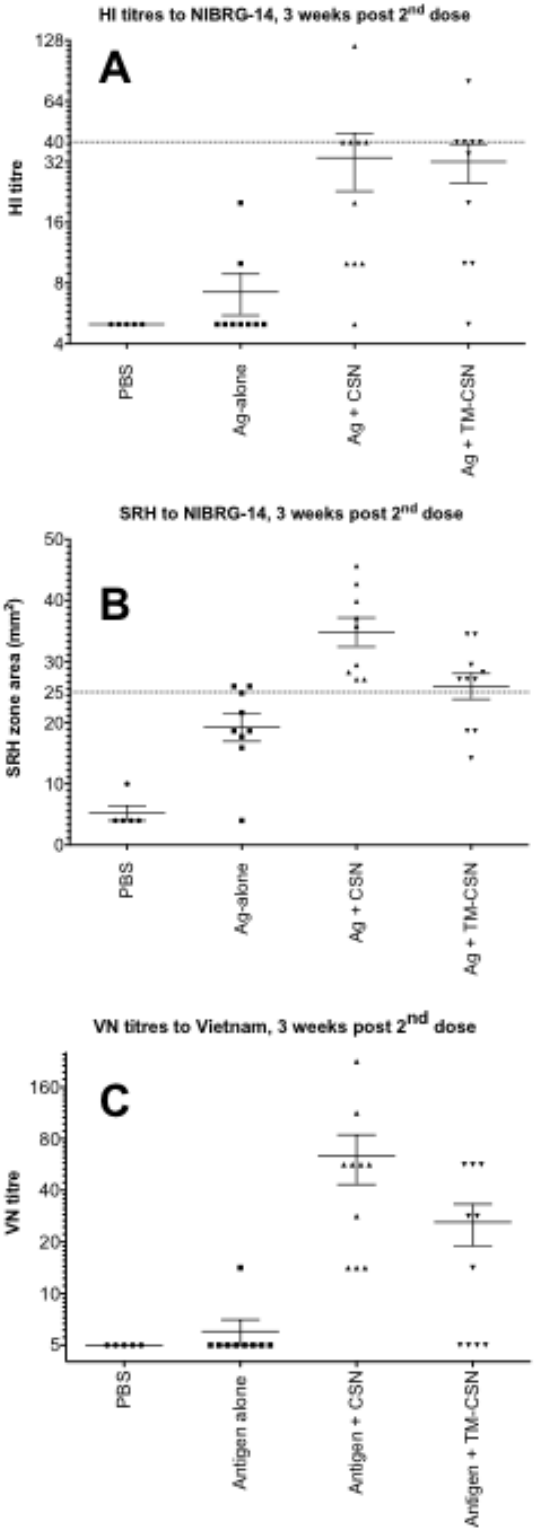


Figure 8. The serological antibody response induced after vaccination.

A) serum haemagglutination inhibition (HI) titres, **B)** single radial haemolysis (SRH) zone areas (mm^2) and **C)** virus neutralisation titres measured at three weeks after the second vaccination against the homologous strain. The lines represent the geometric mean titre (GMT) ±95% CI, and each symbol represents one animal. The limit of detection of the HI assay was 10 and negative titres were assigned an arbitrary value of 5. The dotted lines represent the protective HI titre (40) and SRH (25 mm^2) zone areas. *significant difference ($p<0.05$).

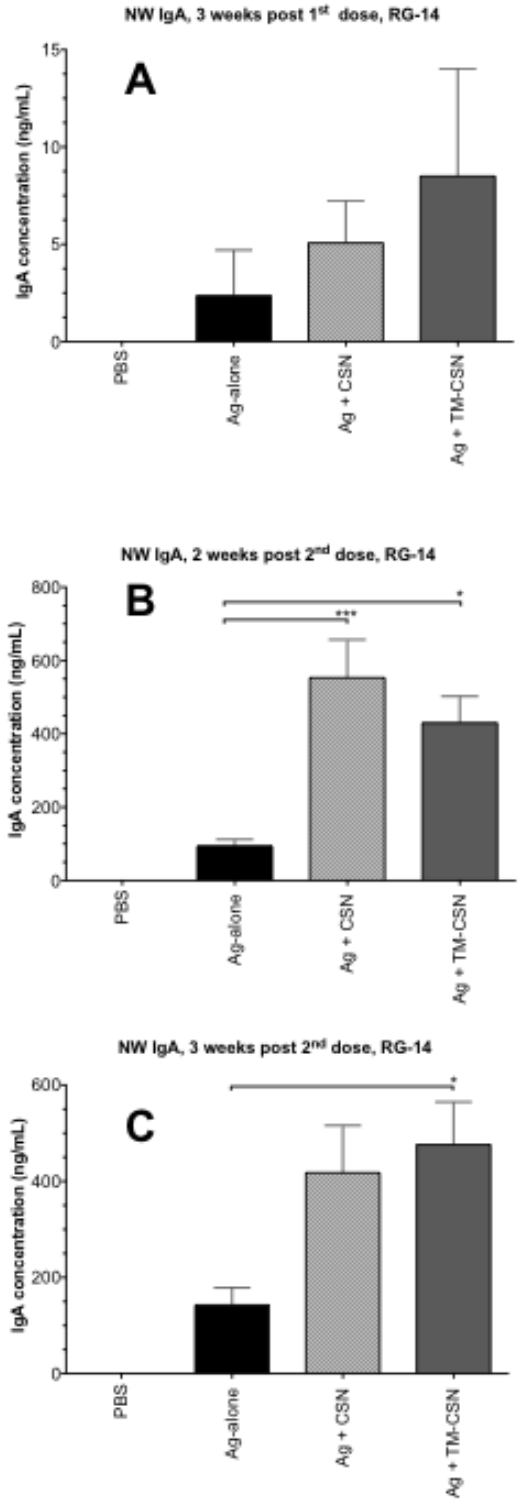


Figure 9. The nasal wash IgA antibody response

A) IgA three weeks post 1st dose, **B)** IgA two weeks post 2nd dose, **C)** IgA three weeks post 2nd dose. The bar data represents the mean antibody concentration (ng/ml) ± SEM the standard error of the mean.
*significant difference ($p<0.05$)

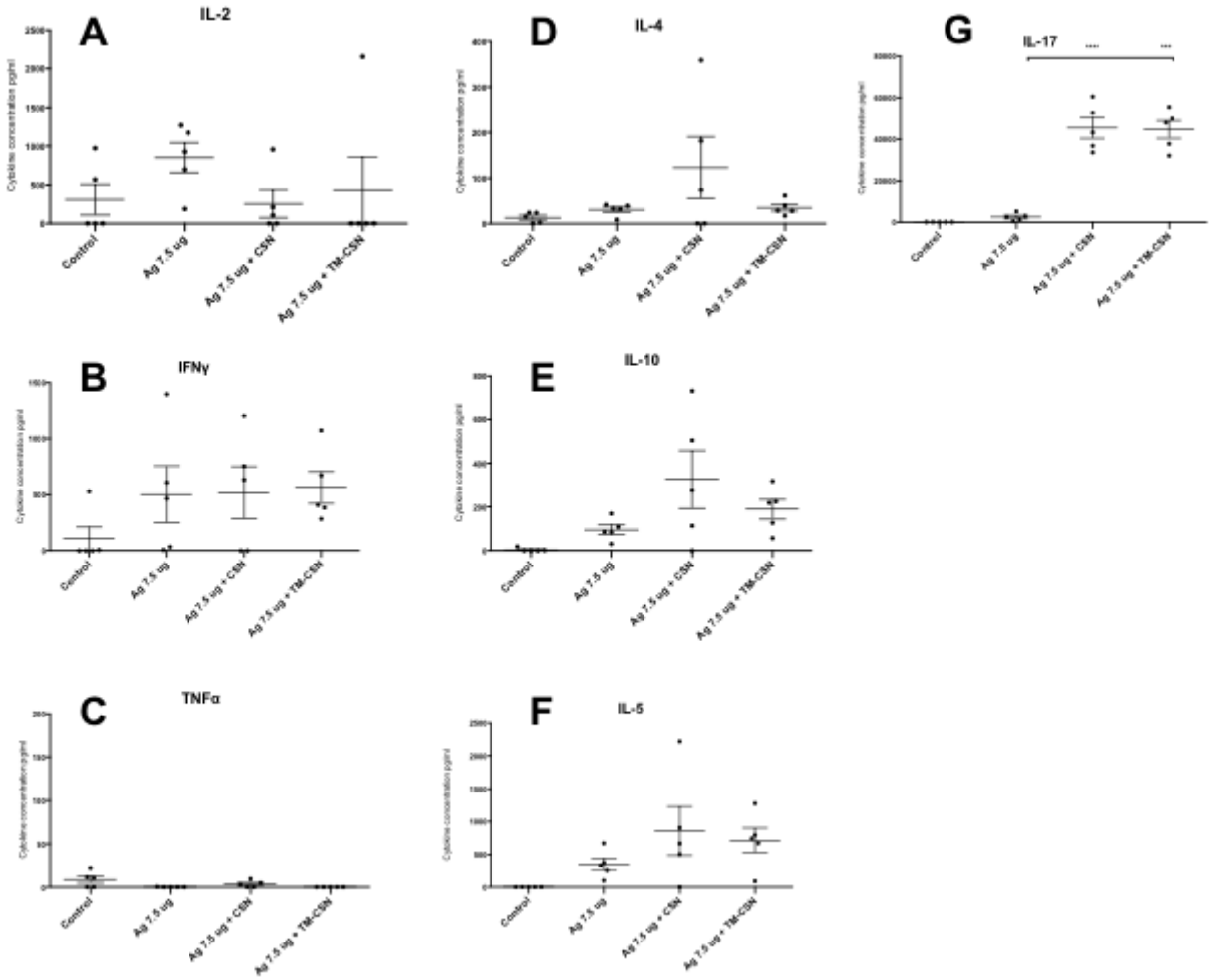


Figure 10. The cytokine response induced in the spleen

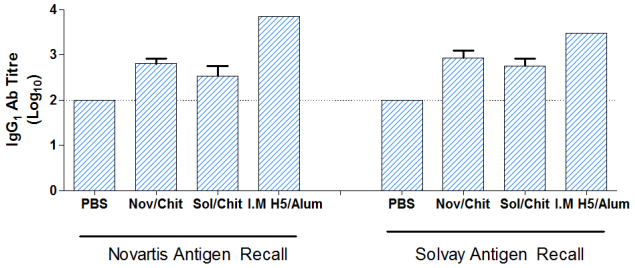
A, B and C) Th1: IL-2, IFN-γ and TNF-α; **D, E and F**) Th2: IL-4, IL-5 and IL-10, **G)** IL-17. The data are presented as the mean cytokine concentration (pg/ml) ± SEM. *significant difference ($p<0.05$).

1.3.4.7 *Comparison of Solvay and Novartis antigens*

Since the source of the antigen was changed during the project, an immunogenicity bridging study was carried out to compare the Solvay and Novartis antigens. Both the antigens elicited similar IgG1titres pre-boost and post-2nd booster vaccination (Figure 11). Slight enhancement in IgG2a titres was observed in pre-boost sera in response to Solvay antigen but comparable IgG2a titres were observed in response to both antigens in the final samples (Figure 12). Both the antigens showed some enhancement of SRH titres compared to the PBS control. Solvay antigen performed slightly better than Novartis antigen in that 2/6 animals reached the protective level (Figure 13). The Solvay antigen also performed slightly better in the homologous VN assay (Figure 14). Moreover, the cytokine data appeared to indicate Solvay antigen to elicit a rather more robust mixed T cell response with respect to Th1 and Th17 and similar Th2-type response.

Overall the results indicated that the antigens from the two sources were sufficiently similar for use in further preclinical and clinical studies..

A



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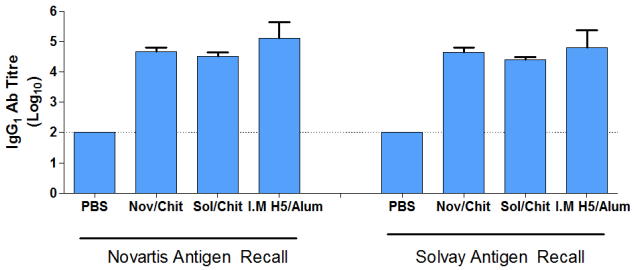
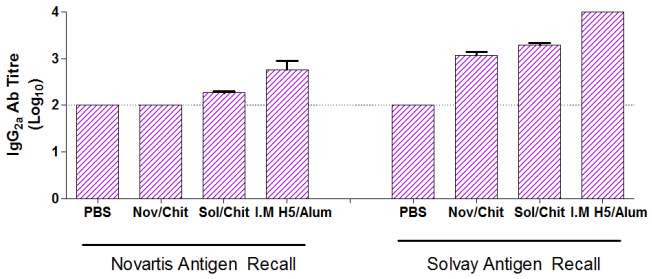


Figure 11. Anti-H5N1 HA IgG_1 antibody responses in (A) preboost and (B) final sera samples.

A



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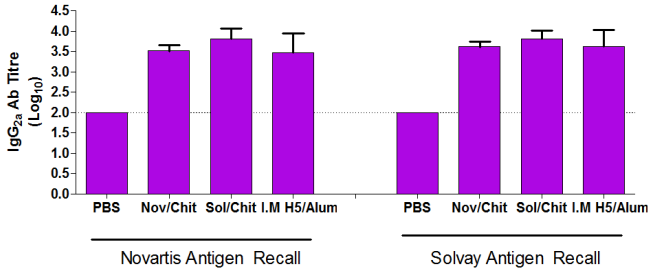


Figure 12. Anti-H5N1 HA IgG_{2a} antibody responses in (A) preboost and final (B) sera samples

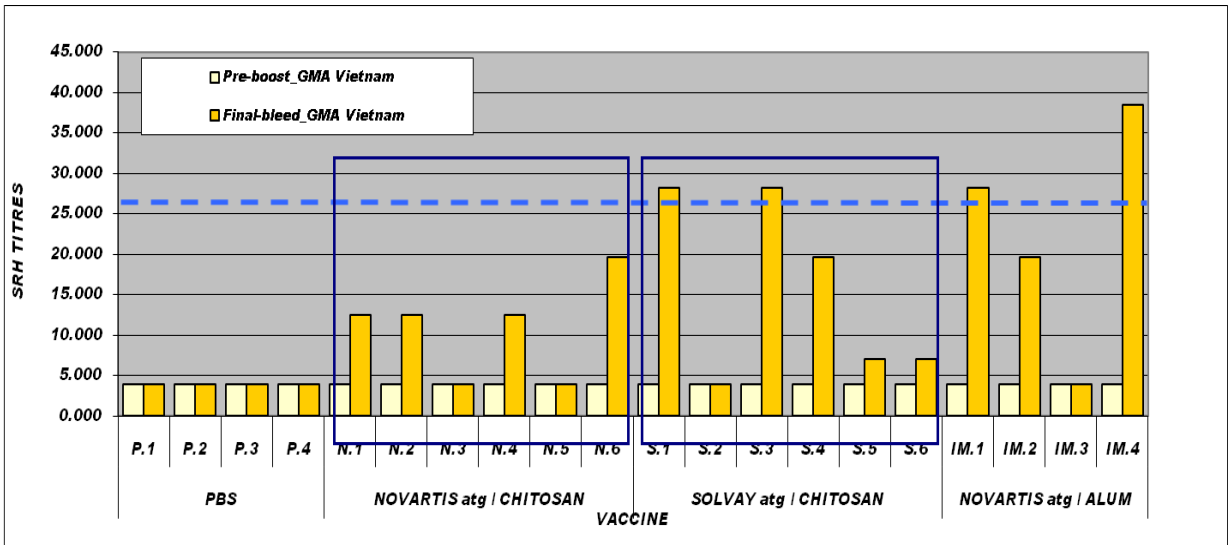


Figure 13. SRH homologous strain responses in preboost and final sera samples

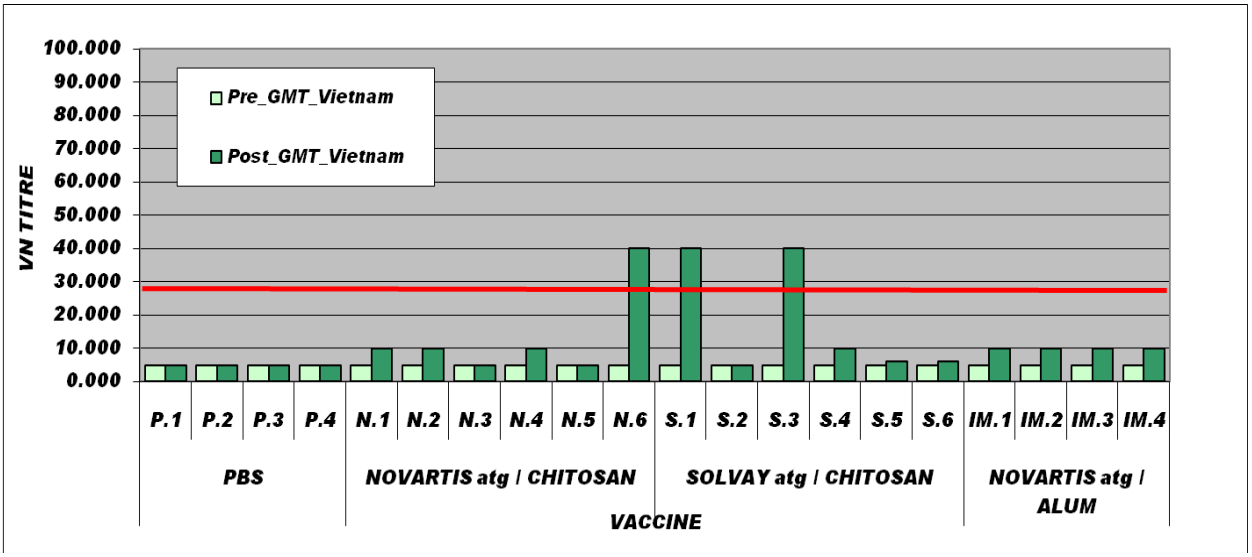


Figure 14. VN homologous strain responses in preboost and final sera samples

1.3.5 *In vivo ferret studies*

1.3.5.1 *The immunogenicity ferret study 1*

The immunogenicity of novel intranasal formulations, both as liquid and powders containing H5N1 antigen, was evaluated in the ferret model. Antibody titres obtained in ferrets were were relatively low and inconsistent, for example the positive control (i.m.) in this study performed poorly as no SRH seroprotection was observed despite some HI seroconversion. Although the results of this study were inconclusivethere were indications that chitosan enhanced immune response and that the liquid formulation possibly was more efficacious than powders. The consortium concluded that the non-responsiveness of powders was probably due to technical issues, i.e. not delivering the powders appropriately in the nasal cavity of the ferrets rather than the antigen immunogenicity. It also appears that.

1.3.5.2 The immunogenicity ferret study 2

A further immunogenicity study was carried out to evaluate liquid and powder formulations containing H5N1 antigen, in combination with two different chitosans (CSN or TM-CSN), via the nasal route in the ferret model. The study included a chitosan-free nasal liquid formulation and a control intramuscular (IM) injection of the antigen.

The overall results of this study were compromised for a number of reasons. The background SRH values (Day 0) were high in all the groups. Furthermore the serum samples for days 0 and 21 were observed to be coloured indicating contamination with plasma proteins, thereby possibly contributing to non-specific cross-reaction. The outcome of the results was further complicated by the administration of antibiotics to animals in two groups as well as a reduced number of animals completing the study in another two groups due to poor health.

The immune responses indicated that chitosan liquid formulations were efficacious in enhancing both the seroconversion and seroprotection rates following two nasal vaccinations, with TM-CSN possibly performing similarly but possibly slightly better than CSN. The positive control (i.m.) group also enhanced immune responses following both one and two vaccinations. However the nasal and the i.m. groups could not be directly compared as the latter group had a limited number of animals.

It was not possible to select the most appropriate formulations for further evaluation from the two ferret stuidies. Hence the consortium decided to carry out a statistically powered study in mice to compare the efficacy of CSN and TM-CSN as mucosal adjuvants in solution formulations (section 1.3.5.3).

1.3.5.3 The challenge study

Following the decision by Novartis not to supply antigen for clinical use, a definitive challenge study to establish efficacy of intranasally administered influenza H5N1 A/Vietnam/1194/2004 vaccine (supplied by Novartis) was conducted in the ferret model using liquid vaccine formulations based on both CSN and TM-CSN. The study was subcontracted to a contract research organisation, Viroclinics. The study protocol was specifically designed by several members of the consortium in collaboration with Viroclinics.

The objective of this study was to investigate the immunogenicity and protective efficacy of intranasally administered adjuvanted vaccines in the ferret H5N1 wild type model. The intranasal candidate vaccines were aqueous solutions containing subunit of inactivated NIBRG-14 influenza vaccine prepared in egg, GMP grade, obtained from Novartis. The

potency of the antigen was defined by its HA content quantified by SRID method. Candidate vaccines were adjuvanted with CSN or TM-CSN prior to testing. For control purposes, a chitosan-free vaccine formulation was tested along with appropriate placebo phosphate buffered saline (PBS) controls. The design of this study is summarised in Figure 15.

The vaccination phase:
tThis study consisted of six groups each comprising 6 ferrets.

- Groups 1 and 2 were intranasally immunised with CSN adjuvanted vaccine
- Group 3 was intranasally immunised with non-adjuvanted vaccine
- Group 4 was intranasally immunised with TM-CSN adjuvanted vaccine
- Groups 5 and 6 were treated with PBS

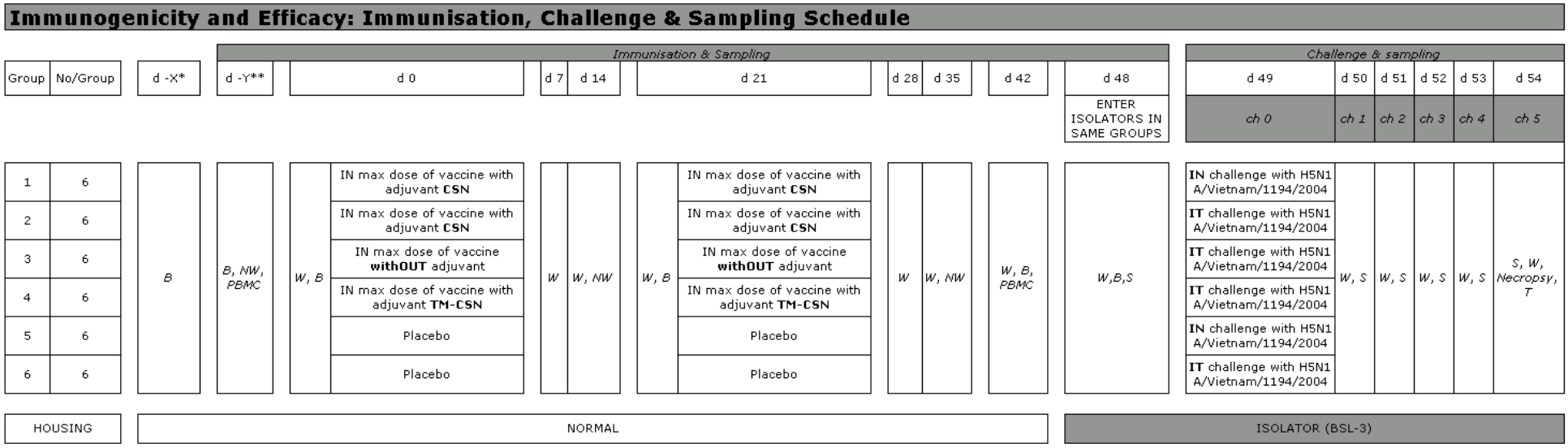
Intranasal vaccine were administered twice, once on study Day 0 and then again on study day 21. During the immunisation phase and just prior to challenge blood samples were taken to evaluate the immune response to vaccination.

The challenge phase:
Animals were challenged individually with a dose of 10^5 $TCID_{50}$ H5N1 A/Vietnam/1194/2004 HPAI virus on study day 49. Animals from Groups 1 and 5 were challenged via the intranasal route and animals of Groups 2, 3, 4 and 6 were challenged via the intratracheal route. Ferrets were monitored for infection related clinical and virological responses and for vaccine induced immunological responses. Animals were sacrificed on study day 54 (i.e. 5 days post challenge) to evaluate pathologic and additional virological parameters.

Summary of results

i. Antibody response induced by vaccine candidates

Antibody responses were tested using three different assay methodologies; SRH, VN and HI. The serum HI antibody response against H5N1 A/Vietnam/1194/2004 was greater in animals immunised with the TM-CSN adjuvanted vaccine compared to CSN and was absent in animals immunised with non-adjuvanted vaccine (Figure 16). Results were similar for SRH and VN assays (Tables 2 – 5). CSN and TM-CSN adjuvanted vaccines also induced cross protective antibodies *in-vitro* against H5N1 viruses from representatives from clade 2.1 and 2.2.



* Blood sampling at the suppplier => animals seronegative for Aleutian disease virus and influenza were brought into the animal facility (standard housing).

** Blood and nasal wash sampling prior to the first immunisation

Abbreviations:

IN = intranasal

IT = intratracheal

B = whole blood for serum

S = throat & nose swabs

T = lung, nasal turbinate and brain tissue after sacrification

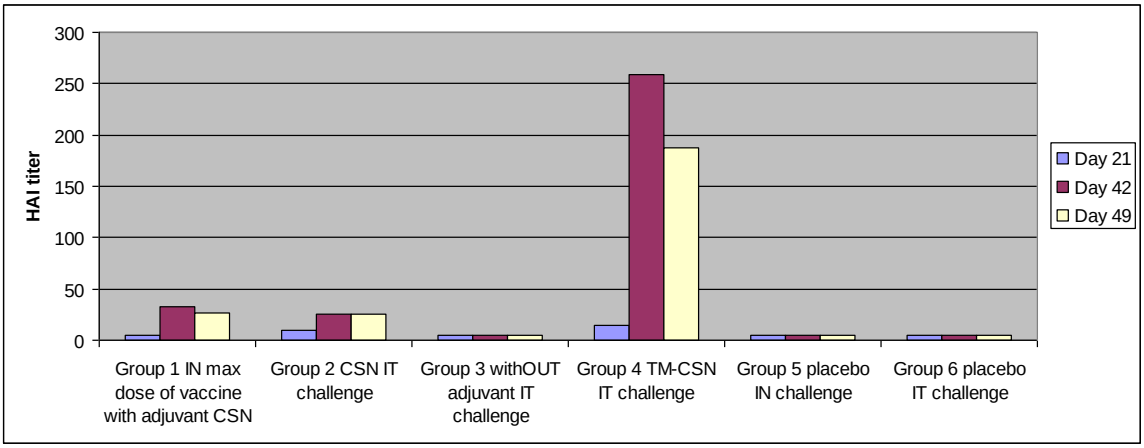
W = body weight

NW = nasal wash

PMBC = whole blood for PBMC

ctrl. = control

Figure 15. Protocol design of the Challenge study



LS Mean Ratio (P)

CSN IT (Gp 2) IT (Gp 3) TM CSN (Gp 4) P IN (Gp 5) P IT (Gp 6)

Figure 16. Haemagglutination inhibition titration performed against A/Vietnam/1194/02004 virus using serum samples of days 21, 42 and 49. Table below the graph shows the statistical analysis using raw data obtained for each animal of each group. The lower limit of quantification (LLOQ) value is 5, however for analysis purposes this value has been replaced by 0.5 LLOQ (2.5). ANCOVA using repeated measures analysis; Group p-value = <0.001, Day p-value = 0.758, Group*Day (interaction); p-value = 0.999.

Table 2. Humoral response against homologous strain at day 49.

Results of single radial haemolysis, micro neutralisation and haemagglutination inhibition tests against A/H5N1/Vietnam/1194/2004 virus are summarised (seropositive and seroprotection).

		Status at Day 49 (serum) **A/H5N1/Vietnam/1194/2004**				
Group	Treatment (immunisation)	SRH		Micro neutralisation		HAI
		Seroprotected	Seropositive	Seroprotected	Seropositive	Seropositive
1	0.075 mg/ml HA + 5 mg/ml CSN	4/6	6/6	4/6	6/6	4/6
2	0.075 mg/ml HA + 5 mg/ml CSN	3/6	3/6	4/6	4/6	3/6
3	0.075 mg/ml HA	0/6	0/6	0/0	0/0	0/0
4	0.075 mg/ml HA + 5 mg/ml TM-CSN	6/6	6/6	6/6	6/6	6/6
5	PBS	0/6	3/6	0/6	0/6	0/6
6	PBS	1/6	2/6	0/6	0/6	0/6

Table 3. Humoral response against Heterologous strain at day 49. Results of single radial haemolysis and micro neutralisation tests against Clade 2.1 A/H5N1/Indonesia/05/2005 virus are summarised (seropositive and seroprotection)

		Status at Day 49 (serum) ***A/H5N1/Indonesia/05/2005***			
Group	Treatment (immunisation)	SRH		Micro neutralisation	
		Seroprotected	Seropositive	Seroprotected	Seropositive
1	0.075 mg/ml HA + 5 mg/ml CSN	2/6	3/6	1/6	2/6
2	0.075 mg/ml HA + 5 mg/ml CSN	1/6	2/6	2/6	2/6
3	0.075 mg/ml HA	0/6	1/6	0/0	0/0
4	0.075 mg/ml HA + 5 mg/ml TM-CSN	4/6	4/6	4/6	5/6
5	PBS	0/6	1/6	0/6	1/6
6	PBS	0/6	0/6	0/6	0/6

Table 4. Humoral response against heterologous strain at day 49. Results of single radial haemolysis test against clade 2.2 A/H5N1/Turkey/Turkey/1/2005 virus are summarised (seropositive and seroprotection).

		Status at Day 49 (serum) **A/H5N1/Turkey/Turkey/1/2005**	
Group	Treatment (immunisation)	SRH	
		Seroprotected	Seropositive
1	0.075 mg/ml HA + 5 mg/ml CSN	2/6	6/6
2	0.075 mg/ml HA + 5 mg/ml CSN	3/6	4/6
3	0.075 mg/ml HA	0/6	1/6
4	0.075 mg/ml HA + 5 mg/ml TM-CSN	6/6	6/6
5	PBS	0/6	3/6
6	PBS	2/6	3/6

Table 5. Seroconversion measured by virus neutralisation assay.

If the antibody titre is greater than 5 and less than 20, then the subject is considered to be seropositive (highlighted in pink). If the antibody titre is equal or greater than 20, then the subject is considered to be seroprotected (highlighted in yellow); ≥20 neutralisation titre is usually recognised as seroprotective cut-off in humans.

		Virus Neutralization CPE based							
GROUPS	FERRETS	Viet_d0	Viet_d21	Viet_d42	Viet_d49	Indo_d0	Indo_d21	Indo_d42	Indo_d49
IN max dose of vaccine with CSN	1	5	5	20	57	5	5	5	5
	2	5	5	14	14	5	5	5	5
	3	5	14	14	14	5	5	5	5
	4	5	14	40	57	5	5	5	5
	5	5	5	40	57	5	5	20	20
	6	5	5	113	113	5	5	14	10
IN max dose of vaccine with CSN	7	5	5	5	5	5	5	5	5
	8	5	5	5	5	5	5	5	5
	9	5	5	57	57	5	5	28	28
	10	5	5	57	57	5	5	5	5
	11	5	5	80	80	5	5	5	5
	12	5	5	80	57	5	5	20	20
IN max dose of vaccine withOUT adjuvant	13	5	5	5	5	5	5	5	5
	14	5	5	5	5	5	5	5	5
	15	5	5	5	5	5	5	5	5
	16	5	5	5	5	5	5	5	5
	17	5	5	5	5	5	5	5	5
	18	5	5	5	5	5	5	5	5
IN max dose of vaccine with TM-CSN	19	5	5	113	57	5	5	28	20
	20	5	5	113	226	5	5	28	28
	21	5	5	28	57	5	5	20	20
	22	5	5	57	57	5	5	28	40
	23	5	5	57	57	5	5	14	14
	24	5	5	113	40	5	5	5	5
Placebo	25	5	5	5	5	5	5	5	5
	26	5	5	5	5	5	5	5	10
	27	5	5	5	5	5	5	5	5
	28	5	5	5	5	5	5	5	5
	29	5	5	5	5	5	5	5	5
	30	5	5	5	5	5	5	5	5
Placebo	31	5	5	5	5	5	5	5	5
	32	5	5	5	5	5	5	5	5
	33	5	5	5	5	5	5	5	5
	34	5	5	5	5	5	5	5	5
	35	5	5	5	5	5	5	5	5
	36	5	5	5	5	5	5	5	5

ii. Results of challenge phase of the study

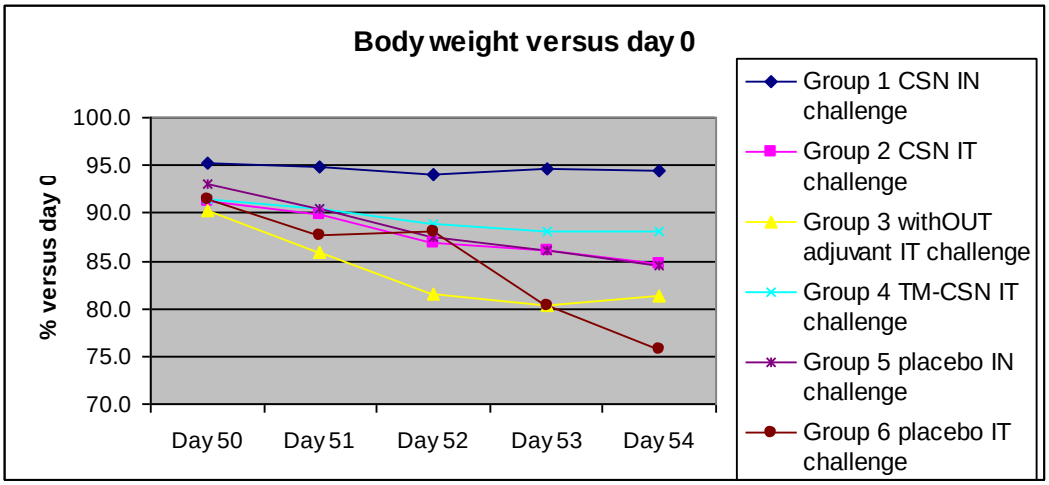
The inoculation route of the challenge with H5N1 A/Vietnam/1194/2004 HPAI virus had significant influence on the clinical, virological and pathological outcome of the virus infection. Intratracheal challenge with H5N1 A/Vietnam/1194/2004 influenza virus was shown to be lethal and to result in high level virus replication in lung and trachea. Intranasal challenge with H5N1 A/Vietnam/1194/2004 influenza virus was not lethal and resulted in high level virus replication in the upper respiratory tract and the majority of animals also exhibited virus replication in the olfactory bulb. In accordance, histopathological findings in the lung was more severe after intratracheal challenge and encephalitis was more common after intranasal challenge. The serum antibody response correlated well with the protection from intratracheal infection with influenza H5N1 A/Vietnam/1194/2004.

Animals immunised with the TM-CSN adjuvanted vaccine were protected against intratracheal infection-related findings in terms of lethality (Table 6), body weight loss(Figure 17), fever (figure 18), virus replication in respiratory and brain tissues and lung pathology (data not shown). Animals immunised with CSN- and in particular TM-CSN-adjuvanted vaccine were protected against intratracheal infection. Performance of the control group immunised with the non-adjuvanted vaccine and intratracheally challenged was, with respect to the evaluated clinical, virological and pathological parameters, low and not substantially different from that of PBS treated animals following intraracheal challenge.
In conclusion the severity of the H5N1 A/Vietnam/1194/2004 HPAI virus infection was, with respect to clinical, virological and pathological outcome, defined by the route of challenge. The outcome of infection was dependent on route of inoculation. Intranasal immunisation with the CSN or TM-CSN adjuvanted vaccines induced serum antibody responses that were protective against inoculation with H5N1 A/Vietnam/1194/2004 HPAI virus, whereas intranasal immunisation with the non-adjuvanted vaccine did not. Antibody responses were cross protective among different clades of H5N1 viruses. The highest level of protection from an intratracheal challenge with H5N1 A/Vietnam/1194/2004 HPAI virus was induced by the TM-CSN adjuvanted vaccine and this corresponded with the highest serum HI antibody responses.

Overall, immunisation with CSN adjuvanted vaccine was less effective than immunisation with TM-CSN adjuvanted vaccine. Immunisation with the CSN adjuvanted vaccine induced comparable levels of protection against an intranasal and intratracheal challenge with H5N1 A/Vietnam/1194/2004 HPAI virus. Moreover the comparison of intratracheal and intranasal challenge with a lethal dose of live virus in ferret is groundbreaking research as it has highlighted the advantages and disadvantages of each route of challenge; the normal practice has been to challenge only via the intratracheal route.

Table 6. Mortality observed after challenge with 10^5 $TCID_{50}$ H5N1 A/Vietnam/1194/2004 virus.

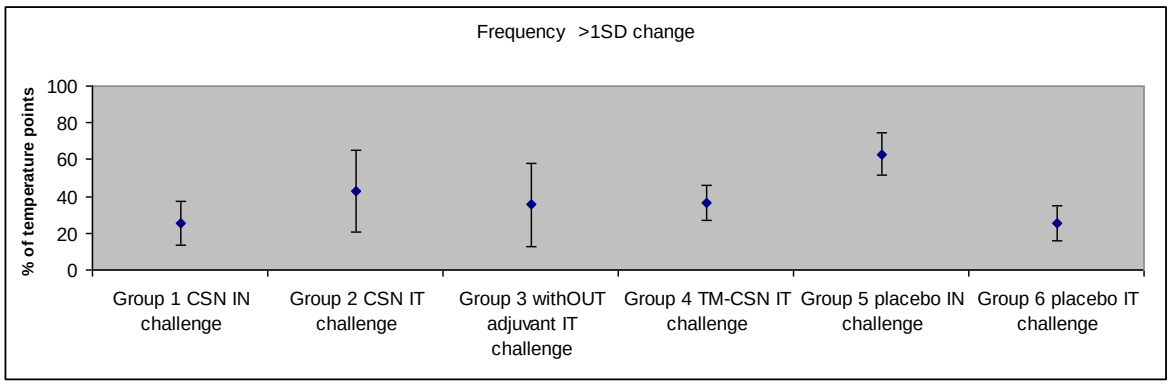
Animal number	Chip ID:	Group number	Immunisation Day 0	Immunisation Day 21	Challenge Day 49	Day of death after challenge					Reason of death
						50	51	52	53	54	
1	7199	1	IN max dose of vaccine with adjuvant CSN	IN max dose of vaccine with adjuvant CSN	IN challenge with H5N1 A/Vietnam/1194/2004					X	euthanised
2	7563									X	euthanised
3	3696									X	euthanised
4	5413									X	euthanised
5	6652									X	euthanised
6	7947									X	euthanised
7	8601	2	IN max dose of vaccine with adjuvant CSN	IN max dose of vaccine with adjuvant CSN	IT challenge with H5N1 A/Vietnam/1194/2004					X	euthanised
8	7901									X	euthanised
9	1613									X	euthanised
10	7272									X	euthanised
11	4087									X	euthanised
12	5554									X	euthanised
13	7965	3	IN max dose of vaccine withOUT adjuvant	IN max dose of vaccine withOUT adjuvant	IT challenge with H5N1 A/Vietnam/1194/2004			X			found death
14	7469									X	euthanised
15	6988									X	euthanised
16	3547									X	euthanised
17	1462								X		euthanised
18	8079									X	euthanised
19	8689	4	IN max dose of vaccine with adjuvant TM-CSN	IN max dose of vaccine with adjuvant TM-CSN	IT challenge with H5N1 A/Vietnam/1194/2004					X	euthanised
20	6552									X	euthanised
21	7345									X	euthanised
22	8738									X	euthanised
23	6938									X	euthanised
24	9542									X	euthanised
25	0108	5	placebo	placebo	IN challenge with H5N1 A/Vietnam/1194/2004					X	euthanised
26	2816									X	euthanised
27	5834									X	euthanised
28	8851									X	euthanised
29	5555									X	euthanised
30	8844									X	euthanised
31	4767	6	placebo	placebo	IT challenge with H5N1 A/Vietnam/1194/2004					X	euthanised
32	9182							X			found death
33	1131							X			found death
34	0131									X	found death
35	6502						X				found death
36	8945								X		found death



% Mean Change in BW

CSN IT (Gp 2) IT (Gp 3) TM CSN (Gp 4) P IN (Gp 5) P IT (Gp 6)

Figure 17 Relative body weight loss against day 0. ANCOVA using repeated measures analysis; Group p-value = <0.001, Day p-value = 0.001, Group*Day (interaction); p-value = 0.998; Baseline weight as covariate p-value =0.315.



AUC difference (P)

Figure 18. Frequency of fever events. Temperature value greater than 1 Standard Deviation from Day 0 Temperature is recorded every 10 minutes. the standard deviation is obtained for each animal using the mean of values from day -5 to day 0. Non-parametric analysis (Kruskal-Wallis test) Group p-value = <0.001. P-values are presented for pair-wise comparisons from Kruskal-Wallis Test on data from the two treatment groups. Difference = Estimated Median Treatment Difference.

1.3.6 *Clinical study*

The relevant consortium members made preparations to perform a clinical proof-of-principle efficacy study. Whilst awaiting the supply of GMP antigen by Novartis, participant 2 wrote an outline of the study protocol and participant 1 started to prepare the CTA in preparation for submission of the application for the planned clinical trial to the regulatory authorities.Considerations were also given to the type of containers that might be used for the delivery of vaccine to the humans and hence a stability study of the antigen in either glass vials or plastic syringes was performed and the suitability of spray devices was confirmed (section 1.3.1.2). Furthermore to optimise B cell assays, participant 7 used test samples from a Phase I clinical trial of an H5N1 (RG14) vaccine using Matrix M as an adjuvant performed at University of Bergen for a project other than NASPANVAC. B cells called antibody secreting cells (ASC) in the ELISPOT assay were successfully optimised and enumerated. Good levels of long term memory B cell response at 12 months post vaccination was also observed with the highest responses being detected in the adjuvanted groups.

When Novartis declined the supply of GMP antigen for a clinical study on 23 Oct 2011, it became apparent that it was not possible to do a clinical study; no alternative supplier could be identified at that point. Hence the consortium unanimously agreed to focus the efforts and resources to perform a challenge study in an established in-vivo efficacy (ferret). This study showed highly promising results which are discussed above (section 1.3.5.3).

1.3.7 *Conclusion*

Although, for reasons not under the control of the consortium the objective of performing a proof-of-principle clinical study was not accomplished, the robust efficacy data in the ferret model along with the immense immunogenicity (both systemic and local antigen specific immunoglobulins, protective/functional antibodies, several cytokines) mouse data including the evaluation of the most efficacious chitosan type, dose response, effect of previous immunity, vaccine regime and the effect of nasally administered chitosan on local brain cytokine production obtained in this project, forms an excellent comprehensive preclinical package for submission to the regulatory authorities to develop a nasal H5N1 vaccine using chitosan as the mucosal delivery platform. Stability data and administration techniques/devices are confirmed to facilitate such a study.

1.4 The potential impact

1.4.1 *Socio-economic impact*

To date, 606 cases of human H5N1 infections have been reported in 15 different countries of which almost 60% have been fatal. However, any initiative that formulated transmission to/between humans could produce catastrophic levels of infection. A number of strategies and approaches are being brought into play to confront an influenza pandemic. Certain antiviral drugs (Tamiflu, Relenza) are commercially available and are the first line of treatment for health care providers. However, for these drugs to be effective the treatment has to commence within 48 hours of the onset of the symptoms. These drugs belong to a class of medicines called neuraminidase inhibitors which prevent the influenza virus from spreading inside the body and are designed to be active against all clinically relevant influenza virus strains. Due to the rapid production of drifted antigens of the H5N1 virus, there are already indications of development of resistance to these drugs. Hence, the use of antiviral drugs may not provide the treatment that would be required for an avian influenza pandemic.

Vaccination is the cornerstone of influenza prophylaxis. In the event of a pandemic, rapid production and deployment of a pandemic vaccine will limit viral spread, hospitalisations, serious complications of disease and ultimately death. The pandemic virus strain could significantly differ from the virus vaccine strain being stockpiled by various governments as a mean of strategic planning. In recent years there has been an increase in manufacturing of seasonal trivalent influenza doses. There are only a few countries with influenza vaccine manufacturing capacity and these will probably reserve the scarce supply of a pandemic vaccine for their own citizens. The WHO aims for equitable and timely access to vaccines for all peoples of the world. However, the reality is that only a few countries manufacture influenza vaccine; therefore populations in other countries most affected by the pandemic may not have access to a pandemic vaccine. During the swine origin influenza pandemic in 2009, the virus spread globally within two months, highlighting the importance of rapid deployment of vaccine. Developing countries had limited access to pandemic vaccines and a needle free mucosal influenza vaccine would have allowed vaccination with limited numbers of public health providers. The NASPANVAC programme investigated a number of adjuvants, alone and as co-adjuvants, that could enhance the immunogenicity of current vaccines to allow dose-sparing formulations and allow the limited vaccine supply to be used as effectively as possible which could have a real positive effect on controlling a pandemic.

The history of pandemic vaccine development since 1997 provides worrying lessons in the difficulties of preparing influenza vaccines containing novel influenza subtypes. The hope is that these lessons will allow the vaccine industry to be ready to immediately start production of the most efficacious vaccine once a pandemic has been declared. Each manufacturer has its own licensed facility and process for manufacturing and these may not be the best vaccine formulation to provide the best protection in a pandemic. There is an urgent need for research and comparative clinical trials comparing different vaccine formulations with or without adjuvant, vaccine strength, number of doses and routes of administration to provide information on what is the appropriately formulated vaccine and optimal use, to save valuable time in preparation of a pandemic vaccine. The preclinical studies carried out under the NASPANVAC project clearly showed that nasally delivered chitosan adjuvanted vaccine enhanced both local and systemic immune responses thereby combating the virus at its point of entry and also systemically, induced specific cytokines giving insight into Th1/Th2 balance as well as their role in regulation of immunity and inflammation, demonstrated heterologous cross-reactive neutralising antibody response against a couple of H5N1 strains and most of all provided protection against the challenge with the live homologous virus. Although the NASPANVAC programme did not manage to demonstrate the efficacy of a nasally delivered pandemic vaccine in humans, the various issues were extensively addressed and investigated in preclinical studies which now form the basis for further exploitation of the project results in terms of Phase II/III clinical trials, applications for regulatory approval in major jurisdictions, implementation of commercial scale manufacture and global commercialization of a nasal vaccine.

1.4.2 *Wider societal implications of the project*

In view of the excellent safety profile of chitosan and the robust preclinical data produced in the NASPANVAC programme are highly encouraging given that traditionally vaccine adjuvants tend to be relatively more toxic moieties. CSN and TM-CSN were shown to elicit protective immunity against lethal H5N1 challenge in a recognised model of human influenza, the ferret. Thus, the risk : benefit ratio for a chitosan-based vaccine may be favourable when compared with that of more potent adjuvants. A H5N1 pandemic could have a major impact on public services, making self reliant treatment and management of the disease mandatory. The NASPANVAC

project has clearly demonstrated the potential to develop a nasal vaccine which could be self administered and also enable rapid mass vaccination with minimum aid from specialist healthcare providers.

1.4.3 *Main dissemination activities and exploitation of results*

a) The dissemination activities to date have been as follows:

1. Jabbal-Gill I. Nasal vaccine innovation. *J Drug Target.* 2010. Dec;18(10):771-86
2. Jabbal-Gill I, Watts P, Smith A. Chitosan-based delivery systems for mucosal vaccines. In press: Expert Opinion on Drug Delivery, 2012.
3. Signe C. Svindland, Åsne Jul-Larsen, Solveig Andersen, Rishi Pathirana, Abdullah Madhun, Emanuelle Montomoli, Inderjit Jabbal-Gill, Rebecca J. Cox. "The mucosal and systemic immune responses elicited by a chitosan adjuvanted intranasal influenza H5N1 vaccine" Poster presented at *The Oxford influenza conference, Influenza* 2010.
4. Svindland SC, Jul-Larsen Å, Pathirana R, Andersen S, Madhun A, Montomoli E, Jabbal-Gill I, Cox RJ. The mucosal and systemic immune responses elicited by a chitosan-adjuvanted intranasal influenza H5N1 vaccine. *Influenza Other Respi Viruses*. 2012 Mar;6(2):90-100. doi: 10.1111/j.1750-2659.2011.00271.x. Epub 2011 Jul 12.
5. Signe C. Svindland[1], Gabriel Kristian Pedersen, Rishi Pathirana*, Geir Bredholt*, Jane Kristin Nøstbakken*, Åsne Jul-Larsen*, Carlos Guzman‡, Emanuele Montomoli[§], Giulia Lapini[§] Inderjit Jabbal-Gill[*§], Michael Hinchcliffe[*§], Rebecca J. Cox[1]*. A study of Chitosan and c-di-GMP as mucosal adjuvants for intranasal influenza H5N1 vaccine. 2012; submitted to International Society for Influenza and other Respiratory Virus Diseases (ISIRV)

b) Publish the mouse data, especially the cytokines and the ferret data demonstrating the efficacy of a nasally delivered chitosan adjuvanted vaccine in prestigious peer-reviewed scientific journals by the end of 2012.

c) Present the efficacy data in ferrets initially at an international meeting in Q4 2012 and others thereafter.

1.5 Address of the project public website

www.naspanvac.com

2. Use and dissemination of foreground

The prototype nasal H5N1 vaccine developed in the NASPANVAC project can best be taken forward for commercial purposes by a major pharmaceutical company that manufactures or has access to H5N1 antigen. The consortium very much hopes that together with direct representations the current and future publications and presentations at international meetings would enable this process.

Section A (public)

TEMPLATE A1: LIST OF SCIENTIFIC (PEER REVIEWED) PUBLICATIONS, STARTING WITH THE MOST IMPORTANT ONES										
NO.	Title	Main author	Title of the periodical or the series	Number, date or frequency	Publisher	Place of publication	Year of publication	Relevant pages	Permanent identifiers[2] (if available)	Is/Will open access[3] provided to this publication?
1	Nasal vaccine innovation	I Jabbal-Gill	J Drug Target.	18(10), Dec 2010	Informa Healthcare	London	2010	pp. 771-786		no
2	The mucosal and systemic immune responses elicited by a chitosan-adjuvanted intranasal influenza H5N1 vaccine.	Svindland SC	Influenza Other Respi Viruses	6(2), Mar 2012	Wiley-Blackwell	Oxford	2012	pp 90-100	PMID21749672	no
3	Chitosan-based delivery systems for mucosal vaccines	I Jabbal-Gill	Expert Opinion on Drug Delivery	In press	Informa Healthcare	London	2012			no

[2] A permanent identifier should be a persistent link to the published version full text if open access or abstract if article is pay per view) or to the final manuscript accepted for publication (link to article in repository).

[3] Open Access is defined as free of charge access for anyone via Internet. Please answer "yes" if the open access to the publication is already established and also if the embargo period for open access is not yet over but you intend to establish open access afterwards.

TEMPLATE A2: LIST OF DISSEMINATION ACTIVITIES								
NO.	Type of activities[4]	Main leader	Title	Date	Place	Type of audience[5]	Size of audience	Countries addressed
1	*Poster at The Oxford influenza conference, Influenza* 2010.	Signe C. Svindland	The mucosal and systemic immune responses elicited by a chitosan adjuvanted intranasal influenza H5N1 vaccine	*2010 – 21-23 September*	*Oxford*	*Oxford Scientific audience*	*300*	*International*

[4] A drop down list allows choosing the dissemination activity: publications, conferences, workshops, web, press releases, flyers, articles published in the popular press, videos, media briefings, presentations, exhibitions, thesis, interviews, films, TV clips, posters, Other.

[5] A drop down list allows choosing the type of public: Scientific Community (higher education, Research), Industry, Civil Society, Policy makers, Medias ('multiple choices' is possible.

Section B (Confidential[6] or public: confidential information to be marked clearly)
Part B1

Not applicable as no applications for patents, trademarks, registered designs, etc. were made.

TEMPLATE B1: LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS, ETC.					
Type of IP Rights[7]:	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Application reference(s) (e.g. EP123456)	Subject or title of application	Applicant (s) (as on the application)

[6] Note to be confused with the "EU CONFIDENTIAL" classification for some security research projects.

[7] A drop down list allows choosing the type of IP rights: Patents, Trademarks, Registered designs, Utility models, Others.

Part B2
Please complete the table hereafter:

Not applicable

Type of Exploitable Foreground[8]	Description of exploitable foreground	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Exploitable product(s) or measure(s)	Sector(s) of application[9]	Timetable, commercial or any other use	Patents or other IPR exploitation (licences)	Owner & Other Beneficiary(s) involved
	Ex: New supercond uctive Nb-Ti alloy			*MRI equipment*	*1. Medical 2. Industrial inspection*	*2008 2010*	*A materials patent is planned for 2006*	*Beneficiary X (owner) Beneficiary Y, Beneficiary Z, Poss. licensing to equipment manuf. ABC*

In addition to the table, please provide a text to explain the exploitable foreground, in particular:

- Its purpose
- How the foreground might be exploited, when and by whom
- IPR exploitable measures taken or intended
- Further research necessary, if any
- Potential/expected impact (quantify where possible)

[19] A drop down list allows choosing the type of foreground: General advancement of knowledge, Commercial exploitation of R&D results, Exploitation of R&D results via standards, exploitation of results through EU policies, exploitation of results through (social) innovation.
[9] A drop down list allows choosing the type sector (NACE nomenclature) : http://ec.europa.eu/competition/mergers/cases/index/nace_all.html

3. Report on societal implications

Replies to the following questions will assist the Commission to obtain statistics and indicators on societal and socio-economic issues addressed by projects. The questions are arranged in a number of key themes. As well as producing certain statistics, the replies will also help identify those projects that have shown a real engagement with wider societal issues, and thereby identify interesting approaches to these issues and best practices. The replies for individual projects will not be made public.

A General Information *(completed automatically when **Grant Agreement number** is entered.*

Grant Agreement Number:	202083
Title of Project:	Intranasal Pandemic Influenza Vaccine
Name and Title of Coordinator:	Dr Inderjit Gill

B Ethics

1. Did your project undergo an Ethics Review (and/or Screening)? • If Yes: have you described the progress of compliance with the relevant Ethics Review/Screening Requirements in the frame of the periodic/final project reports? Special Reminder: the progress of compliance with the Ethics Review/Screening Requirements should be described in the Period/Final Project Reports under the Section 3.2.2 *'Work Progress and Achievements'*	***No***
2. Please indicate whether your project involved any of the following issues (tick box) :	***YES***
RESEARCH ON HUMANS	
• Did the project involve children?	
• Did the project involve patients?	
• Did the project involve persons not able to give consent?	
• Did the project involve adult healthy volunteers?	
• Did the project involve Human genetic material?	
• Did the project involve Human biological samples?	
• Did the project involve Human data collection?	
RESEARCH ON HUMAN EMBRYO/FOETUS	
• Did the project involve Human Embryos?	
• Did the project involve Human Foetal Tissue / Cells?	
• Did the project involve Human Embryonic Stem Cells (hESCs)?	
• Did the project on human Embryonic Stem Cells involve cells in culture?	
• Did the project on human Embryonic Stem Cells involve the derivation of cells from Embryos?	
PRIVACY	
• Did the project involve processing of genetic information or personal data (eg. health, sexual lifestyle, ethnicity, political opinion, religious or philosophical conviction)?	
• Did the project involve tracking the location or observation of people?	
RESEARCH ON ANIMALS	
• Did the project involve research on animals?	✓
• Were those animals transgenic small laboratory animals?	
• Were those animals transgenic farm animals?	

• Were those animals cloned farm animals?	
• Were those animals non-human primates?	
RESEARCH INVOLVING DEVELOPING COUNTRIES	
• Did the project involve the use of local resources (genetic, animal, plant etc)?	
• Was the project of benefit to local community (capacity building, access to healthcare, education etc)?	
DUAL USE	
• Research having direct military use	No
• Research having the potential for terrorist abuse	No

C Workforce Statistics

3. Workforce statistics for the project: Please indicate in the table below the number of people who worked on the project (on a headcount basis).

Type of Position	Number of Women	Number of Men
Scientific Coordinator	2	6
Work package leaders	6	5
Experienced researchers (i.e. PhD holders)	6	6
PhD Students	2	1
Other		

4. How many additional researchers (in companies and universities) were recruited specifically for this project?	**2**
Of which, indicate the number of men:	none

D Gender Aspects

5.	**Did you carry out specific Gender Equality Actions under the project**?	○ ○ ✓	Yes No

6. Which of the following actions did you carry out and how effective were they?

		Not at all effective				Very effective
❑	Design and implement an equal opportunity policy	○	○	○	○	○
❑	Set targets to achieve a gender balance in the workforce	○	○	○	○	○
❑	Organise conferences and workshops on gender	○	○	○	○	○
❑	Actions to improve work-life balance	○	○	○	○	○ ✓
○	Other:					

7. Was there a gender dimension associated with the research content – i.e. wherever people were the focus of the research as, for example, consumers, users, patients or in trials, was the issue of gender considered and addressed?

○ Yes- please specify

✓ ○ No

E Synergies with Science Education

8. Did your project involve working with students and/or school pupils (e.g. open days, participation in science festivals and events, prizes/competitions or joint projects)?

○ Yes- please specify

✓ ○ No

9. Did the project generate any science education material (e.g. kits, websites, explanatory booklets, DVDs)?

○ Yes- please specify

✓ ○ No

F Interdisciplinarity

10. Which disciplines (see list below) are involved in your project?

○ Main discipline[10]: **3.1**

○ Associated discipline[10]: **1.5** ○ Associated discipline[10]:

G Engaging with Civil society and policy makers

11a	**Did your project engage with societal actors beyond the research community?** *(if 'No', go to Question 14)*	○ ✓ ○	Yes No

11b If yes, did you engage with citizens (citizens' panels / juries) or organised civil society (NGOs, patients' groups etc.)?

○ No

○ Yes- in determining what research should be performed

✓ ○ Yes - in implementing the research

○ Yes, in communicating /disseminating / using the results of the project

[10] Insert number from list below (Frascati Manual).

11c In doing so, did your project involve actors whose role is mainly to organise the dialogue with citizens and organised civil society (e.g. professional mediator; communication company, science museums)?	○ ○ ✓	Yes No

12. Did you engage with government / public bodies or policy makers (including international organisations)

○	No
✓ ○	Yes- in framing the research agenda
○	Yes - in implementing the research agenda
○	Yes, in communicating /disseminating / using the results of the project

13a Will the project generate outputs (expertise or scientific advice) which could be used by policy makers?

○	Yes – as a **primary** objective (please indicate areas below- multiple answers possible)
✓ ○	Yes – as a **secondary** objective (please indicate areas below - multiple answer possible)
○	No

13b If Yes, in which fields?

Agriculture		Energy		Human rights	
Audiovisual and Media		Enlargement		Information Society	
Budget		Enterprise		Institutional affairs	
Competition		Environment		Internal Market	
Consumers		External Relations		Justice, freedom and security	✓
Culture		External Trade		Public Health	
Customs		Fisheries and Maritime Affairs		Regional Policy	
Development Economic and Monetary Affairs		Food Safety			
		Foreign and Security Policy		Research and Innovation	✓
Education, Training, Youth		Fraud		Space	
Employment and Social Affairs		Humanitarian aid		Taxation	
				Transport	

13c If Yes, at which level?

- ○ Local / regional levels
- ○ National level
- ○ European level
- ✓ ○ International level

H Use and dissemination

14. How many Articles were published/accepted for publication in peer-reviewed journals?	**3 to date**
To how many of these is open access[11] provided?	**3**
How many of these are published in open access journals?	**none**
How many of these are published in open repositories?	**none**
To how many of these is open access not provided?	**3**
Please check all applicable reasons for not providing open access:	
✓❑ publisher's licensing agreement would not permit publishing in a repository ❑ no suitable repository available ❑ no suitable open access journal available ❑ no funds available to publish in an open access journal ✓ ❑ lack of time and resources ❑ lack of information on open access ❑ other[12]: ……	

15. How many new patent applications ('priority filings') have been made? *("Technologically unique": multiple applications for the same invention in different jurisdictions should be counted as just one application of grant).*		**none**
16. Indicate how many of the following Intellectual Property Rights were applied for (give number in each box).	Trademark	**Not applicable**
	Registered design	**Not applicable**
	Other	**Not applicable**
17. How many spin-off companies were created / are planned as a direct result of the project?		**none**
Indicate the approximate number of additional jobs in these companies:		

18. Please indicate whether your project has a potential impact on employment, in comparison with the situation before your project:

	❑ Increase in employment, or	❑	In small & medium-sized enterprises
✓	❑ Safeguard employment, or	❑	In large companies
	❑ Decrease in employment,	❑ ✓	None of the above / not relevant to the project
	❑ Difficult to estimate / not possible to quantify		

19. For your project partnership please estimate the employment effect resulting directly from your participation in Full Time Equivalent (*FTE = one person working fulltime for a year*) jobs:	*Indicate figure:* Not quantifiable

[11] Open Access is defined as free of charge access for anyone via Internet.
[12] For instance: classification for security project.

Difficult to estimate / not possible to quantify	❑ ✓

I Media and Communication to the general public

20. As part of the project, were any of the beneficiaries professionals in communication or media relations?

○ Yes ○ No ✓

21. As part of the project, have any beneficiaries received professional media / communication training / advice to improve communication with the general public?

○ Yes ○ No ✓

22 Which of the following have been used to communicate information about your project to the general public, or have resulted from your project?

✓	❑	Press Release	❑ ✓		Coverage in specialist press
✓	❑	Media briefing	❑		Coverage in general (non-specialist) press
	❑	TV coverage / report	❑		Coverage in national press
	❑	Radio coverage / report	❑		Coverage in international press
✓	❑	Brochures /posters / flyers	❑ ✓		Website for the general public / internet
	❑	DVD /Film /Multimedia	❑		Event targeting general public (festival, conference, exhibition, science café)

23 In which languages are the information products for the general public produced?

❑	Language of the coordinator	❑ ✓	English
❑	Other language(s)		

***Question F-10*:** Classification of Scientific Disciplines according to the Frascati Manual 2002 (Proposed Standard Practice for Surveys on Research and Experimental Development, OECD 2002):

FIELDS OF SCIENCE AND TECHNOLOGY

1. NATURAL SCIENCES

1.1 Mathematics and computer sciences [mathematics and other allied fields: computer sciences and other allied subjects (software development only; hardware development should be classified in the engineering fields)]

1.2 Physical sciences (astronomy and space sciences, physics and other allied subjects)

1.3 Chemical sciences (chemistry, other allied subjects)

1.4 Earth and related environmental sciences (geology, geophysics, mineralogy, physical geography and other geosciences, meteorology and other atmospheric sciences including climatic research, oceanography, vulcanology, palaeoecology, other allied sciences)

1.5 Biological sciences (biology, botany, bacteriology, microbiology, zoology, entomology, genetics, biochemistry, biophysics, other allied sciences, excluding clinical and veterinary sciences)

2 ENGINEERING AND TECHNOLOGY

2.1 Civil engineering (architecture engineering, building science and engineering, construction engineering, municipal and structural engineering and other allied subjects)

2.2 Electrical engineering, electronics [electrical engineering, electronics, communication engineering and systems, computer engineering (hardware only) and other allied subjects]

2.3. Other engineering sciences (such as chemical, aeronautical and space, mechanical, metallurgical and materials engineering, and their specialised subdivisions; forest products; applied sciences such as

geodesy, industrial chemistry, etc.; the science and technology of food production; specialised technologies of interdisciplinary fields, e.g. systems analysis, metallurgy, mining, textile technology and other applied subjects)

3. MEDICAL SCIENCES

3.1 Basic medicine (anatomy, cytology, physiology, genetics, pharmacy, pharmacology, toxicology, immunology and immunohaematology, clinical chemistry, clinical microbiology, pathology)

3.2 Clinical medicine (anaesthesiology, paediatrics, obstetrics and gynaecology, internal medicine, surgery, dentistry, neurology, psychiatry, radiology, therapeutics, otorhinolaryngology, ophthalmology)

3.3 Health sciences (public health services, social medicine, hygiene, nursing, epidemiology)

4. AGRICULTURAL SCIENCES

4.1 Agriculture, forestry, fisheries and allied sciences (agronomy, animal husbandry, fisheries, forestry, horticulture, other allied subjects)

4.2 Veterinary medicine

5. SOCIAL SCIENCES

5.1 Psychology

5.2 Economics

5.3 Educational sciences (education and training and other allied subjects)

5.4 Other social sciences [anthropology (social and cultural) and ethnology, demography, geography (human, economic and social), town and country planning, management, law, linguistics, political sciences, sociology, organisation and methods, miscellaneous social sciences and interdisciplinary , methodological and historical S1T activities relating to subjects in this group. Physical anthropology, physical geography and psychophysiology should normally be classified with the natural sciences].

6. HUMANITIES

6.1 History (history, prehistory and history, together with auxiliary historical disciplines such as archaeology, numismatics, palaeography, genealogy, etc.)

6.2 Languages and literature (ancient and modern)

6.3 Other humanities [philosophy (including the history of science and technology) arts, history of art, art criticism, painting, sculpture, musicology, dramatic art excluding artistic "research" of any kind, religion, theology, other fields and subjects pertaining to the humanities, methodological, historical and other S1T activities relating to the subjects in this group]