Module 2.6: Nonclinical Written and Tabulated Summary

2.6.1 INTRODUCTION

GlaxoSmithKline Biologicals (GSK) has developed a pandemic vaccine to immunize against disease caused by infection with a pandemic influenza virus. In addition to the influenza antigen A/H5N1, the GSK pandemic influenza vaccine contains a tocopherol-containing oil-in-water emulsion based adjuvant system, AS03.

GSK's AS03-adjuvanted H5N1 influenza vaccine is a monovalent, split-virion, inactivated, adjuvanted vaccine. The influenza strain contained in the vaccine is A/H5N1/Indonesia/5/2005, prepared by reverse genetics. The antigen is presented in a 10mL Type I glass vial (10 doses) and the AS03 adjuvant presented in a 3mL Type I glass vial (10 doses). At the time of vaccine administration, the entire contents of the adjuvant vial are withdrawn with a syringe and injected into the antigen vial. After mixing, the vaccine can be administered intramuscularly as individual doses of 0.5mL. Two doses of the vaccine should be administered with an interval of 21 days.

The H5N1 antigen is produced in Quebec, Canada according to the licensed process for the monovalent inactivated split virus bulks of GSK's seasonal influenza vaccine FluLaval®. FluLaval has a long manufacturing and safety record, as it has been marketed in its present formulation in Canada since 1992 and in the U.S. since 2006.

The vaccine contains the following residuals from the manufacturing process of the drug substance: formaldehyde, ovalbumin, sucrose and sodium deoxycholate. Because the vaccine is presented in a multidose vial, thimerosal is added to the antigen as a preservative at a final concentration of 5µg per dose after mixing. The composition of the vaccine is given in Table 1.

Table 1 Composition of the Reconstituted AS03-adjuvanted Pandemic Influenza Candidate Vaccine

Component	Quantity per Dose	Analytical Reference
Active Ingredients		
Inactivated Split Virions A/H5N1/Indonesia/5/2005	3.75µg HA	GSK Monograph
AS03 Adjuvant		
Oil-in-water Emulsion Squalene DL-α-tocopherol Polysorbate 80 (Tween 80)	 10.69mg 11.86mg 4.86mg	 GSK Monograph Ph. Eur. 0692 Ph. Eur. 0428
Excipients		
Thimerosal	5µg	USP
Sodium Chloride	3.895mg	USP/Ph. Eur. 0193
Disodium Hydrogen Phosphate	0.613mg	USP/Ph. Eur. 0118
Potassium Dihydrogen Phosphate	0.09mg	NF/Ph. Eur. 0920
Potassium Chloride	0.09mg	USP/Ph. Eur. 0185
Water for Injections	ad 0.5mL	USP/Ph. Eur. 0169

Abbreviations: USP, United States Pharmacopeia; NF, United States National Formulary; Ph. Eur., European Pharmacopoeia.

The nonclinical development of AS03-adjuvanted Quebec H5N1 influenza vaccine has focused on two different aspects: (1) the pharmacology profile, which includes immunogenicity in naïve mice, immunogenicity and protective efficacy in naive ferrets, as well as investigation in the mode of action of AS03, and (2) the safety profile.

Immunogenicity and protective efficacy studies were conducted to demonstrate the efficacy of the adjuvanted vaccine with respect to its ability to elicit robust immune response and its protective effects against mortality and morbidity induced by challenge with viral strains homologous and heterologous to the vaccine strain. In parallel, *in vitro* and *in vivo* studies were conducted to better understand the mechanism by which AS03 combined with split antigen promote robust and persistent humoral and cellular responses.

The safety profile of the AS03-adjuvanted Quebec H5N1 influenza vaccine has been evaluated in single dose toxicity, repeated dose toxicity and local tolerance studies in rabbits, and in a safety pharmacology study in rats using antigen adjuvanted with AS03. These studies used Quebec- or Dresden-sourced H5N1 antigens, or A/H3N2 antigens manufactured according to the same process as the H5N1 antigen. Reproductive and developmental toxicity studies were also conducted with Quebec trivalent seasonal vaccine and with the AS03 adjuvanted Dresden H5N1 influenza vaccine. A reproductive toxicity study with the AS03-adjuvanted Quebec H5N1 influenza vaccine is currently underway, as are two additional repeated dose toxicity studies. In addition, three genotoxicity studies have been performed with the AS03 adjuvant alone.

These data are summarized in m2.6.2 through m2.6.7.

Module 2.6: Non Clinical Summary

2.6.2 PHARMACOLOGY WRITTEN SUMMARY

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2.6.2.1 Short Summary

GSK has developed a vaccine for prophylaxis against disease caused by pandemic A/H5N1 influenza virus subtypes. This vaccine contains purified antigen fractions of inactivated monovalent, split virions from A/H5N1 strain. Since the population is immunologically naïve towards pandemic influenza viruses and influenza viruses in general are prone to antigenic drift, a strong immunity needs to be induced by the vaccine. The Company's H5N1 pandemic influenza vaccine is therefore adjuvanted with the AS03 oil-in-water emulsion adjuvant.

Over the past two years, several animal studies were performed in mice and ferrets to investigate the primary pharmacodynamics of the AS03-adjuvanted Quebec H5N1 influenza vaccine. Immunogenicity studies were performed in naive mice to evaluate the impact of different dose of AS03, antigen or adjuvant/antigen ratio on the humoral response (in terms of haemagglutination inhibition and neutralizing antibodies) induced by the vaccine. In addition, the protective efficacy of the adjuvanted vaccine was assessed in ferrets. These protection studies were performed to evaluate the potential of the H5N1 vaccine to reduce disease symptoms (mortality and morbidity) and viral shedding in the lungs and in nasal secretions of ferrets challenged with homologous and heterologous strains. The objectives of these experiments were also to demonstrate the efficacy of an adjuvanted H5N1 vaccine compared to the plain (non-adjuvanted) vaccine. These studies are summarized in Table 1. Overall, the immunogenicity studies in mice and protection studies in ferrets clearly demonstrated a significant increase in humoral immunity due to the presence of the adjuvant. Moreover, vaccination with AS03-adjuvanted H5N1 vaccine was able to protect ferrets from mortality and morbidity caused by infection with a H5N1 virus strain homologous or heterologous to the vaccine strain.

In accordance with the guideline on adjuvant in vaccines for human use (EMEA/CHMP/VEG/134716/2004), the Company has performed an investigation into the mode of action of the AS03 adjuvant system. A series of *in vitro* and *in vivo* experiments were conducted to better understand the mechanism by which AS03 combined with split antigen promote strong and persistent humoral and cellular responses. An overview of the different non-clinical experiments that have been performed is provided in the module 4 of this application. The available data indicate that AS03 does not act as a "delivery system" for the influenza split antigen but rather works as an immunostimulant. AS03 promotes the maturation of APC, increases costimulatory properties and favor the production of cytokines including IL-6, TNFα and IFNγ.

In addition to these studies, a safety pharmacology experiment in rats was also conducted to assess the vaccine effect on cardiovascular and respiratory systems. Administration of AS03-adjuvanted Quebec H3N2 influenza vaccine did not produce any treatment-related effects on any recorded cardiovascular or respiratory parameters, supporting the safety profile of the vaccine composition.

Table 1 List of immunogenicity and protection studies performed in animals with influenza antigens and the AS03 adjuvant

Study type	Test System	Antigen	Adjuvant	Read out
Immunogenicity studies				
Dose range H5N1 (A/Vietnam/1194/04) and AS03	C57Bl/6 mice: naïve	H5N1 5µg, 1µg, 0.2µg, 0.04µg	AS03	- HI titers -IgG concentrations
Dose range H5N1 (A/Indonesia/5/2005) and AS03	C57Bl/6 mice: naïve	H5N1 5µg, 1µg, 0.2µg 0.04µg	AS03, AS03 1/2	- HI titers -IgG concentrations
Protection studies				
H5N1 homologous challenge Vaccination and challenge with A/Indonesia/5/2005	Naïve ferrets	H5N1: 7.5µg, 3.8µg, 1.9µg	AS03, AS03 1/2	- Virus titration - HI titers - NI titers - Clinical signs
H5N1 heterologous challenge Vaccination: A/ndonesia/5/2005 Challenge: A/Hong Kong/156/97	Naïve ferrets	H5N1: 3.8µg, 1.5µg, 0.6µg, 0.24µg,	AS03, AS03 1/2	- Virus titration - HI titers - NI titers - Clinical signs

Abbreviations: HI = Hemagglutinin Inhibition, NI, Neutralizing Antibodies

2.6.2.2 Primary Pharmacodynamics

Two different aspects of the pharmacology profile of the AS03-adjuvanted H5N1 Quebec influenza vaccine have been studied:

1. The ability of the vaccine to elicit an immune response in animals. Immunogenicity studies in naïve mice and immunogenicity and efficacy studies in a naïve ferret model were conducted to demonstrate the immunogenicity as well as the efficacy of the adjuvanted vaccine against mortality and morbidity induced by challenge with viral strains homologous and heterologous to the vaccine strain.
2. The mode of action of the AS03 adjuvant system. *In vitro* and *in vivo* experiments were conducted to explore the mechanism by which AS03 combined with split antigen promote strong and persistent humoral and cellular response.

2.6.2.2.1. Evaluation of the impact of AS03 on the humoral response induced in mice immunized with H5N1 vaccines.

In the event of an influenza pandemic, it is anticipated that the world's population will be immunologically naïve to the newly circulating strain causing the pandemic, and therefore it is anticipated that two vaccine doses will be required to induce protective immunity. To mimic this immunologically naïve state in humans, a preclinical model was developed in unprimed mice to evaluate the immunogenicity of adjuvanted-H5N1 candidate vaccines.

Two experiments were performed to evaluate the impact of the AS03 on the humoral response induced in naïve mice (C57 BL/6) immunized with the AS03-adjuvanted Quebec H5N1 influenza vaccine. The primary endpoint of these experiments was to

determine the need of the AS03 adjuvant and/or the antigen/adjuvant ratio required to obtain in the best immunogenicity of the vaccine antigen.

C57BL/6 a common inbred strain of laboratory mouse was used for both experiments. This strain has certain immunophenotypes that distinguish it from other inbred strains such as BALB/c. For example the immunological responses to the same pathogens in C57BL/6 mice show a mixed Th1-Th2-type profile whereas in contrast BALB/c show a Th2-biased profile.

A. Evaluation of different doses of H5N1 (A/Vietnam/1194/04) with a fixed dose of AS03

The first experiment (study n°5690-■) was conducted in naïve mice to evaluate humoral responses induced by influenza vaccines formulated with various doses of H5N1 (A/Vietnam/1194/04) with and without AS03.

Groups of 10 eight-week-old female unprimed C57BL/6 mice were immunized intramuscularly on study days 0 and 21 with doses of A/Vietnam/1194/04 Quebec H5N1 representing 0.04, 0.2, 1, or 5µg of heamagglutinin, with and without a full dose equivalent (1/10 human dose) of AS03. Whole blood was collected at sacrifice on study day 35, and the amplitude and frequency of the serum antibody response to the immunizing strain was determined by enzyme-linked immunosorbent assay (measurement of IgG virus-specific antibody) and haemagglutination inhibition (HI) using horse erythrocytes (measurement of total functional antibody). The experimental groups are summarized in Table 2.

Table 2 Experimental groups in study n°5690-01 – antigen dose range in mice

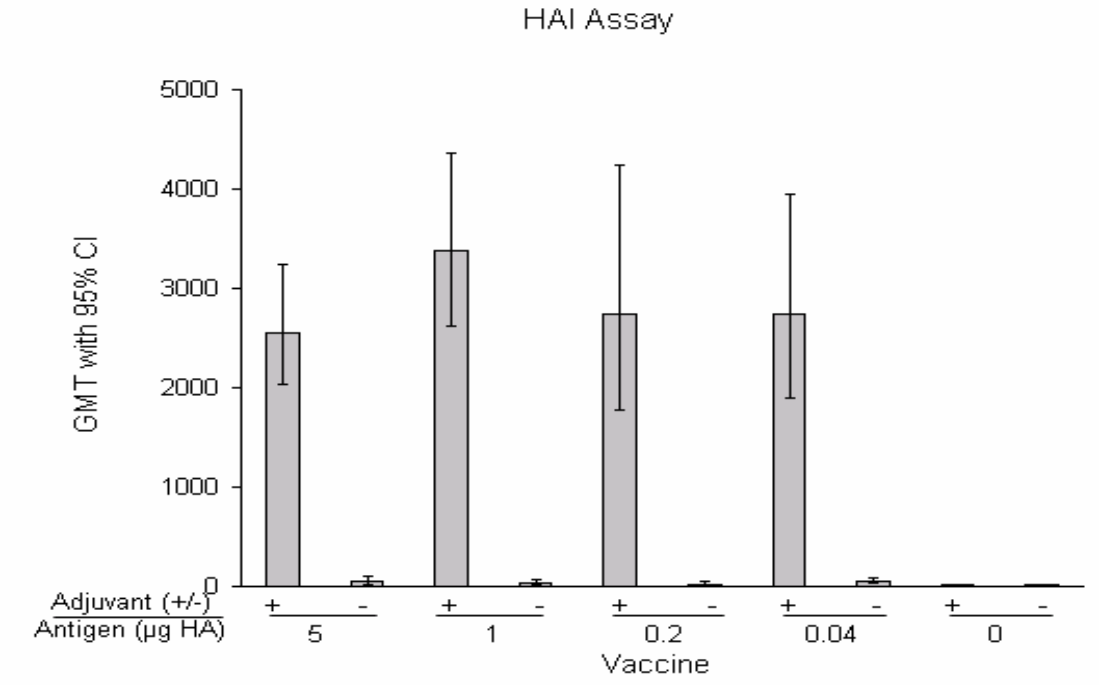
Group	Antigen content	Adjuvant content
1	5 µg H5N1	Full dose equivalent (1/10 HD)
2	5 µg H5N1	-
3	1 µg H5N1	Full dose equivalent (1/10 HD)
4	1 µg H5N1	-
5	0.2 µg H5N1	Full dose equivalent (1/10 HD)
6	0.2 µg H5N1	-
7	0.04 µg H5N1	Full dose equivalent (1/10 HD)
8	0.04 µg H5N1	-
9	-	Full dose equivalent (1/10 HD)
10	PBS	-

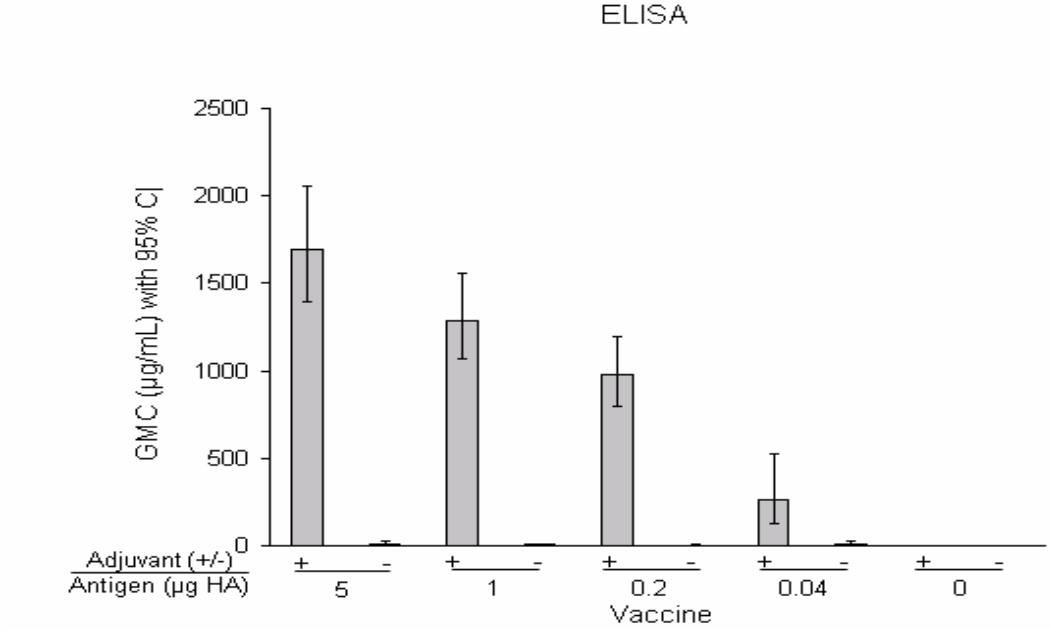
Abbreviation: HD = Human Dose

Results are presented in Figure 1. An increase of anti-A/Vietnam immune responses (anti-H5N1 ELISA and HI titers) was observed when the antigen was administered with, but not without AS03 indicating the benefit of the adjuvantation. An antigen dose response was observed when IgG antibody was measured by enzyme-linked immunosorbent assay, but no dose response was seen when total antibody was measured by haemagglutination inhibition. The latter observation suggests that the epitopes responsible for haemagglutination inhibition are immunodominant and induce antibody responses very efficiently at low doses. All of the additional epitopes in the antigen

mixture are recognized at relatively higher doses resulting in increasing amounts of serum IgG without change in haemagglutination inhibition activity.

Figure 1 Serum haemagglutination inhibition titers (GMT ± 95% CI) and serum IgG concentrations by ELISA (GMC ± 95% CI) in mice immunized with various doses of Quebec-H5N1 antigen (A/Vietnam/1194/04) with and without AS03





B. Evaluation of different doses of H5N1 (A/Indonesia/5/2005) and AS03

To further evaluate the optimal antigen/adjuvant ratio, a new dose range study was performed in mice. In this study (study n° 20[redacted]0787), two doses of AS03 adjuvant (full- and half -dose) were evaluated in combination with several doses (5.0, 1.0, 0.2 or 0.04µg of HA) of H5N1 A/Indonesia/5/2005 antigen.

Briefly, six to eight-week-old female unprimed C57BL/6 mice were immunized intramuscularly on study days 0 and 21 with indicated amounts of haemagglutinin (HA) antigen adjuvanted with full dose equivalent (1/10 human dose) of AS03 or half dose equivalent of AS03 adjuvant. Control animals received 5µg HA without adjuvant, phosphate-buffered saline (PBS) with full dose equivalent AS03, PBS with half dose equivalent AS03, or PBS without adjuvant. Whole blood was collected at sacrifice on study day 35, and the amplitude and frequency of the serum antibody response to the immunizing strain was determined by enzyme-linked immunosorbent assay (measurement of IgG binding antibody) and by haemagglutination inhibition (HAI) using horse erythrocytes (measurement of total functional antibody). Test items administered to each group are described in Table 3.

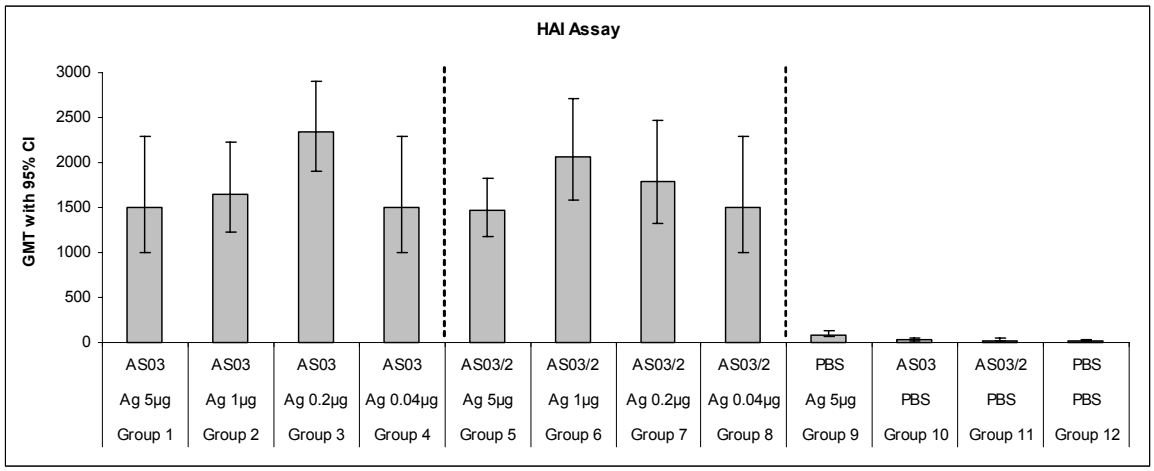
Table 3 Experimental groups in study n°20070787 – antigen and adjuvant dose range in mice

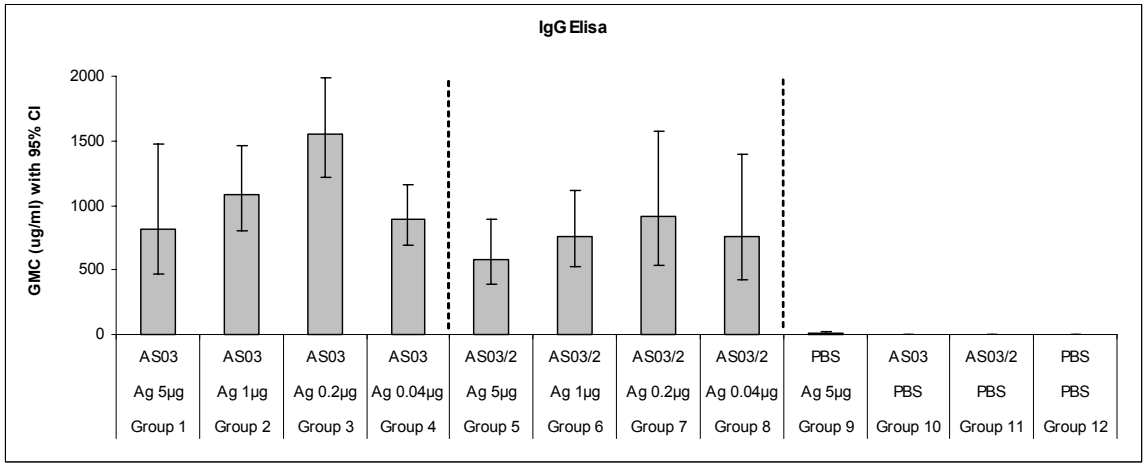
Group No.	N	Antigen Dose (µg HA)	Adjuvant
1	8	5.0	Full dose equivalent (1/10 HD)
2	8	1.0	Full dose equivalent (1/10 HD)
3	8	0.2	Full dose equivalent (1/10 HD)
4	8	0.04	Full dose equivalent (1/10 HD)
5	8	5.0	Half dose equivalent (1/20 HD)
6	8	1.0	Half dose equivalent (1/20 HD)
7	8	0.2	Half dose equivalent (1/20 HD)
8	8	0.04	Half dose equivalent (1/20 HD)
9	8	5.0	–
10	8	PBS	Full dose equivalent (1/10 HD)
11	8	PBS	Half dose equivalent (1/20 HD)
12	8	PBS	–

Abbreviation: HD = Human Dose

Results are presented in Figure 2. For all antigen doses tested, increases of anti-A/Indonesia antibody responses were detected by both serum IgG ELISA and HI assays when the antigen was administered with full dose equivalent and half dose equivalent of AS03, but not when the highest antigen dose was administered without adjuvant indicating a clear effect of the AS03 adjuvant. These data confirm the initial observation made with A/Vietnam/1194/2004 antigen demonstrating that when unadjuvanted, the Quebec H5N1 antigen is a poor immunogen when administered intramuscularly to naïve mice. The results also show that there is a trend for stronger IgG response with full dose equivalent adjuvant groups compared to half dose equivalent adjuvant groups at a given dose of antigen. This trend is not observed using the HAI assay.

Figure 2 Serum Haemagglutination inhibition titers (GMT ± 95% CI) and serum IgG concentrations by ELISA (GMC ± 95% CI) in mice immunized with various doses of Quebec antigen (A/Indonesia/5/2005) with and without AS03





AS03 = full dose AS03, AS03/2 = ½ dose of AS03, PBS = phosphate saline buffer, Ag = antigen H5N1

C. Conclusion

In conclusion, results of experiments performed in naïve mice showed that the immune response induced by AS03-adjuvanted H5N1 vaccines is significantly higher than for non adjuvanted vaccines; thus clearly demonstrating the need for adjuvantation. However, no clear antigen or adjuvant dose effect was observed.

2.6.2.2.2. Protection studies in naive ferrets immunized with the adjuvanted H5N1 influenza vaccine

Ferrets are currently considered to be the most suitable animal model for preclinical evaluation of human influenza vaccines. Indeed, ferrets are natural hosts for influenza and can be easily infected with human isolates. Infection of ferrets with influenza results in systemic and upper respiratory system symptoms including fever, runny nose, and coughing.

The AS03-adjuvanted Quebec H5N1 (A/Indonesia/5/05) influenza vaccine was tested in the ferret model to evaluate the potential of this vaccine to reduce disease symptoms (body temperature, weight loss, and histopathological changes in the respiratory tract and viral loads in the upper (pharynx) and lower (lung) respiratory tract of ferrets challenged with homologous (A/Indonesia/5/05) or heterologous (A/Hong Kong/156/97) strains.

1. Challenge study with a homologous H5N1 strain (A/Indonesia/5/05)

In this protection study ([redacted] study n° 2810117), eight groups of ferrets (n=6) were immunized intramuscularly with three different doses of Quebec H5N1 (A/Indonesia/5/05) split virus antigen (7.5, 3.8 and 1.9µg) in combination with full human dose of AS03 adjuvant (referred to as AS03) or half human dose of AS03 adjuvant (referred to as AS03/2). Two control groups included animals immunized with unadjuvanted antigen (7.5µg HA) or a full human dose of AS03 alone. Ferrets were vaccinated on days 0 and 21. On day 49 all animals were challenged intratracheally with lethal dose of wild-type H5N1 A/Indonesia/5/05 virus, 10^5 $TCID_{50}$ (50% tissue culture infective dose). Throat swabs were collected prior to challenge (day -1) and on days 2, 4 and 5 after challenge to assess virus shedding in the upper respiratory tract. In addition, to evaluate the immune response (HI and neutralizing antibody titers) induced by the vaccine, whole blood was collected pre-immunization (day 0) and on study day 21, 42 and 48. On day 54, animals were euthanized as scheduled and scored for gross-pathology. After death, lung tissue was collected from each animal for measurement of viral loads and histological assessment. Test items administered to each group are described in Table 4. Homologous protection was assessed by analyzing survival rate, pathology, body temperatures, body weight and viral titers in throat swabs and lung tissue.

Table 4 Experimental groups in study n°2810117 – homologous protection study in ferrets

Group	Vaccine composition	Antigen dose	Adjuvant AS03
1	H5N1 with adjuvant	7.5µg	full-dose equivalent
2	H5N1 with adjuvant	3.8µg	full-dose equivalent
3	H5N1 with adjuvant	1.9µg	full-dose equivalent
4	H5N1 with adjuvant	7.5µg	half-dose equivalent
5	H5N1 with adjuvant	3.8µg	half-dose equivalent
6	H5N1 with adjuvant	1.9µg	half-dose equivalent
7	H5N1 alone	7.5µg	-
8	AS03 alone	-	full-dose equivalent

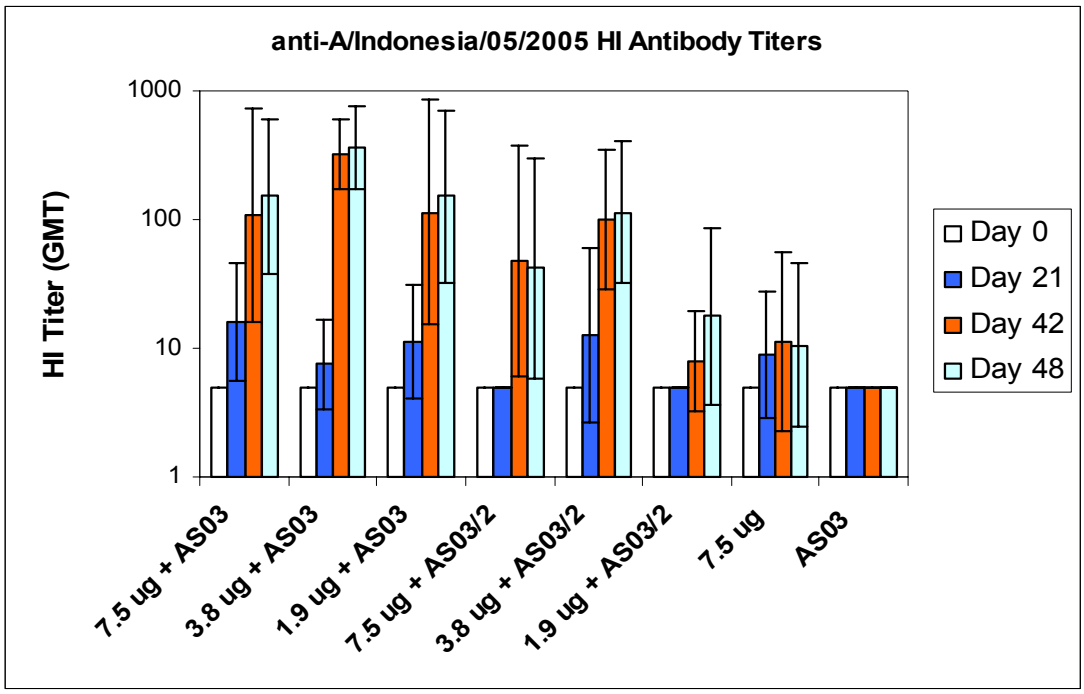
A. Humoral immune responses (HI and neutralizing titers)

The humoral immune response (HI titers and neutralizing antibody titers) to vaccination was measured after each immunization on days 21, 42, and 48 (one day prior to viral challenge on day 49). Serum samples were tested by the haemagglutination inhibition (HI) and virus neutralization assays.

Anti -A/Indonesia/05/2005 (H5N1) haemagglutination antibody titers:

HI antibody titers to the homologous virus are shown in Figure 3. AS03-adjuvanted Quebec H5N1 influenza vaccine induced significantly higher humoral responses compared to ferrets immunized with the unadjuvanted influenza vaccine demonstrating a strong effect of the adjuvant. No antigen dose response effect was observed. However there is a trend for higher immune response in ferrets having received the full-dose of AS03 compared to those who have received half-dose of AS03. There was a clear boosting effect following the second immunization on day 21. HI titers were at or near their peak at the time of viral challenge (day 48).

Figure 3 Prechallenge homologous serum HI antibody titers (GMT ± 95% CI) in ferrets immunized with various doses of Quebec-H5N1 antigen (A/Indonesia/05/2005) formulated with full- or half-dose AS03



AS03 = full dose AS03, AS03/2 = ½ dose of AS03

Anti-A/Indonesia/05/2005 (H5N1) neutralizing antibody titers:

To further characterize the immune response induced by the AS03-adjuvanted Quebec H5N1 influenza vaccines, the ferret sera were tested for their neutralization antibodies response to the H5N1 A/Indonesia/5/05 strain. As shown in Figure 4, AS03-adjuvanted

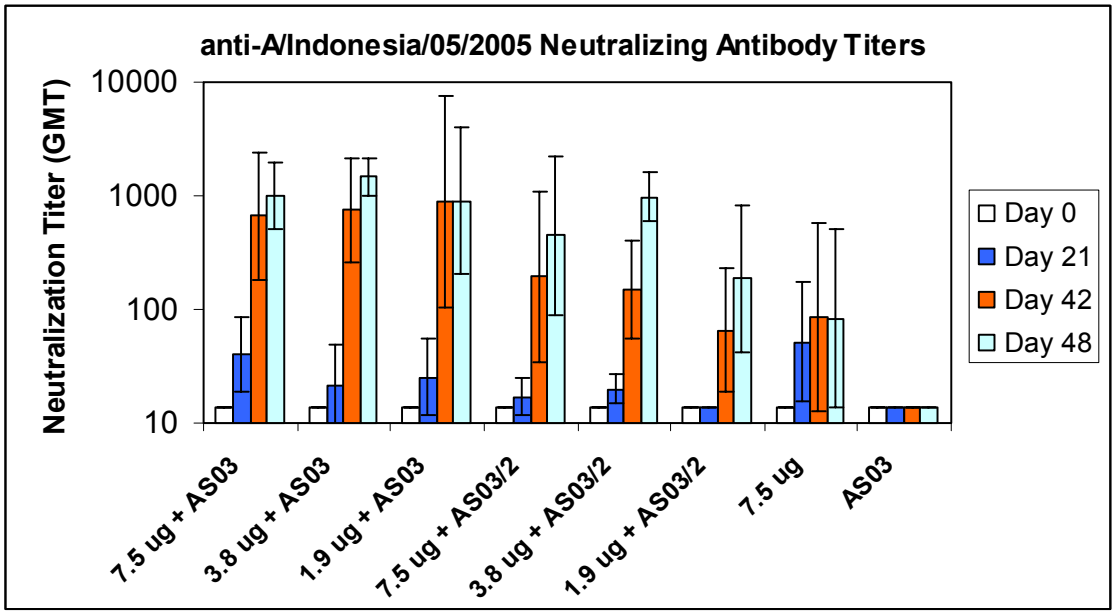
Quebec H5N1 vaccines induced high neutralizing antibodies to the homologous strain while low detectable titers were obtained in ferrets immunized with AS03 alone. As was the case with the HI responses, induction of H5N1 specific antibodies required two immunizations in these naïve animals. No clear antigen dose effect was observed. The neutralization responses were at or near their peak at the time of viral challenge.

In addition to determining the neutralizing antibody response to A/Indonesia (clade 2.1 strain) the cross-neutralization responses were also determined to the following three subtypes drift variants: A/Turkey/Turkey/01/2005 (clade 2.2 strain), A/Vietnam/1194/2004 (clade 1 strain), A/Anhui/01/2005 (clade 2.3 strain). The results are shown in Figure 5. Of the three drift strains tested, the anti-Turkey/Turkey responses appeared the highest. As with the anti-A/Indonesia responses, there was a clear boosting effect following the second immunization, but there was no apparent antigen or adjuvant dose response.

Conclusion

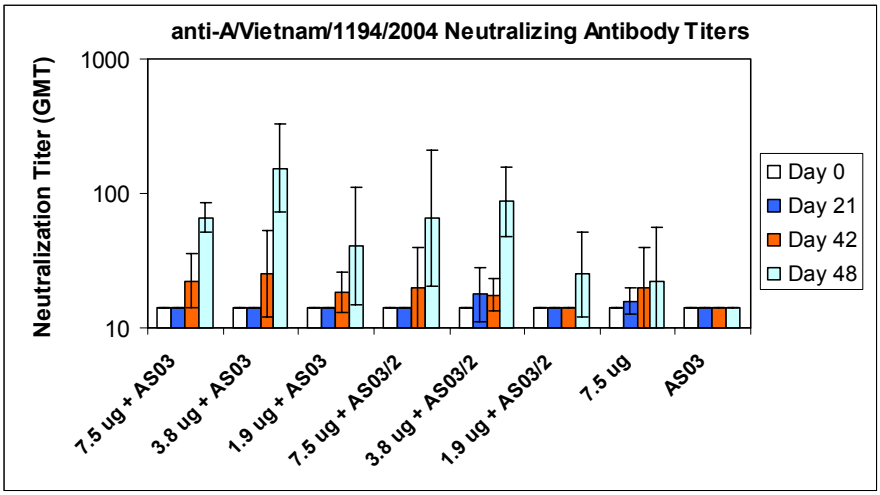
In conclusion, the AS03-adjuvanted Quebec H5N1 influenza vaccine is strongly immunogenic in naive ferrets. In addition to eliciting neutralizing antibodies to the homologous immunizing strain, the candidate vaccine was able to induce cross-neutralizing antibodies to three H5N1 drift variants from three different clades. These results highlight the potential of the adjuvanted H5N1 influenza vaccine to induce, even with a low dose of antigen, cross-protective response in ferrets against a lethal challenge with homologous H5N1 virus.

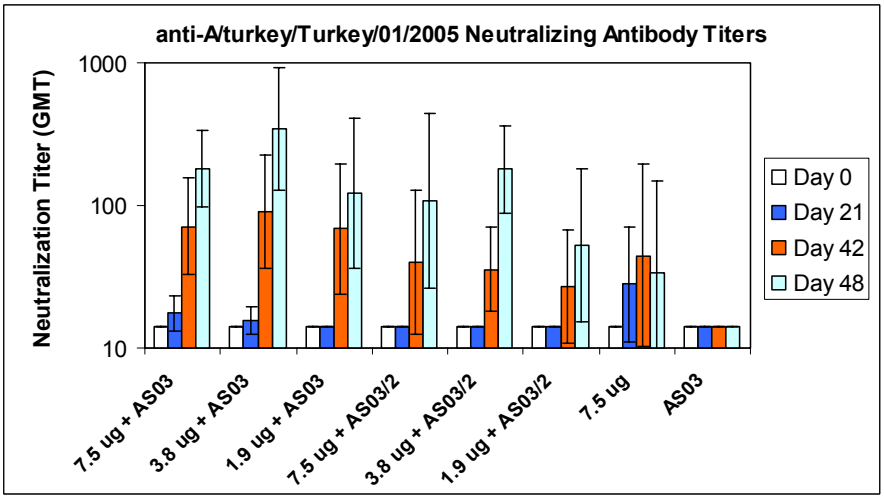
Figure 4 **Prechallenge serum homologous neutralization antibody titers (GMT ± 95% CI) in ferrets immunized with various doses of Quebec-H5N1 antigen (A/Indonesia/05/2005) formulated with full- or half dose AS03**

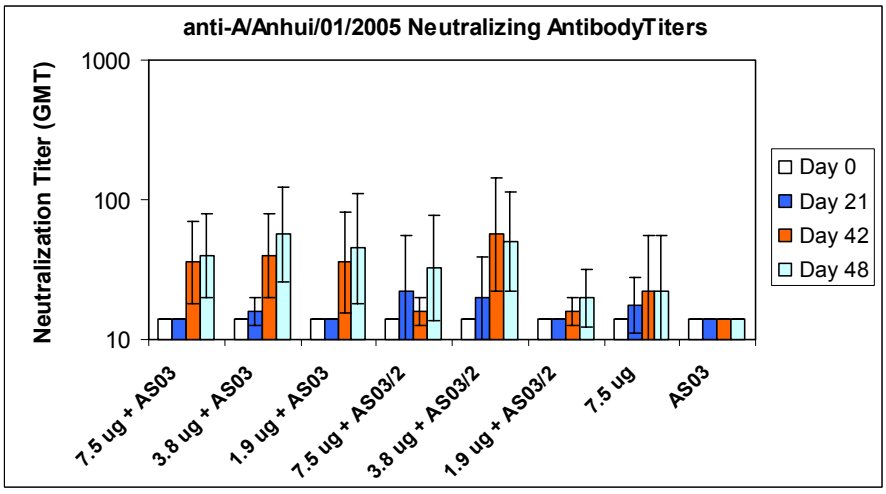


AS03 = full dose AS03, AS03/2 = ½ dose of AS03

Figure 5 **Prechallenge serum heterologous neutralization antibody titers against A/Vietnam/1194/04, A/turkey/Turkey/01/2005, and A/Anhui/01/2005 (GMT ± 95% CI) in ferrets immunized with various doses of Quebec-H5N1 antigen (A/Indonesia/05/2005) formulated with full- or half-dose AS03**







AS03 = full dose AS03, AS03/2 = ½ dose of AS03

B. Mortality and viral detection

All ferrets that received two doses of adjuvanted H5N1 influenza vaccines survived the lethal homologous challenge. Thus, overall, 100% of animals immunized with AS03-adjuvanted Quebec H5N1 influenza vaccine were protected against lethal homologous challenge with A/Indonesia/5/05 and survived to the end of the challenge phase on day 5. A summary of mortality and viral detection is given in Table 5. At 3 days after the challenge three animals were found dead in the 7.5µg unadjuvanted antigen control group, three animals had to be euthanized as they were moribund and one animal in the adjuvant control group was found dead. At 4 days after challenge, 1 animal of the adjuvant control group was found dead. The remaining animals (three ferrets in the H5N1 control group and one in AS03 control group) survived until scheduled euthanasia on day 5.

Table 5 Protection of AS03-adjuvanted Quebec H5N1 (A/Indonesia/05/2005)-vaccinated ferrets against challenge with homologous H5N1 influenza virus: mortality and viral detection.

group	antigen dose (µg HA + adj)	Mortality		Pharyngeal viral shedding Titration		Lung viral load Titration	
		Dead/total	% survival	Viral load*	%**	Viral load*	%**
1	7.5 +AS03	0/6	100 %	- - - - - -	0 %	- - - - - -	0 %
2	3.8 +AS03	0/6	100 %	- - - - - -	0 %	- - - - - -	0 %
3	1.9 +AS03	0/6	100 %	- - - - - -	0 %	- - - - - -	0 %
4	7.5 +AS03/2	0/6	100 %	- - - - - +	17 %	- - - - - +	17 %
5	3.8 +AS03/2	0/6	100 %	- - - - - -	0 %	- - - - - -	0 %
6	1.9 +AS03/2	0/6	100 %	- - - - - +	17 %	- - - + + +	50 %
7	7.5	3/6	50 %	- - - - + +	33 %	- - + + + +	67 %
8	AS03 alone	5/6	17 %	- + + + + +	83 %	+ + + + + +	100 %

*- below detection level, + above detection level
** Percentage of animal with viral load above detection level

Analysis of lung tissues and throat swabs showed that most animals in the control groups (immunized with the adjuvant alone or antigen alone) contained high viral loads as assessed by virus titration analysis. In contrast, 100% of ferrets immunized with full dose AS03-adjuvanted H5N1 vaccine, lung and pharyngeal virus load were below detection level (Table 5). Although low, the virus load observed in the ferret immunized with the half dose AS03-adjuvanted H5N1 influenza vaccine were generally higher than those observed in ferret immunized with full dose AS03-adjuvanted vaccines suggesting that full dose of AS03 may induce greater protection from viral replication in the respiratory tract (lung and pharynx) compared to half dose of AS03. There was no overall antigen-dose dependent effect on viral load observed among ferrets immunized with adjuvanted influenza vaccine.

In ferrets immunized with the adjuvant alone or with the non-adjuvanted H5N1 split vaccine, the absence of protection against viral replication in the lung and viral shedding

in the upper respiratory tract is correlated with the low humoral responses, which resulted in high mortality rate.

C. Gross-pathology and histopathology

There were more animals in the control groups (antigen alone group and AS03 alone group) in poor clinical condition (moribund), and/or found dead, compared to the other groups, which indicated a relationship to the treatment. Moribund animals showed general depression, anorexia, lethargy and exhibited clinical signs of respiratory disease, including dyspnea. All animals that died prematurely, showed signs of atypical pneumonia that could be confirmed by macroscopy and/or microscopy analysis.

Changes in the trachea (inflammation of respiratory epithelium) were observed in all animals of the AS03 control group that died before schedule. These animals also occasionally showed more prominent adverse pulmonary changes compared to the other groups (bronchiolitis obliterans, (peri-)bronch(iol)itis and primary atypical pneumonia). Tracheal epithelial change/squamous epithelium, which might have been a result of (repeated) damage, can also be regarded as a regenerative response subsequent to inflammation/damage. Enlarged lymph nodes were observed in animals of all groups and are considered to be due to a specific immune response to infection in groups receiving antigen. However, there does not appear to be any antigen dose-relationship with enlarged bronchial lymph nodes.

When comparing the adjuvanted vaccine groups (full-dose AS03 vs. half-dose AS03) at comparable antigen dosages, there seems to be slightly less overall pulmonary pathology in animals having received half-dose of AS03 compared to animals having received full dose of AS03.

Overall, these data indicate a treatment-relationship for the AS03-adjuvanted Quebec H5N1 influenza vaccine in reducing morbidity and pulmonary pathology of H5N1 affected animals compared to control animals, with animals having received 3.8µg HA + half-dose AS03 showing lowest incidence/grading of overall pulmonary pathology.

D. Clinical signs

Body weight

Regarding the body weight, the following observations can be made:

- In the groups that received antigen adjuvanted with full dose AS03, modest or no weight loss was observed.
- In the groups that received antigen adjuvanted with half dose AS03, more weight loss (approx. 7% on average) was observed in the groups that received 7.5 and 1.9µg HA than the group that received 3.8µg HA dose of antigen.
- In the antigen alone and adjuvant alone groups, marked weight loss (approx. 15% on average) was observed.

Temperature

Fever was induced in all but one of the animals of the antigen and adjuvant control groups with body temperatures peaking at 41ºC between 12 to 48 hours following the start of challenge. The majority of the animals vaccinated with different doses of adjuvanted vaccine showed no or milder temperature elevations.

E. Conclusion

In conclusion, a range of doses of Quebec H5N1 antigen combined with AS03 adjuvant was able to protect against mortality and to reduce lung virus loads and viral shedding in the respiratory tract after intratracheally challenge with a homologous wild-type H5N1 virus. In addition, the AS03-adjuvanted Quebec H5N1 influenza vaccine was able to induce, even at low doses of antigen, a robust immune response to the homologous vaccine strain as well as against three H5N1 drift variants representing three different virus clades in ferrets. This latter result suggests that the adjuvanted H5N1 vaccine could elicit cross-protection against other H5N1 strain subtypes not present in vaccine. Conversely ferrets immunized with the non-adjuvanted Quebec H5N1 influenza vaccine were not protected from death and showed similar lung viral loads and degree of viral shedding in the upper respiratory tract as those exhibited by ferrets immunized with the adjuvant alone. Moreover, the lack of protection from challenge in animals that received either unadjuvanted vaccine or AS03 alone was associated with poor immune responses.

2. Challenge study with a heterologous H5N1 strain: A/Hong Kong/156/97

Following the efficient cross protective immunity observed in the homologous challenge study, the AS03-adjuvanted Quebec H5N1 A/Indonesia/5/05 influenza vaccine was evaluated in naïve ferrets for its potential to induce cross-protective immunity against the A/Hong Kong/156/97 drifted strain.

In this heterologous challenge study ([redacted] study n° 2810147), four groups of ferrets (n=8) were immunized intramuscularly with two doses of 3.75, 1.5, 0.6 and 0.24µg HA of Quebec H5N1 (A/Indonesia/5/05) virus antigen adjuvanted with half human dose of AS03. One additional group of ferrets was immunized with two doses of 3.75µg HA adjuvanted with full human dose of AS03. The control group included ferrets immunized with an unadjuvanted vaccine containing 3.75µg of HA. Ferrets were vaccinated on days 0 and 21 and challenged intratracheally on day 49 with a lethal dose of H5N1 A/Hong Kong/156/97 virus, 10^5 $TCID_{50}$ (50% Tissue Culture Infective Dose). Nasal/throat swabs were collected prior to challenge (day -1) and on days 2,4 and 5 after challenge to assess virus shedding in the upper respiratory tract. In addition, to evaluate the immune response induced by the vaccine (haemagglutination inhibition titers and neutralizing antibody titers), whole blood was collected pre-immunization (day 0) and on study day 21, 42 and 48. On day 54, animals were euthanized as scheduled and scored for gross-pathology. After death, lung tissue was collected from each animal for measurement of viral loads and histological assessment. Test items administered to each group are described in Table 6. Heterologous protection was assessed by analyzing survival rate, pathology, body temperatures, body weight and viral titers in throat swabs and lung tissue.

Table 6 Experimental groups in study n°2810147 – heterologous protection study in ferrets

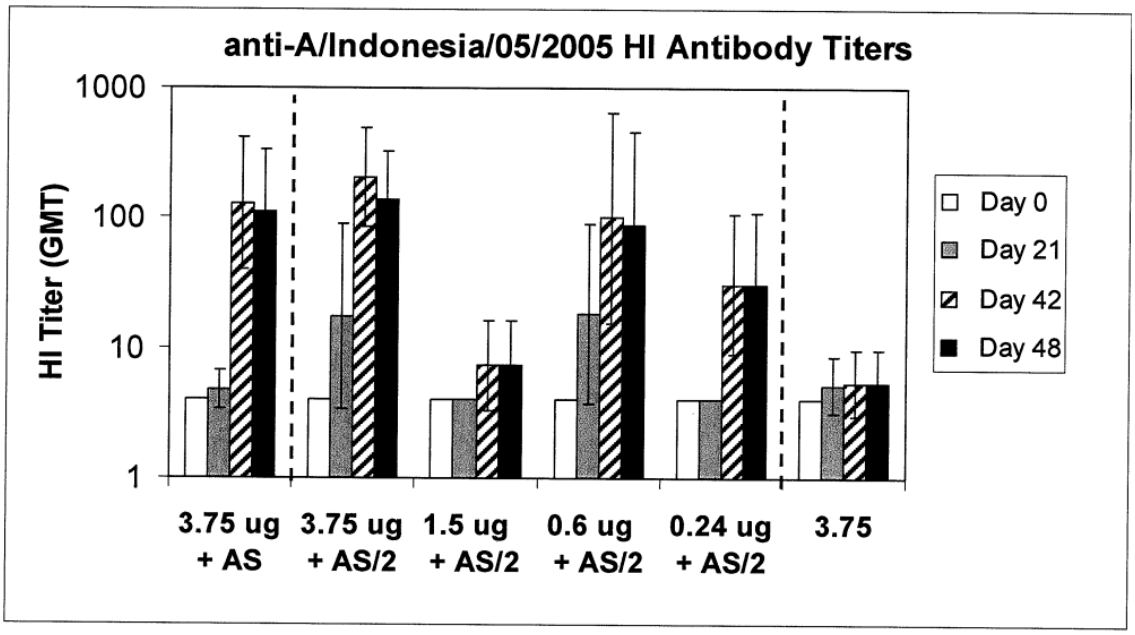
Group	Vaccine composition	Antigen dose	Adjuvant AS03
1	H5N1 with adjuvant	3.75 µg	full-dose
2	H5N1 with adjuvant	3.75 µg	half-dose
3	H5N1 with adjuvant	1.5µg	half-dose
4	H5N1 with adjuvant	0.6 µg	half-dose
5	H5N1 with adjuvant	0.24 µg	half-dose
6	H5N1 alone	3.75 µg	-

A. Humoral immune responses (HI and neutralizing titers)

The humoral immune response (HI and neutralizing antibody titers) to A/Indonesia immunizing strain was measured after each immunization on days 21, 42 and 48 (one day prior to viral challenge on day 49). Serum samples were tested by the haemagglutination inhibition (HI) and virus neutralization assay.

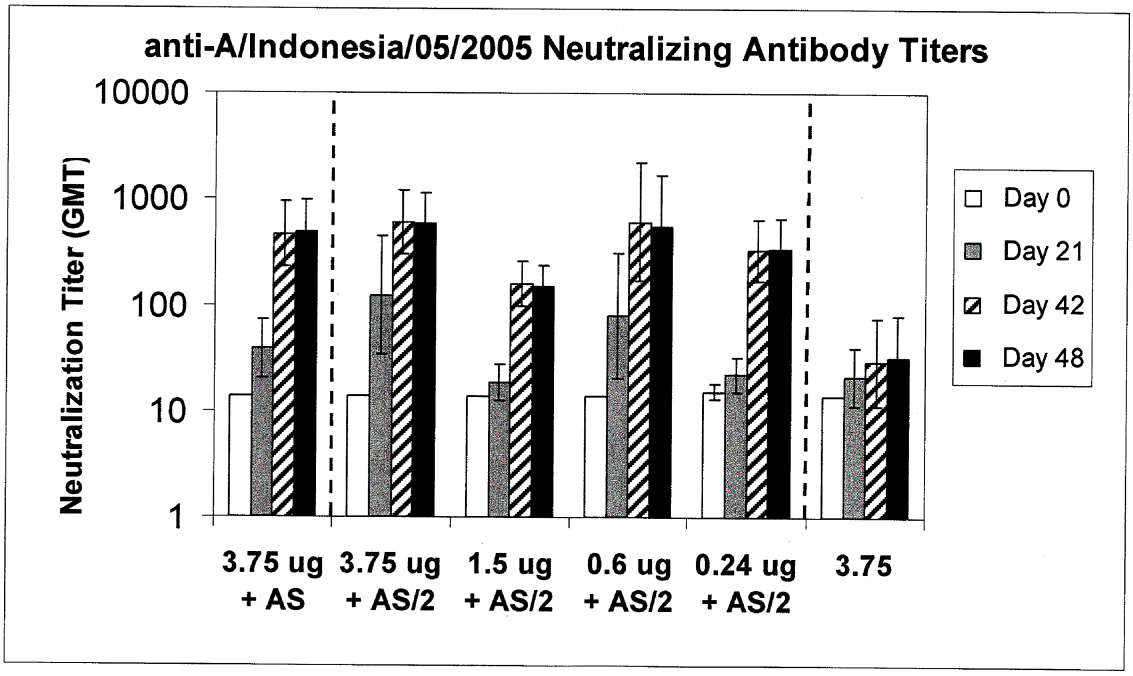
HI and neutralizing antibodies are shown in Figure 6 and Figure 7, respectively.

Figure 6 Pre-challenge homologous serum HI antibody titers (GMT ± 95%CI) in ferrets immunized with a range of A/Indonesia/05/2005 antigen doses formulated with full- or half-dose AS03



AS = full dose AS03, AS/2 = ½ dose of AS03

Figure 7 **Pre-challenge homologous serum neutralization antibody titers (GMT ± 95%CI) in ferrets immunized with a range of A/Indonesia/05/2005 antigen doses formulated with full- or half-dose AS03**



AS = full dose AS03, AS/2 = ½ dose of AS03

For all doses used, high pre-challenge anti-A/Indonesia antibody responses were detected by both HI and neutralization assays when the antigen was administered with full- or half-dose AS03, but were much lower when the antigen was administered without adjuvant, indicating a robust adjuvant effect. Regardless of the dose, a clear boosting effect was seen after the second immunization on day 21 in all groups that received adjuvanted antigen. The neutralization antibody levels were maintained until day 48 (one day prior challenge) confirming that antibody titers were at or near peak at the time of viral challenge. No clear antigen dose dependent effect on HI and neutralizing antibody titres was observed among ferrets immunized with adjuvanted A/Indonesia vaccine.

It should be noted that the 1.5µg HA dose formulated with half-dose AS03 adjuvant (Group 3) elicited the lowest homologous heamagglutinin and neutralization antibody titers of all the adjuvanted HA doses administered in this study. All other adjuvanted groups exhibited several fold higher HI and neutralization titers, even with groups formulated with low antigen content (0.6 and 0.24µg HA). This atypical result with the Group 3 animals would suggest that there might have been an error in the formulation of this vaccine administered either at the first or second dose, error that would result in lower antigen and/or AS03 content. Atypical profiles for this group were also observed with respect to viral shedding levels and cross-pathology following challenge (see following sections); Group 3 animals presenting profiles similar to those of the control

group; thus also pointing towards a lower antigen or AS03 content. Unfortunately, the amount of remaining vaccine material was insufficient so that it was not possible to analyze the content of the vaccine used in Group 3, which would have confirmed the potential formulation error.

B. Mortality and viral detection

A summary of mortality and viral detection is given in Table 5. One animal of group having received 0.6µg HA + AS03/2 (Group 4) and one animal of the control group were found dead before challenge. The cause of death was not related to the treatment.

Altogether, 95% of all the animals immunized with the AS03-adjuvanted Quebec H5N1 influenza vaccines were protected against a lethal challenge with A/Hong Kong/156/97 and survived to the end of the challenge phase on day 5; as opposed to only 43% of the animals that received the unadjuvanted control vaccine.

Three days after start of challenge, two animals were found dead in the control group. At day four after challenge, another animal of the same group was found dead. At day five after challenge, one animal of Group 1, one animal of Group 3 and one animal of Group 6 were found dead. The remaining animals survived until scheduled euthanasia on day 5.

Table 7 Protection of AS03-adjuvanted H5N1-vaccinated ferrets against challenge with heterologous H5N1 influenza virus: mortality and viral detection.

group	antigen dose (µg HA + adj)	Mortality		Pharyngeal viral shedding Titration		Lung viral load Titration	
		Dead/ total	Survival rate	Viral load*	%**	Viral load*	%**
1	3.75 +AS03	1/8	87.5%	- - - -+ + + +	50%	- - - - - - - +	25%
2	3.75 +AS03/2	0/8	100%	- - - - - - + +	25%	- - - + + + + +	62.5%
3	1.5 +AS03/2	1/8	87.5%	+ + + + + + + +	100%	+ + + + + + + +	100%
4	0.6 +AS03/2	0/7	100%	- - + + + + +	71.5%	- - - - + + +	43%
5	0.24 +AS03/2	0/8	100%	- - - + + + + + +	75%	- + + + + + + +	87.5%
6	3.75	4/7	43%	+ + + + + + + +	100%	+ + + + + + + +	100%

*- below detection level, + above detection level
** Percentage of animal with viral load above detection level

Analysis of lung tissue and throat swabs showed that all animals in the control group contained high viral load in both organs as assessed by virus titration analysis. The same virus shedding profile was also observed with Group 3 (1.5µg HA +AS03/2) animals.

Variable shedding of replication competent viruses were observed in both the lung and in the pharynx of all other AS03-adjuvanted groups, with a general tendency (if one excludes Group 3 animals) of having an increasing virus loads in both organs along with a decrease in the antigen dosages. Only two of eight animals of Group 2 shed virus, whereas in the other groups four or more animals were shedding replication competent virus into the upper respiratory tract. Only two of eight animals of Group 1 showed titers of replication competent virus in their lungs whereas three or more animals of Group 2, 4

and 5 were positive. This weak protection of AS03-adjuvanted Quebec H5N1 influenza vaccine from viral replication could be explained by the low antigen dose tested in this study and suggest that the minimal dose required to have a sufficient protection level could be 3.8µg HA adjuvanted with full dose AS03.

As mentioned in the previous section, it is worthwhile to note that the ferrets immunized with 1.5µg HA adjuvanted with half dose AS03, showed the highest lung and upper respiratory tract viral load. These results correlate with the low HI and neutralizing antibody titers observed in this group. These two observations suggest that there might have been an error in the vaccine formulation administered at either the first or second dose.

C. Gross-pathology and histopathology

There were more animals in the control Group 6 (3.75µg HA) with either poor clinical condition (moribund), and/or found dead compared to the other dose groups, which indicated a relation to treatment. Moribund animals showed general depression, anorexia, lethargy and exhibited clinical signs of respiratory disease, including dyspnea. All animals that died pre-terminally, showed signs of atypical pneumonia that could be confirmed by macroscopy and/or microscopy.

Macroscopic analyses showed diffuse dark read area consisting of consolidated affected lung tissue mostly in the control group and in Group 3 (1.5µg HA + AS03/2), the control group being most affected whereas only diffuse grey/read area of lung tissues were observed in Group 1, 2, 4 and 5.

Enlarged bronchial lymph nodes were noted in animals from all groups, with a higher incidence in animals treated with adjuvant (groups 1 to 5) compared to non adjuvanted group (3.75µg HA) and is considered to be contributed by the immunomodulatory effect of the adjuvant.

The changes in the upper respiratory tract (trachea) consisting of subacute inflammation of respiratory epithelium, ulceration and epithelial cell change/squamous metaplasia, was observed most prominently in animals of Group 1 (3.75µg HA + AS03). The tracheal epithelial change/squamous epithelium is considered to be a result of (repeated) damage, which can be regarded also as a regenerative response subsequent to inflammation/damage.

From all groups, animals from both groups having received 3.75µg HA with full dose or half dose of AS03 (Group 1 and 2) showed the lowest grading/incidence of overall adverse pulmonary lesions compared to all other groups. All animals treated with adjuvant also showed a marked difference compared to the animals of the control group which did not receive an adjuvant. This indicates a treatment-relationship for the H5N1 vaccine in reducing morbidity and pulmonary pathology of H5N1 infected animals compared to control animals treated with 3.75µg HA alone, with animals treated with 3.75µg HA + AS03/2 showing slightly lowest incidence/grading of overall pulmonary pathology.

D. Clinical signs

Fever was induced in all animals of control group, with body temperatures peaking at 40°C and sometimes 41ºC (in 5 animals out of 7) between 12 to 48 hours following the start of challenge. Fever at equal or slightly milder temperature elevations was also observed in animals vaccinated with different doses of adjuvanted Quebec H5N1 virus antigen; peaks at 41°C were rarely observed, only Group 3 (5 animals out of 8) and group 5 (2 animals out of 8) did reach that elevation; Group 3 thus showing a profile comparable to the control group with respect to temperature.

Body weights were measured just prior to the challenge infection (Day -1) and at the end of the experiment (moment of premature death or as scheduled on day 5). Animals of Group 1 (3.5µg HA +AS03) showed an average body weight loss of 6.3%. On average the animals of the groups having received half dose AS03-adjuvanted Quebec H5N1 influenza vaccines showed body weight loss of 14 to 21 % and the control group showed an average body weight loss of 13%; it should be noted that the latter occurred in the course of only three days in some cases.

E. Conclusion

Overall, these data show that ferrets vaccinated with A/Indonesia/5/05 H5N1 antigen adjuvanted with AS03 protects ferrets against a heterologous challenge infection with the A/Hong Kong/156/97 H5N1 strain. More specifically, vaccines containing a dose of 3.75µg of A/Indonesia/5/05 adjuvanted with full dose or half-dose AS03, elicited cross protection against the heterologous challenge as assessed by protection from death, reduced viral loads in the upper and lower respiratory tract, reduced clinical signs and reduced microscopic/macroscopic pathology (overall adverse pulmonary lesions). At lower antigen doses (0.6 and 0.24µg HA/dose), the protective effect of the AS03-adjuvanted vaccines was clearly evident with respect to protection against mortality and reduced microscopic/macroscopic pathology. In contrast, a more limited or no effect on the viral loads in the upper and lower respiratory tract were observed similar to the unadjuvanted control vaccine. This protective effect is correlated with the presence by high HI and neutralization heterologous antibody titers prior to challenge.

These data are in line with the results from another heterologous challenge study in ferrets ([redacted] study 2810087) performed with GSK's AS03-adjuvanted influenza vaccines Pandemrix/Prepandrix, which is manufactured with Dresden H5N1 antigen. This study demonstrated that vaccination of ferrets with an AS03-adjuvanted H5N1 influenza vaccine provides cross-protection against infection with a virus strain of a subtype different from that present in the vaccine. In this study, ferrets were administered two doses of AS03-adjuvanted Dresden H5N1 vaccine formulations containing A/Vietnam1194/04 ranging from 1.7 to 15µg HA /dose and adjuvanted with a full dose AS03. All animals were challenged with A/Indonesia/5/05 H5N1 strain. The control groups received either an unadjuvanted A/Vietnam 1194/04 vaccine (15µg HA/dose) or AS03 alone. All animals in the two control groups failed to develop specific or cross-reactive neutralizing antibodies and all died or had to be euthanized within four days of virus challenge. Two doses of adjuvanted split H5N1 vaccine containing ≥1.7µg HA induced neutralizing antibodies in the majority of ferrets to both clade 1 (17/23

(74%) responders) and clade 2 viruses (14/23 (61%) responders), and 96% (22/23) of the vaccinated animals survived the lethal challenge. Furthermore lung virus loads and viral shedding in the upper respiratory tract were reduced in vaccinated animals relative to controls suggesting that vaccination might also confer a reduced risk of viral transmission.

Recent clinical results from study Q-Pan-001 have clearly demonstrated that Quebec- and Dresden-manufactured H5N1 antigens are immunogenically equivalent based on HI GMT for both the vaccine virus and a drifted strain. These data are discussed in the Summary of Clinical Efficacy in m.2.5 of this dossier.

Hence the clinical equivalence between the AS03-adjuvanted Quebec and Dresden H5N1 vaccines as well as the results of heterologous challenge studies in ferrets conducted with these two vaccines both point out that AS03-adjuvanted H5N1 vaccines are able to provide not only protection against the vaccine strain but also cross-protection against other non-vaccine strains.

2.6.2.2.2. Mode of action of AS03

Diverse modes of action have been proposed to explain mechanisms by which adjuvants increase antigen immunogenicity. Adjuvant can be classified into two categories based on their most important mechanism of action (Fraser CK 2007, Guy B 2007)).

1. Delivery systems: target the antigen to APC, enhance the uptake of the antigen by APC and delay the clearance of the antigen through depot effect and slow release
2. Immune potentiators or immunostimulants: act on immune cells involved in immune response, particularly on cells from innate immune system, and activate pathways significant in the induction of adaptive immunity

Non clinical experiments were carried out to better understand the mechanism(s) by which AS03 combined to split antigen promote strong and persistent humoral and cellular responses. Several aspects of the mode of action were studied in mice, from the biodistribution and cellular localization of emulsion and antigen to the impact of emulsion on various aspects of innate immunity known -such as co-stimulation, pro-inflammatory response and chemokines, which are known to play a key role in regulating the quantitative and qualitative aspects of the adaptive immune response. In parallel, the adaptive immune response was characterized in mice, ferrets and humans. The role of α -tocopherol was also explored. This was achieved by comparing AS03 to an emulsion where α -tocopherol was replaced by an equivalent amount of squalene, to reach a final 10% squalene. These studies are summarized in Table 8. The experimental details and results of these studies are fully described in module 4 of this dossier. The findings on the impact of AS03 on innate and adaptive responses are discussed in the sections below.

Table 8 Summary of the studies performed to explore the mode of action of AS03

Aspect of mode of action	antigen	study type
Association between antigen/AS03	Seasonal influenza H5N1	Physico-chemical analysis: isothermal titration calorimetry, sucrose gradient, zeta potential
		Immunogenicity study in naïve mice: humoral and CMI response
Biodistribution of antigen and emulsion	Ovalbumin (Ova-Alexa 647)	Fluorescence and confocal microscopy and Flow cytometry analysis of mice tibialis and draining lymph nodes
Uptake of antigen	Ovalbumin (Ova-Alexa 647)	Flow cytometry analysis of murine monocytic cell line (J774)
Antigen presentation	Ovalbumin	Murine T cell proliferation *in vivo*
Necrosis and apoptosis	Ovalbumin	Proliferation of murine T cell and cell death (*in vitro*) by flow cytometry
Costimulation	Ovalbumin	Cell death (in vivo) by flow cytometry
Production of cytokines and chemokines	no antigen	Proinflammatory cytokine secretion – by human stimulated PBMCs by CBA – by human monocytes by flow cytometry
	HBs	Proinflammatory response in mice by CBA
	Ovalbumin	Cytokine expression by APCs by intracellular staining
AS03-induced adaptive immune response	ovalbumin	Immunogenicity study in naïve mice: production of Th1 and Th2 cytokines
	H5N1	Immunogenicity study in naïve mice: production of Th1 and Th2 cytokines
Role of α-tocopherol	no antigen	Proinflammatory cytokine secretion by human stimulated PBMCs by CBA
	HBs	Immunogenicity study in naïve mice: production of Th1 and Th2 cytokines
	Seasonal influenza	Immunogenicity study in naïve mice: humoral immune response in term of HI titers

CMI= Cell-Mediated Immunity, PBMC = Peripheral Blood Mononuclear Cell, CBA= cytokine bead array, APC= Antigen-Presenting Cell

1) AS03 does not act as a delivery system

No major association between AS03 and antigen neither a depot effect could be observed. This is in line with publications reporting that, in contrast to water in oil emulsions, oil in water emulsions do not or minimally associate with antigen (Aucouturier et al 2001, Podda 2003, Jansen et al., 2006) indicating that the increased immunogenicity was not due to a depot effect (Podda, 2003)

In immunogenicity studies in mice the adjuvant effect of AS03 has been observed even when antigen and adjuvant are injected separately (1h between injections, same area) indicating that the action of AS03 does not depend on strict physical interaction. The immunogenicity was however clearly decreased if the antigen and the adjuvant were injected at two distant injection sites indicating that the action of AS03 depends on spatio-temporal co-localization (e.g. draining to the same lymph node) rather than on strict physical interaction.

2) Immunostimulatory properties of AS03: innate immune response and impact on adaptive response

A possible mechanism of action for an adjuvant includes its impact on cells from the innate immune system and particularly on APC which are able to provide signals to T and B cells and as such play a key role in modulating adaptive immune response (Banchereau, 2000). The results have shown that AS03 induces chemokines production, promotes immune cell recruitment at injection site and optimize antigen presenting cell functions (increased costimulatory molecules, cytokines). This increase of innate immune response has been shown to correlate with an increase of adaptive responses. Interestingly, emulsion with α-tocopherol was shown to induce an increase of pro-inflammatory cytokines and chemokines which correlates with an increase of antibody responses.

Results have shown that activated APC present the antigen to T cells and provide T and B cells with increased and prolonged signals. Indeed, an increase of CD80, CD86 and CD40 together with the panel of cytokines (IL-6, TNFα, IL-1β, and IFNγ) has been detected in vitro or ex-vivo (serum, monocytes, dendritic cells). These are known to play a key role in promoting amplification, differentiation and survival of T and B cells. Combination of antigens with AS03 has been shown to induce strong and persistent immune responses in mice, ferrets and humans. A similar quality and amplitude of responses was observed even with low amounts of antigen.

In a clinical trial performed with Dresden H5N1 influenza antigen, strong CD4+ T cell responses with effector and memory phenotype were observed together with persistent antibodies and memory B cells. These results indicate that (i) the panel of signals increased by AS03 (co-stimulatory molecules, cytokines) were indeed able to promote effector and memory responses in humans, (ii) the vaccine is able to promote memory response required for long term protection. Importantly, cross-reactive responses were observed (HI, CD4+ T cells in mice and in humans) and protection against heterologous strains were observed in ferrets indicating that AS03 based vaccine is able to amplify protective immune responses

3) Th1/Th2 profile of the immune response induced

Cytokines represent one of the principal factors that determine the differentiation of naïve CD4+ T cells into Th1 and Th2 (reviewed by Constant and Bottomly 1997, Murphy 2000, Swain 1995). Among the most studied cytokines, the presence of IL-4 causes Th precursors to differentiate into Th2, whereas IL-12, IL-18 and to a lesser extent IFNγ drive Th1 differentiation (Murphy 1998, Bradley LM 1996,). IL-6, IL-1β and TNF-α in association with increased co-stimulation have been shown to promote T cell response without inducing strong bias toward Th1 or Th2 responses (Joseph SB et al 1998, Palmer EM 1997). Additionally, IL-6 has been reported to favor Th2 development through the induction of IL-4 (pro-Th2) (Rincon 1997) and the inhibition of IFN-γ receptor mediated signals (pro-Th1) (Dielh S 2000). In contrast, TNF-α has been shown to favor Th1 response through differentiation of human monocytes into pro-Th1 dendritic cells (Iwamoto 2007) and by interfering with Th2 development through direct inhibition of Th2 cytokines (So 2004). In agreement with these sets of investigations, the pattern of

cytokines produced upon AS03/antigen vaccination (IL-6 and TNF-α, some IFN-γ) seems to not strongly bias the response toward a Th1 or Th2 response but rather to a mixed response.

4) Immunostimulant role of α -tocopherol

The experiments conducted with model antigen (ovalbumin, hepatitis B surface antigen) combined with AS03 indicated that emulsion with α-tocopherol induces an increase of pro-inflammatory cytokines which correlates with an increase of antibody responses as compared to an AS03 emulsion without α-tocopherol. Importantly, the presence of tocopherol in the emulsion was shown to limit the induction of Th2 type response inducing a more balanced Th1/Th2 response than the emulsion without α-tocopherol.

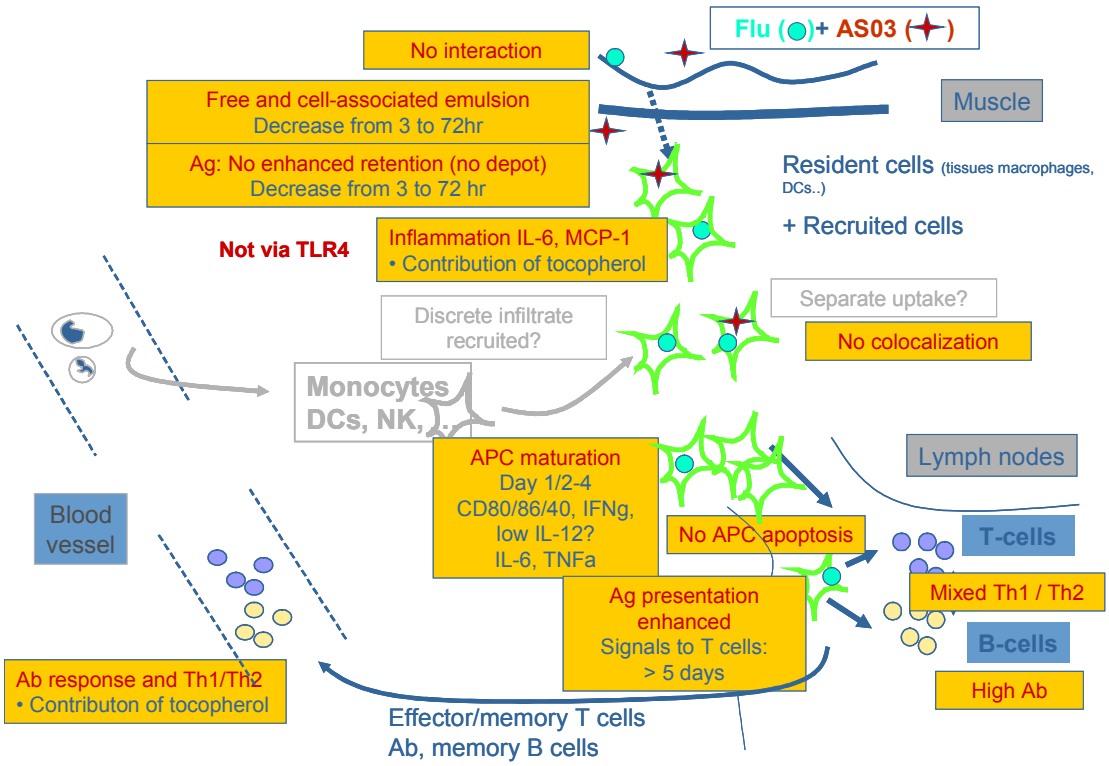
The additional experiments conducted with influenza antigens did confirm that the addition of α-tocopherol to a simple oil-in-water emulsion results in superior immunogenicity. These non clinical results support the addition of α-tocopherol in an oil-in-water emulsion like the AS03 adjuvant.

Conclusion

In conclusion, the available data indicate that AS03 does not act as a "delivery system" for the influenza split antigen but rather works as an immunostimulant (see Figure 8). AS03 promotes the maturation of APC, increases costimulatory properties and favor the production of cytokines including IL-6, TNFα and IFNγ.

These signals provided by APC to T and B cell are likely to play a role in the induction of the adaptive immune response to the AS03 adjuvanted H5N1 vaccine and they also may be involved in the induction of long lasting response.

Figure 8 **Adaptive responses induced by influenza antigen adjuvanted with AS03**



2.6.2.3 Secondary Pharmacodynamics

No secondary pharmacodynamic studies were performed, in accordance with the Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/465/95) and Guideline on Adjuvants in Vaccines for Human Use (EMEA/CHMP/VEG/134716/2004).

2.6.2.4 Safety Pharmacology

A safety pharmacology study ([redacted] study n° [redacted] 0016/072148) was performed in 8 nine-week-old male Wistar rats. Under surgical anesthesia, the rats underwent cannulation of the femoral artery, jugular vein, and trachea: and insertion of subcutaneous ECG electrodes. Under continuous anesthesia by infusion pump, rats were treated by intravenous bolus with 1mL/kg of saline placebo (n = 4) or Quebec A/Wisconsin/67/05 influenza adjuvanted with AS03 (n = 4). The final concentration of the influenza antigen was 30µg/mL and the AS03 concentration represented the full strength intended for human. Assuming a 250g rat, a 1mL/kg dose represents an approximately 100-fold excess over the mL/kg exposure of a 50Kg human receiving a 0.5mL intramuscular dose. Parameters monitored included systolic, diastolic, and mean arterial blood pressures, heart rate, electrocardiogram, respiratory rate, tidal volume, and minute volume. Over 120 minutes after infusion there was a tendency for minute volume to increase in all

animals, and a single animal in the actively treated group showed a transient inverted P wave on ECG. Both occurrences are non-specific and often observed in such experiments; and there was no evidence of any treatment-specific changes in cardio-respiratory performance.

2.6.2.5 Pharmacodynamic Drug Interactions

No pharmacodynamic drug interaction studies were performed, in accordance with the Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/465/95) and Guideline on Adjuvants in Vaccines for Human Use (EMEA/CHMP/VEG/134716/2004).

2.6.2.6 Discussion and Conclusions

The ability of the AS03 adjuvant to increase the immune response in immune naïve animals (i.e. as in a pandemic setting) was shown in mouse experiments. Adjuvantation with AS03 was required to induce detectable humoral immunity against the Quebec H5N1 antigen. The results in mice showed that very low doses of antigen can be used when adjuvanted with AS03. However, in those mouse experiments, the magnitude of the immune response is not clearly dose dependent.

Induction of protective immunity by the AS03-adjuvanted Quebec H5N1 influenza vaccines was demonstrated in the ferret homologous challenge model (H5N1 Indonesia strain). Animals vaccinated with different doses of H5N1 antigen, adjuvanted with AS03, survived the infection with homologous H5N1 virus. There was no dose effect; animals vaccinated with the adjuvanted vaccines showed 100% protection against death, reduced viral loads in the lower and upper respiratory tract, reduced clinical sign symptoms and reduced gross/histopathological effects, whatever the dose of antigen and the dose of adjuvant. Nearly all control animals (AS03 alone) succumbed to the virus challenge, presented high viral loads in lungs and in the trachea, with high temperature and fever together with clear gross and histopathological effects. Although low, the viral loads observed in the ferrets immunized with the half dose AS03-adjuvanted H5N1 influenza vaccine were generally higher than those observed in ferrets immunized with full dose AS03-adjuvanted vaccines suggesting that full dose of AS03 appeared to induce greater protection from viral replication in the respiratory tract (lung and pharynx) compared to half dose of AS03. Serological testing indicated a direct correlation between vaccines induced HI and neutralizing antibody titers in protected animals compared to antigen and adjuvant controls.

Since the specific nature of the final pandemic strain cannot be predicted, priming of the immune response by the vaccine towards other pandemic virus strains is a prerequisite for prepandemic vaccines. Our data from ferret homologous challenge study demonstrated that the AS03-adjuvanted Quebec H5N1 A/Indonesia/5/05 candidate vaccine was efficient in terms of induction of specific and cross-reactive humoral responses.

Cross-protection has been established with the H5N1 A/Indonesia/5/05 (clade 2.1) adjuvanted vaccine following challenge of ferrets with a lethal dose of heterologous

influenza strain A/Hong Kong/156/97 (clade 0). After challenge with a heterologous drifted strain, the majority of animals immunized with AS03-adjuvanted H5N1 split vaccine were protected against lethal challenge with H5N1/A/Indonesia/5/05 and overall 95% survived to the end of the challenge phase on Day 5. More specifically, vaccines containing a dose of 3.75µg of A/Indonesia/5/05 adjuvanted with full dose or half-dose AS03, elicited cross protection against the heterologous challenge as assessed by protection from death, reduced viral loads in the upper and lower respiratory tract, reduced clinical signs and reduced microscopic/macroscopic pathology (overall adverse pulmonary lesions). These data are in line with the results from a heterologous challenge study performed in ferrets with GSK's other AS03-adjuvanted influenza vaccines manufactured with Dresden H5N1 antigen (Pandemrix/Prepandrix) which demonstrated that the AS03-adjuvanted H5N1 vaccine was efficient in terms of induction of specific and cross-reactive humoral (IgG ELISA titers, HI titers and neutralizing antibody titers) immune responses.

All together, these protection data in a stringent challenge model in association with an excellent clinical profile highlight the potential of the AS03-adjuvanted Quebec H5N1 influenza vaccine as an effective tool in pandemic preparedness.

Non clinical experiments were carried out to better understand the mechanism(s) by which AS03 combined to split antigen H5N1 promote strong and persistent humoral and cellular responses. Several aspects of the mode of action have been studied in mice, from the biodistribution and cellular localization of emulsion and antigen to the impact of emulsion on various aspects - such as costimulation, pro-inflammatory response and chemokines - of innate immunity which are known to play a key role in regulating the quantitative and qualitative aspects of the adaptive immune response. The available data indicate that AS03 does not act as a "delivery system" for the influenza split antigen but rather works as an immunostimulant. AS03 promotes the maturation of APC, increases costimulatory properties and favor the production of cytokines including IL-6, TNFα and IFNγ.

With respect to the safety pharmacology profile, intravenous administration of an AS03-adjuvanted Quebec seasonal influenza monovalent vaccine did not produce any treatment-related effects on any recorded cardiovascular or respiratory parameters in the rat. Combined with the other non-clinical safety studies, these results demonstrate that the vaccine composition is safe under the experimental settings evaluated.

Module 2.6: Non Clinical Summary

2.6.3 PHARMACOLOGY TABULATED SUMMARY

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2.6.3.1 Pharmacology: Overview

Overview **Test Article:**

Type of Study	Test System	Method of Administration	Testing Facility	Study Number	Location	
					Vol	Page
Primary Pharmacodynamics	C57Bl/6 mice	Intramuscular	GSK Bio	5690-01	4.2.1.1.3	
	C57Bl/6 mice	Intramuscular	GSK Bio	20070787	4.2.1.1.4	
	Ferrets	Intramuscular	[redacted]	2810117	4.2.1.1.5	
	Ferrets	Intramuscular	[redacted]	2810147	4.2.1.1.6	
	Ferrets	Intramuscular	[redacted]	2810087	4.2.1.1.7	
	Mode of action of AS03		GSK Bio	Summary	4.2.1.1.8	
Secondary Pharmacodynamics	N/A					
Safety Pharmacology	Rat/Wistar	Intravenous	[redacted]	[redacted] 0016/072148	4.2.1.3.1	
Pharmacodynamic Drug Interactions	N/A					

2.6.3.2 Primary Pharmacodynamics

See Item 2.6.3.1

2.6.3.3 Secondary Pharmacodynamics

Not applicable to vaccines.

2.6.3.4 Safety Pharmacology

Flu adjuvanted candidate vaccine (Quebec A/H3N2 15 µg/AS03): Cardiovascular and Respiratory Evaluation in the Anaesthetised Rat

Organ Systems Evaluated	Species/ Strain	Method of Admin.	Doses[1] (mg/kg)	Gender and No. per Group	Noteworthy Findings	GLP Compliance	Study Number
Cardiovascular and respiratory	Rat/Wistar	Intravenous	0.1 ml/rat	4M	None	Yes	[redacted] 0016/072148

1 Single dose unless specified otherwise

2.6.3.5 Pharmacodynamic Drug Interactions

Not applicable to vaccines.

Module 2.6: Non Clinical Summary

2.6.4 PHARMACOKINETICS WRITTEN SUMMARY

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2.6.4.1 Brief Summary

The only pharmacokinetic study performed by the Company according to the Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/465/95) and the Guideline on Adjuvants in Vaccines for Human Use (EMEA/CHMP/VEG/134716/ 2004) was the biodistribution in mice of the AS03 adjuvant in both muscle and draining lymph nodes (DLN). The biodistribution studies show a weak co-localization of antigen and emulsion in similar sub-cellular compartments upon administration in mice. The results of this study are described in the section 2.6.4.4.

2.6.4.2 Methods of Analysis

For the biodistribution of antigen and emulsion study, the emulsion was tracked by inserting chloromethyl-benzamidodialkylcarbocyanine (CM-dil) fluorochrome in the oil droplets during the production of the emulsion. Due to the inability to label split influenza, a labelled model antigen, Ovalbumin-Alexa 647 (OVA) was used.

For these studies, mice were immunized with labelled AS03 and labelled antigen. C57Bl/6 mice were injected in the tibialis with 5µg of Ova-Alexa fluor 647 and 25µL of AS03 emulsion where the fluorochrome CM-dil was inserted in the lipid phase. The total volume of the vaccine was 50µL. Tibialis and inguinal draining lymph node (DLN) were collected at indicated time points after immunization and frozen. The location of the labelled emulsion (AS03) was analyzed by both fluorescence microscopy and by confocal microscopy, whereas for technical reasons the antigen (ova) could be localized only by confocal microscopy and by flow cytometry.

2.6.4.3 Absorption

Not applicable, according to the Note for Guidance on Preclinical Pharmacological and Toxicological testing of vaccines (CPMP/465/95) and the Guideline on Adjuvants in Vaccines for Human Use (EMEA/CHMP/VEG/134716/ 2004).

2.6.4.4 Distribution

Biodistribution of antigen and emulsion

As can be seen from Figure 1, the signal of AS03-CM-dil decreased in both muscle and draining lymph nodes (DLN) from 1 to 48hr, suggesting the emulsion is drained away and/or degraded over time.

In the muscle, labelled AS03 and antigen could both be detected by confocal microscopy between fibres (Figure 2, A and B). As seen at 3hr post-injection, some association of the emulsion and the antigen with the cellular infiltrate between muscle fibres could be observed (Figure 2, D – yellow arrow). However, part of the emulsion was also free (not associated with cell as seen by the absence of nuclear labelling), sometimes observed in

the form of droplets (Figure 2 and Figure 3). These results show that, although some co-localization can be observed between OVA antigen and labeled emulsion, the majority of the antigen does not co-localize with AS03. These findings indicate that the primary mode of action of AS03 is not due to formation of an antigen depot.

In the draining lymph node, AS03 could be found at 1hr and 3hr post-injection in the subcapsular sinus, suggesting that part of the emulsion was directly and rapidly drained from the injection site to the lymph node (Figure 1). At 48hr, the emulsion was uniquely found in the paracortical zone, likely to be the T cell zone. In this area the emulsion was not uniformly spread and showed zones of intense labeling and zones of fainter labeling (Figure 4). In both cases patches of emulsion could be seen associated with cells. These patches were rarely co-localized with the antigen. The antigen could be observed in other intracellular vesicles, possibly endosomes.

In conclusion, as previously seen, the weak co-localization of antigen and emulsion in similar sub-cellular compartments ruled out a depot effect as the AS03 mode of action and suggested that the emulsion is impacting the immune response by a mechanism unrelated to a direct antigen entrapment.

Antigen biodistribution was performed with AS03 in the presence and absence of α - tocopherol. The results of antigen distribution are shown in Figure 5. No impact of the presence of tocopherol was observed. Indeed, the localization of the antigen and the emulsion in both the muscle and the DLN were found to be comparable in mice immunized with AS03 or with AS03 without tocopherol.

Figure 1 **Labeled AS03-CM dil (red color) in muscle and DLN 3h and 48h after immunization (Fluorescent microscopy).**

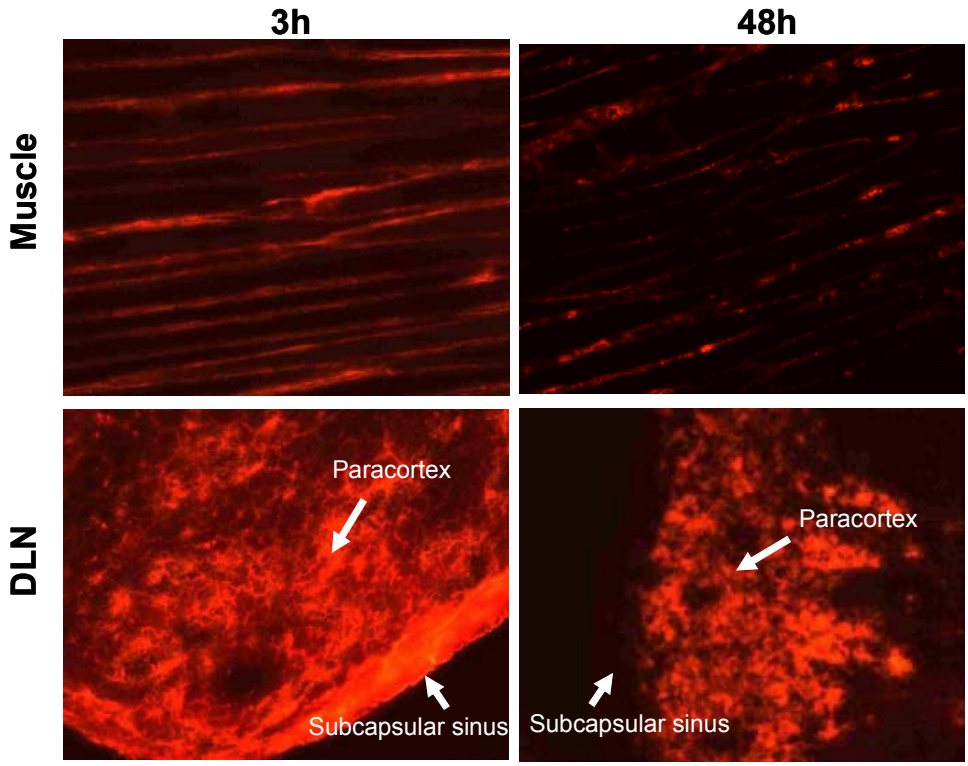
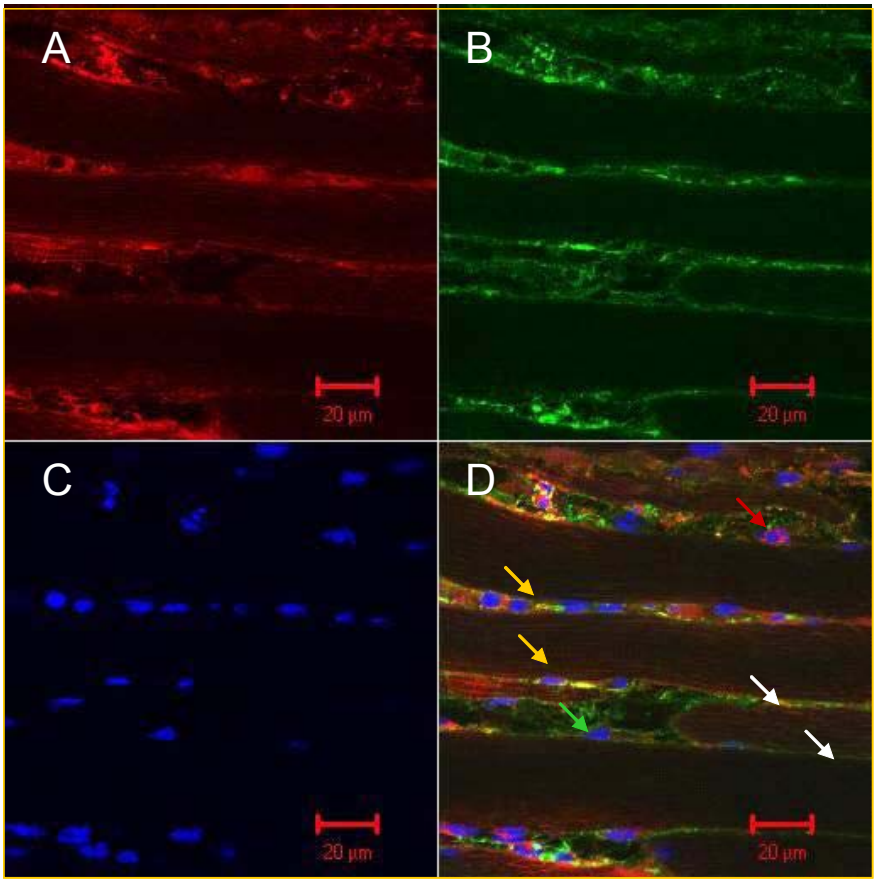
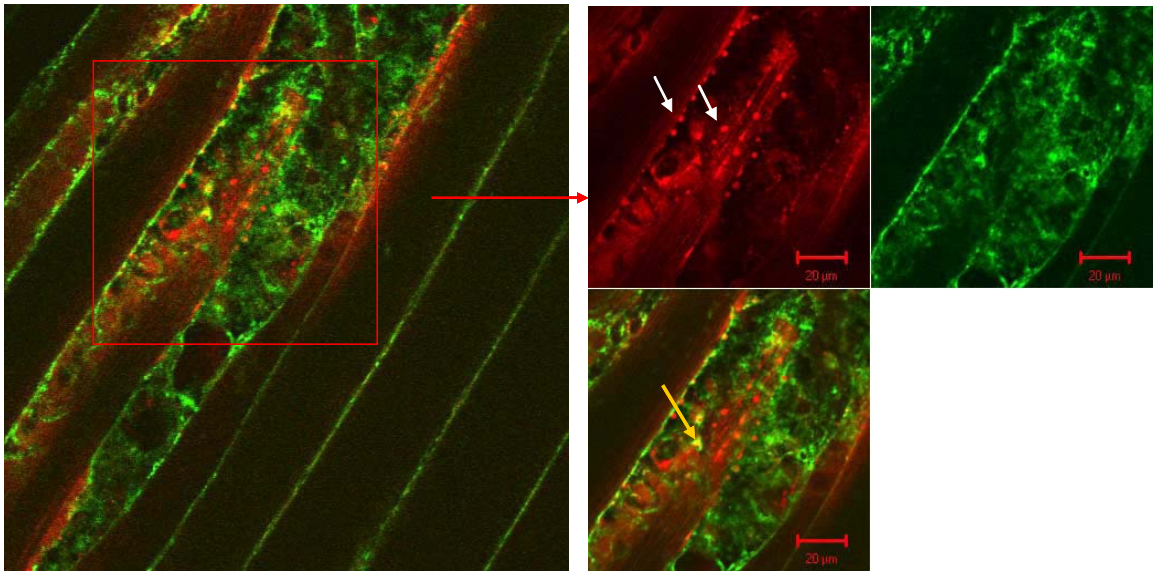


Figure 2 **Biodistribution in the muscle of Ova- Alexa fluor 647 (100µg/ml) + AS03-CM-Dil. Tibialis were examined 3h after injection. (A) AS03-CM-*Dil (red color) (B)* Ova-*alexafluor 647 (green color) (C)* Nuclei-*Yopro (blue color) (D) Overlay***



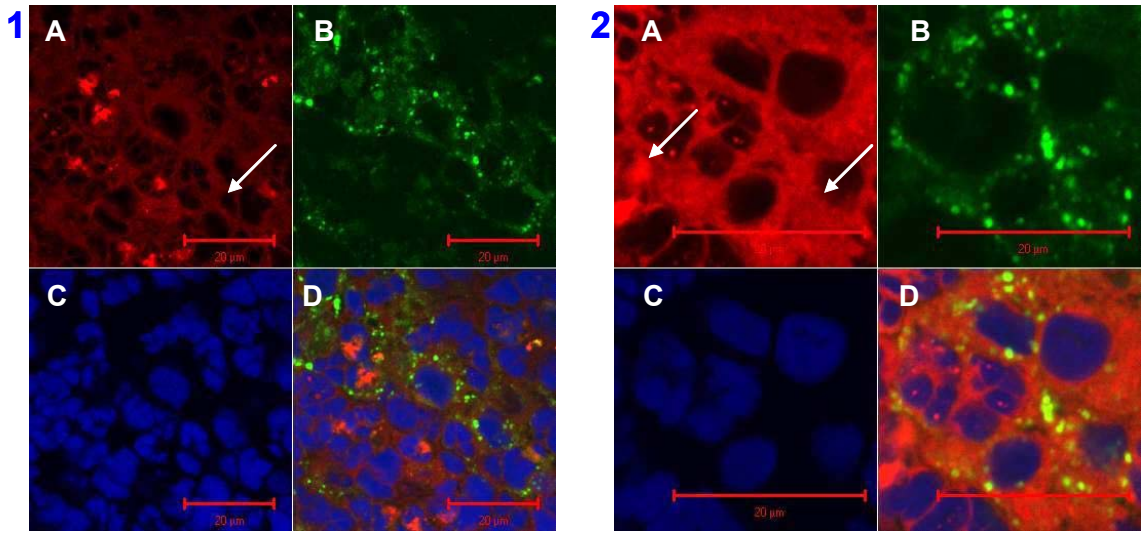
White arrows: antigen and adjuvant not associated with cell infiltrate – Green and red arrows: respectively antigen and adjuvant associated with cells – yellow arrows: colocalization of antigen and AS03. (63x) (the red bar represents 20µm).

Figure 3 **Biodistribution in the muscle of Ova-Alexa fluor 647 (100µg/ml-green color) + AS03-CM-DiI (red color). Tibialis were examined 3h after injection**



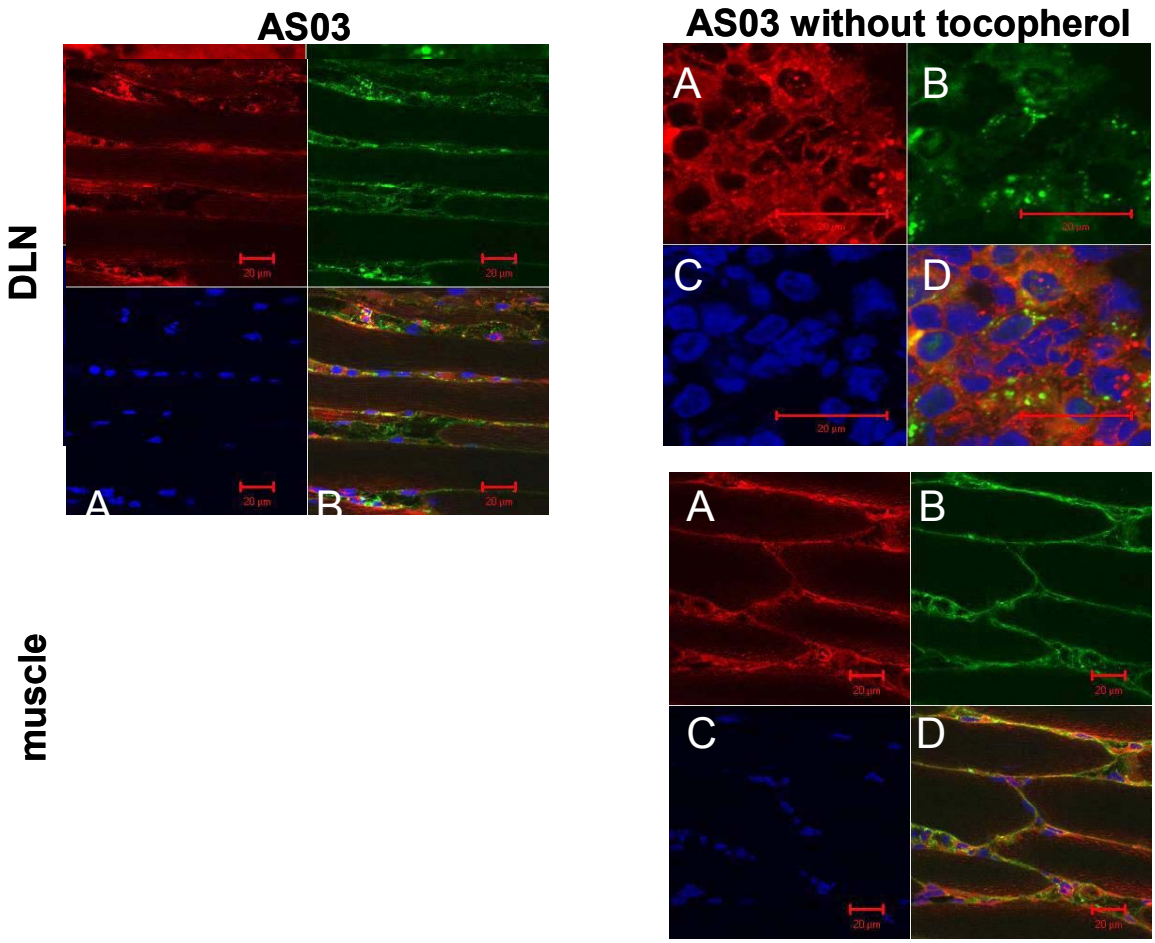
White arrows: AS03 patch with a droplet shape, yellow arrows: colocalization of antigen and AS03; (63x) (the red bar represents 20µm)

Figure 4 **Biodistribution in the DLN of Ova- Alexa fluor 647 (100µg/ml) + AS03-CM-DiI. Tibialis were examined 3h after injection. (A) AS03-CM-*DiI (red color) (B)* Ova-*alexafluor 647 (green color) (C)* Nuclei-*Yopro (blue color) (D) Overlay***



The white arrow points to the zone of higher intensity for AS03-CM-dil. 1. (63x) (the red bar represents 20µm); 2. enlargement of 1 (126x) (the red bar represents 20µm)

Figure 5 **Impact of tocopherol on the localization of antigen and AS03 in DLN and in muscle. Tibialis were examined 3h after injection. (A) AS03-CM-*DiI (red color) (B)* Ova-*alexafluor 647 (green color) (C)* Nuclei-*Yopro (blue color) (D) Overlay***



2.6.4.5 Metabolism (interspecies comparison)

Not applicable

2.6.4.6 Excretion

Not applicable

2.6.4.7 Pharmacokinetic Drug Interactions

Not applicable

2.6.4.8 Other Pharmacokinetic Studies

Not applicable

2.6.4.9 Discussion and Conclusions

The biodistribution studies show a weak co-localization of antigen and emulsion in similar sub-cellular compartments upon administration in mice.

2.6.4.10 Tables and Figures

Figures were integrated in the text

Module 2.6: Non Clinical Summary

2.6.5 PHARMACOKINETICS TABULATED SUMMARY

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2.6.5.1 Pharmacokinetics

Table 1 Overview.

Test Article: AS03

Type of Study	Test System	Method of Administration	Testing Facility	Study Number	Antigen
Absorption	N/A	N/A	N/A	N/A	N/A
Distribution of model antigen (ova) and emulsion (fluorescence and confocal microscopy and flow cytometry) • Distribution and kinetics of labelled AS03 3 and 48hr after injection • Biodistribution of labelled AS03 within muscle and lymph node draining	mice	intramuscular	GSK Bio	Experience n°20060566 n°20070111	Ovalbumin (Ova-Alexa 647)
Metabolism	N/A	N/A	N/A	N/A	N/A
Excretion	N/A	N/A	N/A	N/A	N/A
Pharmacokinetic Drug Interactions	N/A	N/A	N/A	N/A	N/A
Other	N/A	N/A	N/A	N/A	N/A

N/A: Not available

2.6.5.2 Analytical Methods and Validation Reports

Not applicable.

2.6.5.3 Pharmacokinetics: Absorption After a Single Dose

Not applicable.

2.6.5.4 Pharmacokinetics: Absorption after Repeated Doses

Not applicable.

2.6.5.5 Pharmacokinetics: Organ Distribution

Not applicable.

2.6.5.6 Pharmacokinetics: Plasma Protein Binding

Not applicable.

2.6.5.7 Pharmacokinetics: Study in Pregnant or Nursing Animals

Not applicable.

2.6.5.8 Pharmacokinetics: Other Distribution Study

See Table 2 Biodistribution of model antigen and AS03 emulsion in mice

2.6.5.9 Pharmacokinetics: Metabolism In Vivo

Not applicable.

2.6.5.10 Pharmacokinetics: Metabolism In Vitro

Not applicable.

2.6.5.11 Pharmacokinetics: Possible Metabolic Pathways

Not applicable.

2.6.5.12 Pharmacokinetics: Induction/Inhibition of Drug-Metabolizing Enzymes

Not applicable.

2.6.5.13 Pharmacokinetics: Excretion

Not applicable.

2.6.5.14 Pharmacokinetics: Excretion into Bile

Not applicable.

2.6.5.15 Pharmacokinetics: Drug-Drug Interactions

Not applicable.

2.6.5.16 Pharmacokinetics: Other

Not applicable.

Table 2 Biodistribution of model antigen and AS03 emulsion in mice

Test Article:	**adjuvant** CM-dil AS03	**antigen** Ova-Alexa fluor 647
Species Method of Administration Dose injected Analyte Assay	C57Bl/6 mice IM 25µl of AS03 CM-dil fluorescent microscopy	C57Bl/6 mice IM 5µg Alexa fluor 647 fluorescent microscopy
Labelled AS03-CM dil in muscle and draining lymph nodes (DLN) 3h and 48h after immunization (fluorescent microscopy) Study n° 20[redacted]0566 Biodistribution of labelled AS03 within muscle. Study n° 20[redacted]0111 Biodistribution of labelled AS03 in the lymph-node draining the site of injection. Study n° 20[redacted]0111	The signal of AS03-CM-dil decreases in both muscle and draining lymph nodes (DLN) from 1 to 48h, suggesting the emulsion is drained away and/or degraded over time. At 3h post-injection, some association of the emulsion and the antigen with the cellular infiltrate between muscle fibres can be observed. But part of the emulsion is also free (not associated with cell as seen by the absence of nuclear labelling) In the draining lymph node, AS03 can be found at 1hr and 3hr post-injection in the subcapsular sinus, suggesting that part of the emulsion is directly and rapidly drained from the injection site to the lymph node. At 48h, the emulsion is uniquely found in the paracortical zone, likely to be the T cell zone. In both cases patches of emulsion can be seen associated with cells. Interestingly these patch rarely co-localized with the antigen. The antigen can be observed in other intracellular vesicles, possibly endosomes	

Module 2.6: Nonclinical Written and Tabulated Summary

2.6.6 TOXICOLOGY WRITTEN SUMMARY

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2.6.6.1 Brief Summary

In accordance with the CPMP Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95), the Guideline on Adjuvants in Vaccines for Human Use (EMEA/CHMP/VEG/134716/2004), the FDA Guidance for Industry: Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications, CBER, Feb. 2006, and the WHO guidelines on non-clinical evaluation of vaccines and in accordance with the Note for Guidance on Reproductive Toxicology: Detection of Toxicity to Reproduction for Medicinal Products (CPMP/ICH/386/95), non-clinical toxicology studies were undertaken with the AS03-adjuvant alone and in combination with Quebec-manufactured (FluLaval process) and Dresden-manufactured (Fluarix process) influenza antigens:

- Single-dose toxicity, local tolerance, and repeated dose toxicity studies have been performed in rabbits using antigen produced by the FluLaval process with AS03. They also included phosphate buffered saline and AS03 alone as controls. These studies used either an A/H3N2 antigen as model system, or the A/H5N1 (pre) pandemic antigen. In addition to the standard N+1 dose approach, one of the repeated dose studies was designed specifically to mimic two separate immunization series repeated at a prolonged interval, as might occur in a prime-boost strategy in case of pandemic or with annual vaccination.
- Two studies assessing the reproductive toxicity potential of each component of the AS03-adjuvanted Quebec H5N1 influenza vaccine candidate were performed in rats. The first evaluated AS03-containing materials (either AS03 alone or AS03-adjuvanted Dresden H5N1 antigen (A/Vietnam/1194/04), the second assessed the toxicity of inactivated influenza antigens used in the manufacture of trivalent seasonal influenza vaccine (either Fluarix (Dresden process) or FluLaval (Quebec process)).
- Genotoxicity studies in rats have been performed with AS03 alone.

These studies are summarized in Table 1 and the results are described in the following sections. All studies were conducted according to good laboratory practice (GLP) requirements.

In addition, GSK plans to conduct a reproductive toxicity study with the AS03-adjuvanted Quebec H5N1 influenza vaccine to further confirm the results observed in the two reproductive toxicity studies conducted with the separate vaccine components (either seasonal Quebec antigen alone or the AS03 adjuvant alone). Two additional repeat-dose toxicity studies are also underway to evaluate two dose levels of the Quebec H5N1 antigen with and without AS03 adjuvant.

Table 1 Toxicity Studies with AS03-adjuvanted Influenza Antigen or with AS03 Alone.

Study Name	Route of Administration	Species or Substrate	Tested Material
Single-dose Toxicity			
[redacted] 1536-06196	IM	New Zealand White rabbits	Q-H3N2 + AS03
[redacted] 2990/355	IM	New Zealand White rabbits	Q-H5N1 (30µg) + AS03
Repeat-dose Toxicity			
[redacted] 1990/956	IM	New Zealand White rabbits	D-H5N1 + AS03
[redacted] 1536-06194	IM	New Zealand White rabbits	Q-H3N2 + AS03 D-H3N2 + AS03
[redacted] 2990/356[a]	IM	New Zealand White rabbits	Q-H5N1 (30µg) + AS03
[redacted]8550[a]	IM	New Zealand White rabbits	Q-H5N1 (3.8µg) + AS03
Genotoxicity			
[redacted] 768632	*In vitro*	*Salmonella typhimurium, Escherichia coli*	AS03
[redacted]/785	*In vitro*	L5178Y mouse lymphoma assay	AS03
[redacted]/0002	IV	Rat Crl:CD	AS03
Reproductive and Developmental			
[redacted]/0007/063710	IM	Sprague-Dawley rats (Crl:CD (SD) IGS BR)	D-H5N1+ AS03
[redacted]/0009/064374	IM	Crl:CD (SD) IGS BR rats	Fluarix FluLaval
[redacted] 1536-08129[a]	IM	Crl:CD (SD) IGS BR rats	Q-H5N1+ AS03
Local Tolerance			
[redacted] 1536-06196	IM	New Zealand White rabbits	Q-H3N2 + AS03
[redacted] 2990/355	IM	New Zealand White rabbits	Q-H5N1 (30µg) + AS03

Q-H3N2 or Q-H5N1: Influenza antigen prepared from Quebec manufacturing facility (FluLaval process).
D-H5N1: Influenza antigen prepared from Dresden manufacturing facility (Fluarix process).
[a]Ongoing study.

With respect to nonclinical safety, the toxicology studies in rats and rabbits all have shown that administration of Quebec-manufactured antigens together with AS03 do not induce any significant local or systemic toxicity effects. Reproductive toxicity of the AS03-adjuvanted Quebec H5N1 influenza vaccine is supported by the results of the reproductive toxicity studies completed in rats with materials mimicking the individual components of the pandemic vaccine, namely FluLaval trivalent seasonal vaccine (which contains HA antigens manufactured by the same process as the Quebec H5N1 antigen), as well as AS03 alone or AS03-adjuvanted Dresden-manufactured antigen. No evidence of reproductive or developmental toxicity was seen in these studies.

Finally, genotoxicity studies showed no evidence of mutagenicity of AS03 either *in vitro* in bacterial or mammalian cells or *in vivo* following intravenous infusion in rats.

2.6.6.2 Single-dose Toxicity

The single-dose toxicity was assessed as part of the local tolerance studies performed in rabbits.

The first of these studies was conducted with a AS03-adjuvanted vaccine containing 15µg HA/dose of Quebec H3N2 antigen (A/Wisconsin/67/2005) as model strain. There were no deaths or clinical signs observed during the single-dose local tolerance study in the rabbit ([redacted] Study Number 1536-06196 Flu adjuvanted candidate vaccine (Pandemic Influenza Candidate Vaccine with AS03B Adjuvant [Pan2 (Q-Flu/AS03B)]: A Local Tolerance Study in New Zealand White Rabbits). Refer to m2.6.6.7 and m4.2.3.1.1 for details of the study.

A second single-dose toxicity evaluation was obtained as part of an additional local tolerance study conducted with an AS03-adjuvanted vaccine containing 30µg HA/dose of Quebec H5N1 antigen (A/Indonesia/ 2005/05) ([redacted] Number 2990/355 Quebec Seasonal and Pandemic Influenza Candidate Vaccines: Intramuscular Single Dose Toxicity and Local Tolerance Study in the Rabbit). The results of this study confirm the results of the previous single-dose toxicity study, in that there were no unscheduled deaths in the study, and there was no associated toxicity after treatment with the AS03-adjuvanted Quebec H5N1 influenza vaccine. This study is discussed in further detail in m2.6.6.7 and m4.2.3.1.2.

2.6.6.3 Repeat-dose Toxicity

Four repeat-dose toxicity studies have been completed or are underway, each one representing a progression in the development of the Quebec H5N1 project. The earliest of these studies was carried out using the Dresden H5N1 antigen produced using the process for Fluarix seasonal vaccine. This was followed by a second study, in which rabbits were primed with Dresden or Quebec H3N2 adjuvanted antigen and boosted with adjuvanted Quebec H3N2 antigen. Each of these studies was conducted with materials representative of the AS03-adjuvanted Quebec H5N1 vaccine constituents, namely the H5N1 strain (first study) and Quebec manufacturing process-derived components (second study). No toxic effects were observed in either of these studies.

Two additional repeat-dose toxicity studies are underway or were recently initiated to confirm the results obtained in the first two repeat-dose toxicity studies. These studies evaluate AS03-adjuvanted vaccine containing the Quebec H5N1 antigen at two different dosages. In the study underway at [redacted] (Study Number 2990/356), rabbits are administered repeated 30µg HA doses of AS03-adjuvanted Quebec H5N1 antigen. In the study initiated at [redacted] ([redacted]), rabbits will be administered repeated 3.8µg HA doses of AS03 adjuvanted Quebec H5N1 antigen, which is the anticipated human dose. These studies will be discussed in detail in the following sections.

A. Repeat-dose Toxicity with Dresden H5N1 Antigen Adjuvanted with AS03

The objective of this study was to evaluate the potential local and/or systemic toxic effects induced by four intramuscular injections of the candidate vaccines (H5N1 influenza vaccine and H5N1 influenza vaccine adjuvanted with AS03), in the rabbit and to assess the reversibility of any changes over a four-week recovery period.

Groups of SPF New Zealand White rabbits were dosed with the test articles listed in Table 2 on study days 1, 15, 29, and 43.

Table 2 Study Design of Repeat Dose Toxicity Study

Group Number	Test Article	Dose Level	Dose Volume (mL)	Number of Animals	
				Male	Female
1	Saline	0	0.5	10	10
2	AS03	Full human dose AS03	0.5	10	10
3	Dresden H5N1	30µg HA unadjuvanted	0.5	10	10
4	Dresden H5N1 + AS03	30µg HA + full human dose AS03	1.0	10	10

For animals in groups 1, 2, and 3, the doses on days 1, 15, and 29 were administered into the left anterior thigh, and on day 43 the dose was administered to the right anterior thigh. For animals in group 4, the doses on days 1, 15, and 29 were administered to the left anterior and posterior thigh (0.5mL at each site), and the dose on day 43 was delivered to the right anterior and posterior thigh.

Rabbits were evaluated for clinical signs, body weight, food consumption, body temperature, ophthalmoscopy, haematology, clinical chemistry, macroscopic observations, and microscopic observations (including stage dependent evaluation of spermatogenesis).

There was one death in the H5N1/AS03 group during the study, but the death was considered unrelated to local or systemic effects of H5N1 pandemic influenza candidate vaccine.

Administration of the candidate vaccine (H5N1/AS03) and adjuvant (AS03) on four occasions was principally associated with transient erythema and/or edema at the injection site up to 48 hours after dosing. This was not noted to the same extent in the groups administered H5N1, suggesting that it is an effect of AS03, rather than H5N1 (antigen alone).

Increases were also noted in fibrinogen, white blood cell count (neutrophils in particular) in the groups administered H5N1/AS03 and AS03, suggestive of an acute and transient inflammatory response, which correlates with the erythema and/or edema noted in-life during the treatment period and the fasciitis and perivascular cuffing noted microscopically.

Increases noted in the adjusted spleen weight of all treated groups at the day 46 kill were consistent with the microscopic findings of lymphoid hyperplasia. After a 28-day recovery period, the average spleen weights in these groups were lower than the controls and there were no microscopic findings, indicating complete reversal.

A higher incidence and severity of fasciitis was observed at the injection site of rabbits given AS03 or H5N1/AS03, when compared with the H5N1 and saline control groups. This was characterized by a mixed inflammatory cell infiltration in facial planes around muscle bundles and sometimes in deep subcutaneous tissue. Partial reversal of findings seen at day 46 was evident on completion of the 28-day recovery period (i.e., a lesser grade of fasciitis/fibrosis with a resolving of the inflammatory process and lower incidence of perivascular lymphoid cuffing).

In conclusion, aside from increases in fibrinogen and white blood cell count in the groups administered H5N1/AS03 and AS03, suggestive of a transient inflammatory response, there were no systemic effects due to treatment with AS03, H5N1 or H5N1/AS03. Local effects were restricted to erythema and/or edema noted at the injection sites of some animals administered AS03 or H5N1/AS03 up to 48 hours after dosing, suggestive of an effect from the adjuvant rather than the vaccine, as it was not apparent in the H5N1 (antigen) treatment group.

For the study report, refer to m4.2.3.2.1, [redacted] Study Number 1990/956 Pandemic Influenza Candidate Vaccines (Split H5N1 and Split H5N1/AS03): Repeated (4 Occasions) Intramuscular Administration Toxicity Study in the Rabbit with a 4 Week Recovery Period.

B. Repeat-dose Toxicity with Quebec H3N2 Antigen Adjuvanted with AS03

In the second repeat-dose toxicity study, the A/Wisconsin/67/2005 (H3N2) seasonal strain was used as a model virus to evaluate the toxicity in rabbits of antigens produced by the FluLaval process. This study had two phases, the first evaluated the toxicity of a three-dose primary series (N+1 doses for the human primary series) and the second phase evaluated the toxicity of administration of a second, booster series of doses containing the same influenza A/H3N2 antigens. The second phase also examined whether there is differential toxicity after boosting with a Quebec antigen in animals that had received either Dresden or Quebec antigen with AS03 as the primary series. Also examined in this second phase was the question of whether differential toxicity could be observed when the priming and booster antigens were homologous, or of the same subtype but antigenically drifted relative to each other.

One hundred sixteen rabbits (58 per sex) were randomly assigned to receive saline placebo (group 1), AS03 alone (group 2), 15µg of Quebec antigen + AS03 (group 3, A/Wisconsin/67/2005), or 15µg of Dresden antigen + AS03 (group 4, A/New York/55/2004). The first three groups were treated by IM injection in alternating thighs, on study days 1, 15, and 29; group 4 was treated on days 1 and 15 only. On study day 32, and again on day 43, three animals per sex per group in each of groups 1 through 3 were sacrificed and necropsied. The remaining animals proceeded to the boosting phase of the experiment. In the booster phase, groups 1 and 2 received two doses at study days 71 and

85 of saline placebo or AS03, respectively. Groups 3 and 4 received 15µg of drift-variant Quebec antigen (A/Wisconsin/67/2005) + AS03 on the same days. Acute sacrifices and necropsies of five animals per sex per group were performed on day 88; recovery time point sacrifices and necropsies were carried out on day 115. The study groups are described in Table 3.

Table 3 Study Design of Repeat-dose Toxicity

Group	Treatment		Dose Level		Days of Dosing		Number of Animals	
	Priming	Boost	Priming	Boost	Priming	Boost	Male	Female
1	PBS	PBS	0.5mL	0.5mL	SD1, 15, and 29	SD 71 and 85	16	16
2	AS03	AS03	0.25mL with PBS	0.25mL with PBS	SD1, 15, and 29	SD 71 and 85	16	16
3	Q-H3N2* + AS03	Q-H3N2* + AS03	15µg H3N2 with AS03	15µg H3N2 with AS03	SD1, 15, and 29	SD 71 and 85	16	16
4	D-H3N2** + AS03	Q-H3N2* + AS03	15µg H3N2 with AS03	15µg H3N2 with AS03	SD1, and 15	SD 71 and 85	10	10

Note: SD 29 dosing was only for animals that were sacrificed on SD 32 and 43.
PBS, Phosphate buffered saline; SD, Study day.
*Q-H3N2: A/Wisconsin/67/2005.
**D-H3N2: A/New York/55/2004.

Parameters evaluated during the in-life portion of the study included mortality, cage-side clinical observations, body weights and weight changes, food consumption, dermal Draize observations, and opthalmoscopy. At sacrifice, clinical pathology, gross pathology, organ weights and histopathology were examined.

There were no effects of AS03 or either adjuvanted vaccine formulation on mortality, cage-side observations, body weight or weight changes, or dermal Draize observations. Isolated and transient differences in food consumption were noted, but formed no consistent or recurrent pattern.

Clinical laboratory testing revealed slight acute increases in serum globulins and decreases in albumin/globulin ratios in animals receiving AS03 or either, Dresden or Quebec antigens with AS03. These changes were interpreted as consistent with acute inflammation, and tended to resolve at recovery time-points. Transient minimal increases in platelet counts, without changes in hematocrit, hemoglobin, or white blood cell counts, were noted on day 4 in animals receiving AS03 with or without influenza antigens, but these resolved by day 32 and did not recur.

There were no apparent treatment effects on gross pathology findings or organ weights. On histologic evaluation at day 32, all treatment groups demonstrated subacute inflammatory cell infiltrates at injection sites. These consisted of lymphocytes, plasma cells, macrophages, heterophils, and mast cells in the subcutis, epimysium and muscle. Focal hemorrhage, minimal to mild myocyte necrosis, and sciatic perineural fibrosis and

inflammation were noted. These findings were less severe (but not absent) in the saline control animals, but their frequency and severity did not differ between the animals that received AS03 alone and those that received AS03 and Quebec antigen. At the day 43 recovery sacrifice, lesions were similar to, or slightly less severe than, day 32.

After the booster series (administered days 71 and 85), the same spectrum of microscopic findings was noted at the injection sites, but findings were less severe in animals from group 2 (adjuvant only) than in animals from groups 3 and 4 that received a vaccine antigen as well as adjuvant in their booster treatment. Groups 3 and 4 did not differ and lesion severity decreased substantially in all groups at the recovery sacrifice on day 115. All other microscopic findings in other tissues were considered incidental and consistent with the age and strain of rabbit used.

In summary, there were no clinically apparent effects on the health of the test animals. Minor and transient changes in clinical pathology parameters suggesting an acute phase response were associated with the receipt of adjuvant, with or without antigen. A subacute inflammatory response at the injection site was induced by both the adjuvant alone or with influenza Quebec or Dresden antigens. The presence of antigen was immaterial after the primary series, but appeared to increase lesion severity after the booster series. Nevertheless, microscopic findings tended to resolve at the recovery sacrifice after both immunization series, and there were no treatment-related effects aside from the injection sites.

For the study report please refer to m4.2.3.2.2, [redacted] Study Number 1536-06194 (Pandemic Influenza Vaccines with AS03B Adjuvant: A 115-Day Repeat Dose Intramuscular Toxicity Study in New Zealand White Rabbits.

C. Repeat-dose Toxicity with Quebec H5N1 Antigen (30µg HA/Dose) Adjuvanted with AS03

An additional repeat-dose toxicity study is currently underway at [redacted] (Study Number 2990/356). In this study, potential local or systemic toxic effects induced by multiple intramuscular injections of the candidate vaccines with or without AS03 adjuvant into New Zealand White rabbits were evaluated over a period of four weeks post-injection.

Table 4 Study Design of Repeat-dose Toxicity Study (30µg HA/dose)

Group Number	Test Article	Dose Level	Dose Volume (mL)	Number of Animals	
				Male	Female
1	Saline	0	0.5	10	10
2	QIV[a] + AS03½	60µg HA + one-half human dose AS03	0.5	10	10
3	QIV	60µg HA unadjuvanted	0.5	10	10
4	Quebec H5N1 + AS03	30µg HA + full human dose AS03	0.5	10	10

[a]QIV is a quadrivalent seasonal influenza vaccine under development at GSK.

The test articles are administered to the rabbits by intramuscular injection on study days 1, 15, and 29. The first two injections for each animal are in the left anterior thigh, and the third dose is administered to the right anterior thigh. The following evaluations were performed: in-life observations, body weights, food consumption, body temperatures, ophthalmoscopy, hematology, clinical chemistry, organ weights, and macroscopic and microscopic observations.

The protocol for this study is provided in m4.2.3.2.3, [redacted] Study Number 2990/356 Quebec Seasonal and Pandemic Influenza Candidate Vaccines: Repeated (3 Occasions) Intramuscular Administration Toxicity Study in the Rabbit with a 4 Week Recovery Period.

D. Repeat-dose Toxicity with Quebec H5N1 Antigen (3.8µg HA/Dose) Adjuvanted with AS03

In a second repeat-dose toxicity study initiated at [redacted] ([redacted] Study Number [redacted]8550), rabbits (10/sex/group) will be injected intramuscularly on study days 0, 14, and 28 with either saline or AS03-adjuvanted Quebec H5N1 antigen. A description of the test articles is provided in Table 5.

Table 5 Study Design of Repeat-dose Toxicity Study (3.8µg HA/dose)

Group Number	Test Article	Dose Level	Dose Volume (mL)	Number of Animals	
				Male	Female
1	Saline	0	0.5	10	10
2	Q-H5N1 + AS03	3.8µg HA + AS03 full human dose	0.5	10	10

Test articles on day 0 will be injected into the left hind leg (hamstring muscle); on day 14 into the left hind leg (calf muscle), and on day 28 into the right hind leg (calf muscle). One-half of the animals in each group will be sacrificed three days after the last injection, and the remainder will be sacrificed 29 days after the last injection. Clinical signs, ophthalmoscopy, body weight, food intake, body temperature, injection site reactions, hematology, clinical chemistry of the blood, examination at necropsy for gross macroscopic changes, organ weights, histopathology of the injection site, local draining lymph nodes and major organs will be evaluated for possible adverse effects.

The protocol for this study is provided in m4.2.3.2.4, Repeated Dose Toxicity Study with a Quebec Pandemic Influenza Candidate Vaccine (Flu Q-Pan3.8/AS03A) Administered Intramuscularly (Three Times) to Male and Female Rabbits.

2.6.6.4 Genotoxicity

As noted in EMEA/CPMP Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95), mutagenicity studies are not required for vaccines.

However, in accordance with EMEA/CHMP Guideline on Adjuvants in Vaccines for Human Use (EMEA/CHMP/VEG/134716/2004), *in vitro* and *in vivo* genotoxicity studies were performed with AS03 alone.

- AS03 was tested for mutagenic potential in a bacterial reverse mutation (Ames) assay ([redacted] Study Number 21354).
- AS03 was tested for mutagenic potential in an *in vitro* mammalian cell mutation assay based on the detection and of forward mutations in mouse lymphoma L5178Y cells ([redacted] Study Number [redacted] 785/052587).
- AS03 was tested *in vivo* in a rat micronucleus study to assess the potential of AS03 to induce an increase in micronuclei in bone marrow cells of Sprague-Dawley (CD) rats ([redacted] Study Number [redacted] 0002/062069).

Bacterial reverse mutation (Ames) assay ([redacted] Study Number 21354)

AS03 (batches 1011 and 1017) was tested for mutagenic potential in a bacterial reverse mutation (Ames) assay at exposure concentrations ranging from 1.7 to 500µL per plate, with or without S9. Concurrent positive controls demonstrated the sensitivity of the assay. No mutagenic activity was observed in any of the bacterial strains when exposed to AS03. It was concluded that AS03 was not mutagenic under the conditions of this assay at up to the limit of solubility.

For the study report please refer to m4.2.3.3.1, [redacted] Study Number 21354, [redacted] 20[redacted], Testing for Mutagenic Activity with Salmonella typhimurium TA 1535, TA 1537, TA 98, TA 100 and E. coli WP2uvrA.

***In vitro* mammalian cell mutation assay ([redacted] Study Number [redacted] 785/052587)**

Three AS03 formulations with three different concentrations of alpha-tocopheryl quinone, a degradation product of alpha-tocopherol, were tested for mutagenic potential in an *in vitro* mammalian cell mutation assay based on the detection of forward mutations in mouse lymphoma L5178Y cells.

The three formulations of AS03 emulsions with increasing concentrations of alpha-tocopheryl quinone were:

- "AS03 New" with α -tocopheryl quinone at a concentration of less than 0.05mg/mL
- "AS03 Old" with α-tocopheryl quinone at a concentration of 0.25mg/mL
- "AS03/alpha-tocopheryl quinone" with α-tocopheryl quinone at a concentration of 4.94mg/mL (100-fold higher than standard amount found in AS03)

All three test substances were supplied pre-formulated and the highest concentration of each formulation was initially tested in a preliminary toxicity test. A 50mg/mL solution of each test formulation was dosed at 10% in medium (final concentration 5000µg/mL). It was found in some cases that the dose tested was too toxic and did not provide enough

data to select dose levels for the main tests. Therefore, an additional preliminary toxicity test was conducted following the same procedure except that ten concentrations were used up to a maximum of 2500µg/mL. The cells were exposed for either 3 hours or 24 hours in the absence of exogenous metabolic activation (S9 mix) or 3 hours in the presence of S9 mix. The concentrations of each test formulation assessed for mutant frequency in the main test were based upon these data, the objective being to assess concentrations which span the complete toxicity range of approximately 10-100% relative total growth (RTG). In the first and second main tests in the absence and presence of S9 mix, the maximum concentration of AS03 New, AS03 Old, and AS03/alpha-tocopheryl quinone plated for the determination of mutant frequency were 5000, 2000 and 4000µg/mL respectively, where RTG was reduced to 66%, 15% and 18% correspondingly, relative to the concurrent solvent control.

There were no increases in induced mutant frequency at any of the concentrations tested within acceptable levels of cytotoxicity. In all tests, the concurrent solvent control and the positive controls were within acceptable ranges.

It was concluded that none of the AS03 preparations, including the one with a 100-fold higher concentration of alpha-tocopheryl quinone over normal, demonstrated mutagenic potential in this *in vitro* cell mutation assay.

For the study report, please refer to m4.2.3.3.1: [redacted] Study Number [redacted] 785/052587 [redacted] 20[redacted] AS03 New, AS03 Old and AS03/alpha tocopheryl quinone *in vitro* Mutation Test Using Mouse Lymphoma L5178Y Cells.

***In vivo* rat micronucleus study ([redacted] Study Number [redacted] 0002/062069)**

A rat micronucleus study was designed to assess the potential of AS03 to induce an increase in micronuclei in bone marrow cells of Sprague-Dawley (CD) rats. This study compared three formulations of AS03 with increasing concentrations of alpha-tocopheryl quinone:

- "AS03 New" with α -tocopheryl quinone at a concentration of less than 0.05mg/mL
- "AS03 Old" with α-tocopheryl quinone at a concentration of 0.25mg/mL
- "AS03/alpha-tocopheryl quinone" with α-tocopheryl quinone at a concentration of 4.94mg/mL (100-fold higher than standard amount found in AS03)

A preliminary toxicity test confirmed that the test substances were tolerated with acceptable levels of bone marrow toxicity when administered, pre-formulated, at a dose volume of 2mL/kg/day. This dose volume was defined as the maximum for the micronucleus test as higher dose volumes of 5 and 10mL/kg/day caused substantial bone marrow toxicity. As no substantial differences in toxicity were observed between the sexes, the main test was performed using male animals only. All animals in the vehicle control group and each of the test substance groups were dosed intravenously by bolus injection in the lateral tail vein at a dose volume of 2mL/kg/day on two consecutive occasions, approximately 24 hours apart. The negative control group received the vehicle, phosphate buffered saline (PBS), and the positive control group received a single

oral administration of cyclophosphamide at 20mg/kg approximately 24 hours prior to termination.

Bone marrow smears were obtained from seven male animals in the negative control and in each of the test substance groups 24 hours after administration of the second dose. In addition, bone marrow smears were also obtained from five male animals in the positive control group 24 hours after a single dose. One smear from each animal was examined for the presence of micronuclei in 2000 immature erythrocytes. The proportion of immature erythrocytes was assessed by examination of at least 1000 erythrocytes from each animal. A record of the incidence of micronucleated mature erythrocytes was also kept.

No statistically significant increases in the frequency of micronucleated immature erythrocytes and no substantial decrease in the proportion of immature erythrocytes were observed in rats treated with the three AS03 preparations at any treatment level, compared to vehicle control values. The positive control compound, cyclophosphamide, produced significant increases in the frequency of micronucleated immature erythrocytes.

It was concluded that the test substances, AS03 New, AS03 Old and AS03/alpha-tocopheryl quinone (this latter test article containing a 100-fold higher concentration of alpha-tocopheryl quinone over normal (AS03 New)), did not show any evidence of causing an increase in the induction of micronucleated immature erythrocytes or causing bone marrow cell toxicity in rats, when administered intravenously by bolus injection, at a dose volume of 2mL/kg/day, on two consecutive days.

For the study report please refers to m4.2.3.3.2: [redacted] Study Number [redacted] 0002/062069 [redacted] 20[redacted] AS03 New, AS03 Old and AS03/alpha tocopheryl quinone Rat Micronucleus Test.

2.6.6.5 Carcinogenicity (Including Supportive Toxicokinetics Evaluations)

As noted in EMEA/CPMP Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95), this is not required for vaccines.

2.6.6.6 Reproductive and Developmental Toxicity

Absence of potential reproductive effect of the AS03-adjuvanted Quebec H5N1 influenza vaccine is supported by data from the reproductive toxicity studies conducted with each individual component of the vaccine candidates, namely the reproductive toxicity studies with the FluLaval seasonal influenza vaccine ([redacted] Study Number [redacted]0009/064374) and with the AS03-adjuvanted Dresden manufacturing H5N1 (A/Vietnam/1194/04) influenza vaccine ([redacted] Study Number 1536-08129).

These two studies did assess the effect of (1) both Fluarix and FluLaval influenza vaccines, and (2) both AS03 alone and AS03 with H5N1 antigen produced with Fluarix-

process, on embryo-fetal and peri- and post-natal development in naïve or pre-immunized Crl:CD® (SD) IGS BR rats following intramuscular administration.

In order to confirm this absence of reproductive toxicity with the AS03-adjuvanted Quebec H5N1 (A/Indonesia/5/2005) influenza vaccine, the Company has initiated a new reproductive toxicity study (1536-08129). The study design is described in the section below.

A. Reproductive Toxicity of Fluarix and FluLaval Influenza Vaccines

A reproductive toxicity study was conducted to assess the effect of both Fluarix and FluLaval trivalent seasonal influenza vaccines on embryo-fetal and peri- and post-natal development in naïve or pre-immunized Crl:CD® (SD) IGS BR rats following intramuscular administration.

Experimental groups of 48 female rats were allocated to study and treated with a single intramuscular injection at 28 days before pairing. The females were paired with untreated males and 44 females in each group with a positive indication of mating were then treated on days 6, 8, 11 and 15 after mating. One half of the female animals in each group were sacrificed on day 20 post-mating (embryo-fetal phase), for reproductive assessment and fetal examination. Fetuses were inspected macroscopically and subsequently by detailed internal visceral and skeletal examinations. The remaining F0 females were allowed to rear their young to day 25 of age (littering phase). The F1 offspring in the littering phase were examined at day 25 of age for clinical condition, survival, sex ratio, bodyweight and pre-weaning reflex development. Among the F0 females, group 1 animals received saline placebo, while group 2 animals received the Fluarix vaccine and group 3 animals received the FluLaval vaccine. All animals receiving active treatment were dosed with 1/5 of the full human dose of 2006-7 trivalent vaccine, 0.1mL at each injection (a 40-fold excess over the usual human dose, on a weight-adjusted basis, at each exposure). F1 offspring received no direct administration of the test articles.

Neither influenza vaccine had an adverse effect on clinical condition, body weight, or food consumption of F0 females. There were no injection site reactions. Treatment did not affect mating performance or fertility, the length of gestation, or the ability to bear live litters.

Embryo-fetal survival, growth, and development were not adversely affected by the active treatments, and the clinical condition, survival, and growth of the F1 offspring between birth and Day 25 were also unaffected. A single female in the FluLaval group was sacrificed prematurely when her litter failed to thrive. This female had pale, inactive mammary tissue at necropsy, an outcome deemed unrelated to treatment. Among F1 offspring, treatment with either Fluarix or FluLaval had no effect on development of air or surface righting reflexes, or the ability to show startle or papillary reflexes.

There were no macropathologic findings in the F0 females or F1 offspring that were considered related to treatment.

For the study report please refer to m4.2.3.5.2, [redacted] Study Number: [redacted]0009/064374 Influenza Vaccines (Fluarix and FluLaval) Study of Effects on Embryo-fetal, Pre- and Post-natal Development in CD Rats by Intramuscular Administration (including pre-mating immunization phase).

B. Reproductive Toxicity of AS03 and AS03-adjuvanted Dresden H5N1 Antigen Influenza Vaccine

In this study ([redacted]/007/063710), the impact of the aluminum-adjuvanted Dresden H5N1 whole virus influenza vaccine (whole H5N1/Al), the AS03-adjuvanted Dresden H5N1 split virus influenza vaccine (split H5N1/AS03) and the AS03 adjuvant alone was assessed following intramuscular administration, on embryo-fetal and peri- and post-natal development in Crl:CD® (SD) IGS BR rats, naïve or pre-immunized. Initially, six groups of 48 female rats were allocated to study and treated with a singular intramuscular injection on Day -30 (before pairing). The females were paired with stock males of the same strain. Forty four females were required to have positive indication of mating for each group and the excess animals were killed on Day 6 after pairing. The 44 animals in each group with a positive indication of mating were treated on Days 6, 8, 11 and 15 after mating. For each group, 22 animals were killed on Day 20 after mating (embryo-fetal phase) and the remaining 22 animals were allowed to rear their young to Day 25 of age (littering phase). Animals allocated to Group 2 received AS03 (adjuvant Control), animals in Group 3 received Saline (premating) and then Split H5N1/AS03, and animals in Group 4 received split H5N1/AS03 at dosages of 200µL/occasion. Group 5 animals received saline (pre-mating) and then whole H5N1/Al, and animals in Group 6 received whole H5N1/Al at dosages of 100µL/occasion. A similarly constituted control group (Group 1) received 200 µL of saline on the same occasions before pairing and after mating.

Embryo-fetal phase F0 animals were killed on Day 20 after mating, for reproductive assessment and fetal examination. Littering phase F0 animals were allowed to litter, rear their offspring to weaning and were killed on Day 25 of lactation. The F1 offspring received no direct administration of the test substances; any exposure was in utero or via the milk.

During the study, clinical condition, bodyweight, food consumption, gestation length and parturition observations, and macroscopic pathology investigations were undertaken on F0 females. Fetuses on the embryo-fetal phase of the study were examined macroscopically at necropsy and subsequently by detailed internal visceral examination or skeletal examination. For offspring on the littering phase of the study, clinical condition and survival, sex ratio, bodyweight and pre-weaning reflex development were assessed. Serum samples were obtained from all F0 females on Days -33 and -5 before pairing; from excess animals at Day 6 after mating; from embryo-fetal phase animals at Day 20; from littering phase females at Day 25 of lactation; from all fetuses of 11 litters per group at Day 20 of gestation; from up to two male and two female offspring in all litters at Day 4 and Day 25 of age. Serum samples were sent to the Sponsor for antibody analysis and the results were reported as a separate study.

The results demonstrate that treatment of F0 females with AS03, split H5N1/AS03 or whole H5N1/Al did not adversely affect their clinical condition, bodyweight or food consumption throughout the study. One female in the AS03 group of the embryo-fetal phase was killed prematurely but this death was unrelated to treatment. The only reaction to treatment observed was an increased incidence of pale areas at the injection sites of animals dosed with either AS03 or split H5N1/AS03, and an increased incidence of pale and raised areas at the injection sites of animals dosed with whole H5N1/Al.

Treatment did not affect the mating performance or fertility of the F0 females or, the length of gestation or their ability to give birth to a live litter. Embryo-fetal survival, growth and development were not adversely affected by treatment. The clinical condition, survival and growth of the F1 offspring between birth and Day 25 of age were not adversely affected by treatment.

Treatment with split H5N1/AS03 or whole H5N1/Al did not affect the reflex development of the F1 offspring prior to weaning. Among offspring derived from dams treated with AS03, 13 offspring (7 litters) did not show the air righting reflex prior to Day 21 of age and this finding may be related to treatment. Treatment with AS03 did not affect the attainment of the surface righting reflex or the ability of the offspring to show the startle response reflex and the pupil reflex.

There were no macropathology findings in the F1 offspring considered to be related to treatment.

There were no effects of AS03 or AS03-adjuvanted influenza antigens on mortality, clinical observations, body weight or weight changes. Clinical laboratory testing revealed slight acute increases in serum globulins and/or fibrinogen, as well as platelet counts and/or white blood cell counts, at acute sacrifice time-points in animals receiving either AS03 or AS03-adjuvanted influenza antigen. These changes were interpreted as consistent with acute inflammation, and were transient. Although increased spleen weights and hypertrophy of lymph nodes draining the injection site were noted in some studies, the predominant histopathologic findings included mild to moderate subacute inflammatory infiltrates, fasciitis, perivascular and perineural inflammation and/or fibrosis at the injection sites. These findings tended to decrease in frequency and severity at recovery time-points, and there were no other treatment-related histopathologic findings in other organs. The presence of the AS03 adjuvant was the major determinant of severity after the primary series, and the presence of antigen in addition to adjuvant was associated with some increased frequency and severity of local inflammatory findings after the booster series.

For the study report please refers to m4.2.3.5.2, [redacted] Study Number: [redacted]0007/063710, Pandemic Influenza Candidate Vaccines (whole H5N1/Al and Split H5N1/AS03) Study of Effect on Embryo-fetal, Pre- and Post-natal Development Study in CD Rats by Intramuscular Administration (including pre-mating immunization phase).

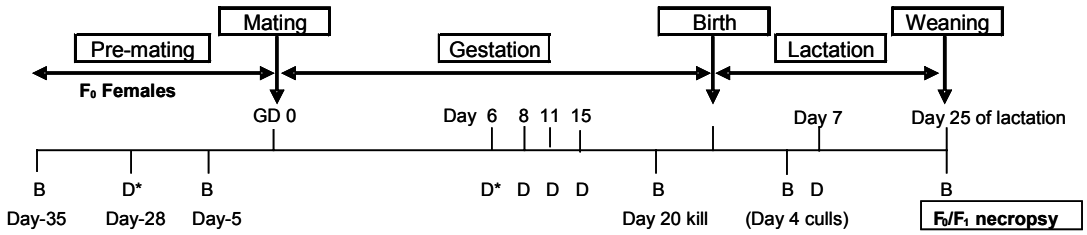
C. Reproductive Toxicity Study with AS03-adjuvanted Quebec H5N1 (A/Indonesia/5/2005) Influenza Vaccine

GSK has initiated an additional reproductive toxicity study with the AS03-adjuvanted Quebec H5N1 influenza vaccine to further confirm the results observed in the two reproductive toxicity studies conducted with materials representative of the two individual components of the pandemic vaccine.

The study design (see Figure 1) is identical to other reproductive toxicity studies done over the past decade by the Company, with the exception that a dose has been added during the lactation period. GSK recently added this additional dose into reproductive toxicity programs for vaccines. The description of the treatment groups to be included in this study is given in Table 6. The rats allocated to the study group receiving H5N1/AS03 vaccine will receive 2/5 of the full human dose of pandemic vaccine, which corresponds to an 80-fold overdose on a body weight basis (assuming a 250g rat and 50kg human, the rat dose is 0.2mL/0.25kg = 0.8mL/kg vs. 0.5mL/50kg = 0.01mL/kg).

In this study, forty-eight (48) animals per group will be allocated and treated, in order to obtain 44 females with a positive indication of mating. 44 females per group will be treated during gestation. The requisite number of animals for each group will be continued after mating and dosed during gestation; excess animals will be killed on Day 6 of gestation. Of the 44 animals continued for each group, half will be killed on Day 20 after mating and the remaining 22 animals will be allowed to rear their young to Day 25 of age.

Figure 1 Study Design of Reproductive Toxicity Study with AS03-adjuvanted Quebec H5N1 (A/Indonesia/5/2005) Influenza Vaccine



GD, Gestation day; D, Dosing event; B, Blood sampling.

Table 6 Identity of Treatment Groups

Group	Treatment	Treatment Days	Number of Females	Animal Numbers
1	Saline	Day -28 prior to pairing, then Days 6, 8, 11, 15 after mating, then Day 7 lactation	48	1-48
2	AS03	Day -28 prior to pairing, then Days 6, 8, 11, 15 after mating, then Day 7 lactation	48	49-96
3	Saline premating and then split H5N1/AS03	Day -28 prior to pairing, then Days 6, 8, 11, 15 after mating, then Day 7 lactation	48	97-144
4	Split H5N1/AS03	Day -28 prior to pairing, then Days 6, 8, 11, 15 after mating, then Day 7 lactation	48	145-192

The study protocol ([redacted] Number, 1536-08129 Pandemic Influenza Vaccines with AS03 Adjuvant (Q-Pan): Study for Effects on Fertility and Embryo/Fetal Development in Pregnant Sprague Dawley Rats) and its addenda comply with ICH S5A Detection of Toxicity to Reproduction for Medicinal Products [ICH 1994], EMEA/CPMP Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95) and FDA's Guidance for Industry: Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications [FDA, 2006].

2.6.6.7 Local Tolerance

Two local tolerance studies have been conducted, each in conjunction with a single dose toxicity study. The first of these studies used an AS03-adjuvanted vaccine containing 15µg HA/dose of Quebec H3N2 antigen produced with the *FluLaval* process as a model antigen to evaluate the toxicity effect of Quebec-sourced antigens. The second study was conducted with an AS03-adjuvanted vaccine containing 30µg HA/dose of Quebec H5N1 antigens. These studies are discussed in the following sections.

A. Quebec-sourced, AS03-adjuvanted H3N2 Antigen (15µg HA/Dose)

In order to evaluate the acute local tolerability of a Quebec-sourced influenza antigen adjuvanted with AS03, 18 rabbits were randomly assigned to one of three groups (3/sex/group) to received either a saline control, AS03 alone at the full human dose, or Quebec-manufactured A/Wisconsin/67/2005 H3N2 antigen at a dose of 15µg HA combined with a full human dose of AS03 (see Table 7). On a body weight basis, the vaccine dosage administered to the rabbit (2.5kg) corresponds to 20-fold the dosage administered to a human (50kg). All animals received a single 0.5mL dose of the appropriate article via an intramuscular injection into the thigh muscle on Day 1. Clinical observations, body weight, and injection site dermal Draize scores were collected over three days. On Day 4, all animals were euthanized and necropsied. Tissues were collected and the injection site and any gross lesions were evaluated microscopically.

Table 7 Design of Local Tolerance Study

Group	Treatment	Dose Level	Dose Volume (mL)	Number of Animals	
				Male	Female
1	PBS	0	0.5mL	3	3
2	AS03	Full human dose AS03	0.5mL	3	3
3	Q-H3N2 + AS03	15µg HA + full human dose AS03	0.5mL	3	3

Q-H3N2: Quebec-manufactured A/Wisconsin/67/2005 H3N2 antigen.

All animals survived until scheduled sacrifice and there were no adverse clinical observations; dermal responses did not differ between controls and experimental groups. There were no adverse observations noted at necropsy. Minimal or mild subacute inflammation of the subcutaneous and/or epimysial tissue was noted in animals receiving the adjuvant, with or without influenza antigen. There were no microscopic findings specifically associated with the presence of influenza antigen in the test article. In general, a single intramuscular injection of influenza vaccine containing 15µg of HA and a full human dose of AS03 was well tolerated by New Zealand White rabbits.

For the study report, please refer to m4.2.3.6, [redacted] Study Number 1536-06196 Flu Adjuvanted Candidate Vaccine (Pandemic Influenza Candidate Vaccine with AS03B Adjuvant [Pan2 (Q-Flu/AS03B)]: A Local Tolerance Study in New Zealand White Rabbits.

B. Quebec-sourced, AS03-adjuvanted H5N1 Antigen (30µg HA/Dose)

An additional study was conducted using the Quebec H5N1 (A/Indonesia/5/2005) antigen as a test article to evaluate the acute toxicity and local tolerance of the adjuvanted (pre) pandemic vaccine. Also included in the study was a quadrivalent seasonal vaccine containing either no AS03 adjuvant or one-half the human dose of AS03. These candidate vaccine formulations were compared to a saline control group. Specific pathogen free New Zealand White rabbits were dosed as described in Table 8. The rabbits received a single intramuscular injection to the right anterior thigh.

Table 8 Design of Local Tolerance Study

Group	Treatment	Dose Level	Dose Volume (mL)	Number of Animals	
				Male	Female
1	Saline	0	0.5	3	3
2	QIV[a]/AS03½	30µg HA + one-half human dose AS03	0.5	3	3
3	QIV[a]	30µg HA unadjuvanted	0.5	3	3
4	Q-H5N1 + AS03	30µg HA + full human dose AS03	0.5	3	3

[a]QIV is a quadrivalent seasonal influenza vaccine formulation currently under development.

The animals were dosed on study day 1. The following evaluations were performed on the rabbits: in-life animal observations, injection site reactions, body weight, macroscopic observations and microscopic observations (at injection sites only). The animals were sacrificed at study day 4 (3 days after injection).

In-life observations showed a single male treated with the unadjuvanted vaccine had a very slight erythema at the injection site 72 hours after dosing. No other incidence of erythema or edema was observed. No differences in body weight or weight change were observed in any treatment group.

After sacrifice, there were no macroscopic findings that would indicate local or systemic effects in any of the four treatment groups.

Microscopically, minor inflammation at the injection site was noted in all groups. The incidence of fasciitis and cellulitis were similar in the adjuvanted groups (2 and 4), and higher than in the non-adjuvanted groups (1 and 3). Incidence and severity of these reactions were similar in the two non-adjuvanted groups, indicating that the occurrence of these types of inflammation may be associated more with the adjuvant than the antigen. Granulomatous myositis was observed in males in the adjuvanted groups at a higher rate and severity than animals dosed with non-adjuvanted vaccine. In females, the incidence and severity were similar in all of the vaccine groups, and higher in these groups than in the control group.

These results indicate that there is no associated toxicity after treatment in any of the treatment groups, including the AS03-adjuvanted Quebec H5N1 vaccine group. The complete study report is included in m4.2.3.1, [redacted] Study Report 2990/355 Quebec Seasonal and Pandemic Influenza Candidate Vaccines: Intramuscular Single Dose Toxicity and Local Tolerance Study in the Rabbit.

2.6.6.8 Other Toxicity Studies

Not applicable.

2.6.6.9 Discussion and Conclusions

The safety of the AS03 adjuvant either alone or combined with Dresden-manufactured or with Quebec-manufactured antigen has been studied in *in vivo* and *in vitro* toxicity studies. Relevant non-clinical safety and toxicology data have been generated in several systems:

- Single-dose toxicity, local tolerance and repeated dose toxicity studies have been performed in rabbits using the AS03-adjuvanted Quebec influenza A/H3N2 antigen (A/Wisconsin/67/2005) and the AS03-adjuvanted Quebec influenza H5N1 antigen (A/Indonesia/5/2005). The evaluations also included toxicity data generated with vaccines with or without adjuvant that contain Dresden H5N1 antigen.

- Reproductive toxicity studies have been performed with AS03-containing material, either AS03 alone or AS03-adjuvanted Dresden manufactured H5N1 antigen and with Dresden and Quebec seasonal trivalent influenza vaccines.

- *In vitro* and *in vivo* genotoxicity studies have been performed with AS03 alone.

The single-dose toxicity was assessed as part of two local tolerance studies performed in rabbits, one with seasonal influenza H3N2 antigen (A/Wisconsin/67/2005) produced with the Quebec manufacturing process as a model strain for antigen process-related constituents, and the other with H5N1 antigen (A/Indonesia/5/2005), also produced with the Quebec manufacturing process. Local tolerance testing of single intramuscular injections of H3N2 antigen with AS03, at doses exceeding by 20-fold on a body weight basis the intended human doses, resulted in dermal Draize scores indistinguishable from saline control and no other clinical observations. Minimal or mild subacute inflammation of the subcutaneous and/or epimysial tissue was noted at the injection site in animals receiving the adjuvant, with or without influenza antigen. There were no microscopic findings specifically associated with the presence of influenza antigen in the test article. From this study, it could be concluded that a single IM injection of influenza vaccine containing 15µg of HA and a full human dose of AS03 was well tolerated by New Zealand White rabbits.

These results were confirmed by the second single-dose toxicity/local tolerance study, which tested a vaccine manufactured with Quebec H5N1 antigen. In this study, which used saline and a developmental seasonal influenza vaccine as comparators, there was no associated toxicity after treatment with any of the candidate vaccines with or without AS03 adjuvant. Minor inflammation at the injection site was noted in all groups, which is indicative of the injection method. The H5N1 and seasonal adjuvanted vaccines both were associated with fasciitis, cellulitis, and, in males, granulomatous myositis, however, there was no clear difference in severity between the two adjuvanted vaccines. This suggests that the inflammation was associated with the adjuvant, and not with any particular antigen.

The two completed repeat-dose toxicity studies also confirm a benign toxicity profile for the AS03-adjuvanted vaccines. In the initial study, four repeated exposures to AS03, Dresden H5N1 vaccine or AS03-adjuvanted Dresden H5N1 vaccine produced no systemic effects due to treatment. Local effects were restricted to erythema and/or edema noted at the injection sites of some animals administered AS03 or H5N1/AS03 up to 48 hours after dosing, suggestive of an effect from the adjuvant, rather than the vaccine, as it was not apparent in the H5N1 (antigen alone) treatment group. Three days after the last injection (day 46), a higher incidence and severity of fasciitis, characterized by a mixed inflammatory cell infiltration, was observed at the injection site of rabbits given AS03 or H5N1/AS03 compared to saline treated controls. Partial reversal of these findings was evident on completion of the 28-day recovery period.

In the repeated-dose toxicity study of AS03-adjuvanted Quebec manufactured H3N2 antigen, the AS03 adjuvant and the AS03-adjuvanted Quebec antigen (A/Wisconsin/67/2005) were administered with each dose greater by 20-fold on a body weight basis than the dose intended for human testing. This repeated dose study was

designed specifically to mimic two separate immunization series repeated at a prolonged interval, as might occur in a prime-booster strategy or with annual vaccination. Results of this study demonstrated that there were no clinically apparent effects of the adjuvant or influenza antigen on the health of the test animal, suggesting that repeated intramuscular injection with AS03 adjuvant and/or Quebec manufacturing influenza antigen was generally well tolerated in male and female New Zealand White rabbits.

Altogether, these two repeat-dose studies showed that there were no clinically apparent effects on the health of the test animals. Minor and transient changes in clinical pathology parameters suggesting an acute phase response were associated with the receipt of adjuvant, with or without antigen. These local inflammatory effects are expected, as the role of the adjuvant is to increase the immunogenicity of the vaccine, and as a consequence a slightly increased reactogenicity is observed. Additional repeat-dose toxicity studies are underway to confirm the conclusions of the initial studies; these additional studies will assess the impact of repeated exposure to AS03-adjuvanted Quebec H5N1 vaccines formulated at two antigen dosages (3.8 and 30µg HA/dose).

A reproductive toxicity study was conducted to assess the effect of both Dresden and Quebec trivalent seasonal influenza vaccines on embryo-fetal and peri- and post-natal development in naïve or pre-immunized rats following IM administration. All animals receiving active treatment were dosed with 1/5 of the full human dose trivalent vaccine, 0.1mL at each injection (a 40-fold excess over the usual human dose, on a weight-adjusted basis, at each exposure). Overall, there was no evidence suggesting any reproductive or developmental toxicity of split influenza antigens produced in Quebec or in Dresden in rats.

A second reproductive toxicity study conducted in rats dosed with AS03 alone or in combination with Dresden-manufactured H5N1 antigen indicated no impact of either treatment on F0 female clinical condition, food consumption, weight, mating performance, fertility, ability to produce a live litter, or pre- or post-natal development of F1 offspring. An isolated finding with regard to the development of the air righting reflex in the offspring of females treated with AS03 alone was not replicated in the offspring of animals receiving the complete vaccine (AS03 with H5N1 antigen).

GSK has initiated an additional reproductive toxicity study with the AS03-adjuvanted Quebec H5N1 influenza vaccine to further confirm the results observed in the two reproductive toxicity studies conducted with materials representative of the individual components of the pandemic vaccine.

No mutagenic effects were observed when AS03 was tested *in vitro* in the Ames assay or mouse lymphoma assay. AS03 was also negative when tested *in vivo* in the rat micronucleus assay. These studies demonstrate that AS03 is not mutagenic in the conditions tested.

In summary, these data indicate that there were no systemic effects due to repeated treatment with AS03, seasonal and pandemic influenza vaccine, or AS03 adjuvanted influenza vaccine. Overall, there was no evidence suggesting mutagenicity, reproductive, local or developmental toxicity of AS03 alone or in combination with influenza antigens.

2.6.6.10 Tables and Figures

Tables were integrated in the text.

Module 2.6: Nonclinical Written and Tabulated Summary

2.6.7 TOXICOLOGY TABULATED SUMMARY

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2.6.7.1 Toxicology

Table 1 Overview of Completed Toxicology Studies

Type of Study/Test Article	Species and Strain	Method of Administration	Duration of Dosing	Doses (mg/kg)	GLP Compliance	Testing Facility	Study Number	Safety Factor Evaluated
Single-dose Toxicity								
15µg HA (H3N2 A/Wisconsin) Q-Antigen + AS03	New Zealand White rabbits	Intramuscular	Single dose	Full human dose (0.5mL)	Yes	[redacted]	1536-06196	- 20-fold excess (0.5mL/2.5kg in rabbits vs. 0.5mL/50kg in human) -79-fold HA content excess compared to (pre-)pandemic vaccine dose (15µg/2.5Kg in rabbits vs. 3.8µg/50Kg in human)
30µg HA (H5N1 A/Indonesia) Q-antigen + AS03	New Zealand White rabbits	Intramuscular	Single dose	Full human dose (0.5mL)	Yes	[redacted]	2990/355	- 20-fold excess (0.5mL/2.5kg in rabbits vs. 0.5mL/50kg in human) -158-fold HA content excess compared to (pre-)pandemic vaccine dose (30µg/2.5Kg in rabbits vs. 3.8µg/50Kg in human)
Repeat-dose Toxicity								
15µg HA (H3N2 A/Wisconsin) Q-Antigen + AS03	New Zealand White rabbits	Intramuscular	Four injections	Full human dose (0.5mL)	Yes	[redacted]	1536-06194	-20-fold excess (0.5mL/2.5kg in rabbits vs. 0.5mL/50kg in human) - 79-fold HA content excess compared to (pre-)pandemic vaccine dose (15µg/2.5Kg in rabbits vs. 3.8µg/50Kg in human)
15µg HA (H5N1 A/Vietnam D-antigen + AS03	New Zealand White rabbits	Intramuscular	Four injections at two-week intervals	Full human dose (0.5mL)	Yes	[redacted]	1990/956	-20-fold excess (0.5mL/2.5kg in rabbits vs. 0.5mL/50kg in human) - 79-fold HA content excess compared to (pre-)pandemic vaccine dose (15µg/2.5Kg in rabbits vs. 3.8µg/50Kg in human)

Type of Study/Test Article	Species and Strain	Method of Administration	Duration of Dosing	Doses (mg/kg)	GLP Compliance	Testing Facility	Study Number	Safety Factor Evaluated
Genotoxicity								
AS03	*Salmonella typhimurium* TA 1535, TA 1537, TA 98 and TA 100 and *Escherichia coli* WP2*uvr*A (Ames test)	In vitro			Yes	[redacted]	21354	NA
AS03	L5178Y mouse lymphoma assay	In vitro			Yes	[redacted]	[redacted]/785/052587	
AS03	Rat Crl:CD	Intravenous	Two doses with a 24 hr interval	2mg/kg	Yes	[redacted]	[redacted]0002/062069	
Carcinogenicity	Not applicable							
Reproductive and Developmental Toxicity								
D-H5N1 antigen +AS03	Crl:CD® (SD) IGS BR rats	Intramuscular	Pre-mating 4 doses during gestation	2/5th of human dose (0.2mL)	Yes	[redacted]	[redacted]/0007/063710	- 80-fold excess compared to seasonal vaccine (0.2mL/0.25kg in rats vs. 0.5mL/50kg in human) - 631-fold HA content excess compared to (pre-)pandemic vaccine dose (12µg/0.25Kg in rats vs. 3.8µg/50Kg in human)
AS03 alone				4/5th of human dose (0.4mL)	Yes	[redacted]	[redacted]/0007/063710	- 160-fold excess (0.4mL/0.25kg in rats vs. 0.5 mL/50kg in human)
Fluarix and FluLaval trivalent influenza vaccine	Crl:CD® (SD) IGS BR rats	Intramuscular	Pre-mating 4 doses during gestation	1/5th of human dose (0.1mL)	Yes	[redacted]	[redacted]/0009/064374	- 40-fold excess (0.1mL/0.25kg in rats vs. 0.5mL/50kg in human) - 474-fold HA content excess compared to (pre-)pandemic vaccine dose (9µg/0.25Kg in rats vs. 3.8µg/50Kg in human)

Type of Study/Test Article	Species and Strain	Method of Administration	Duration of Dosing	Doses (mg/kg)	GLP Compliance	Testing Facility	Study Number	Safety Factor Evaluated
Local Tolerance								
15µg HA (H3N2 A/Wisconsin) Q-antigen + AS03	New Zealand White rabbits	Intramuscular	Single dose	Full human dose (0.5mL)	Yes	[redacted]	1536-06196	- 20-fold excess (0.5mL/2.5kg in rabbits vs. 0.5mL/50kg in human) -79-fold HA content excess compared to (pre-)pandemic vaccine dose (15µg/2.5Kg in rabbits vs. 3.8µg/50Kg in human)
30µg HA (H5N1 A/Indonesia) Q-antigen + AS03	New Zealand White rabbits	Intramuscular	Single dose	Full human dose (0.5mL)	Yes	[redacted]	2990/355	- 20-fold excess (0.5mL/2.5kg in rabbits vs. 0.5mL/50kg in human) -158-fold HA content excess compared to (pre-)pandemic vaccine dose (30µg/2.5Kg in rabbits vs. 3.8µg/50Kg in human)

2.6.7.2 Toxicokinetics: Overview of Toxicokinetics Studies

No toxicokinetic data were generated, since determination of circulating levels of antigens is not required according to Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95).

Evidence of vaccine exposure as measured by serological analysis (e.g., induction of antibodies) is presented in m4.2.1.1, Primary Pharmacodynamics.

2.6.7.3 Toxicokinetics: Overview of Toxicokinetics Data

Not applicable.

2.6.7.4 Single-dose Toxicity

The single dose toxicity was assessed as part of each of the local tolerance studies (refer to m2.6.7.14) performed in rabbits, the first with Quebec H3N2 antigen (A/Wisconsin/67/2005), and the second with Quebec H5N1 (A/Indonesia/5/2005) antigen. Antigens were adjuvanted with AS03 in both studies. Study designs and results are summarized in Table 2.

Table 2 Single dose Toxicity Study Design

Species/Strain	Method of Administration (Test Article)	Doses (µg)	Gender and No. per Group	Observed Maximum Non-lethal Dose	Approximate Lethal Dose	Noteworthy Findings (After Dosing)	Study Number
New Zealand White Rabbit	IM (Q-H3N2 antigen, AS03 adjuvanted)	15µg HA	3 females and 3 males/group	No lethality observed	No lethality observed	None	[redacted], [redacted]. Study No. 1536-06196
New Zealand White Rabbit	IM (Q-H5N1 antigen, AS03 adjuvanted)	30µg HA	3 females and 3 males/group	No lethality observed	No lethality observed	One male rabbit showed very slight erythema at injection site 72h after dosing	[redacted] 2990/355

2.6.7.5 Repeat-dose Toxicity: Non-pivotal Studies

Repeat-dose non-pivotal toxicity studies were not performed.

2.6.7.6 Repeat-dose Toxicity Pivotal Studies

A repeat-dose toxicity study ([redacted] Study Number 1536-06194) of the Quebec manufactured antigen with and without AS03 was undertaken in New Zealand White rabbits. This study included seasonal antigens (H3N2) prepared by both the Dresden (Fluarix) and Quebec (FluLaval) processes. Study design and results for this study are summarized in Table 3 to Table 5.

An additional repeat-dose toxicity ([redacted] Study Number 1009/956) study was conducted using the Dresden H5N1 antigen with and without AS03 in New Zealand White rabbits. The study design and results for this study are presented in Table 6 through Table 8.

These two studies assessed the potential toxicity effect of Quebec-manufactured antigen or H5N1 antigen, with or without adjuvant, when administered by repeated intramuscular injection to male and female New Zealand White rabbits.

In order to confirm this absence of toxicity with repeated administration of AS03-adjuvanted Quebec H5N1 (A/Indonesia/5/2005) influenza vaccine, GSK has initiated two additional repeat-dose toxicity studies with the AS03-adjuvanted Quebec H5N1 influenza vaccine administered at two different dosages ([redacted] study 2990/356, 30µg HA dose; and [redacted] study 8550, 3.8µg HA dose).

Table 3 Summary of Repeat-dose Toxicity Study ([redacted] Study No. 1536-06194)

<table>
<tr><td colspan="3">Report Title: Pandemic Influenza Vaccines with AS03B Adjuvant: A 115-Day Repeat-dose Intramuscular Toxicity Study in New Zealand White Rabbits</td></tr>
<tr><td colspan="2">Duration of Dosing: 4 Occasions (days 1, 15, 71 and 85)
Days 1,15 and 29 for animals that were sacrified on SD32 and 43 (3 animals/group)
Duration of Recovery: 4 weeks</td><td>Test Articles (Batch No.):
Adjuvant, AS03B, DA3BA004A
Candidate Vaccine 1, split Q- H3N2/AS03B (1M6071WC)
Candidate Vaccine 2, split D-H3N2/AS03B (AFLUBDA420)
Sterile Phosphate Buffered Saline-PBS (S-0037-089)</td></tr>
<tr><td>Species/Strain: New Zealand White rabbits</td><td>Route/Frequency: Intramuscular injection on days 1, 15, 71 and 85</td><td>Date of First Dose: [redacted] 20[redacted] (Males)
[redacted] 20[redacted] (Females)</td></tr>
<tr><td>Initial Age: 14 to 15 weeks</td><td>Vehicle/Formulation: Sterile physiological saline</td><td>Study in Compliance with GLP: Yes</td></tr>
<tr><td colspan="3">Data Collected: Cage-side observations (included observation for mortality, moribundity, general health and signs of toxicity), clinical observations (included evaluation of skin and fur characteristics, eye and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor and behavior patterns), body weight, food consumption, ophthalmoscopy, clinical chemistry, organ weights, dermal Draize observations, clinical pathology and immunology.</td></tr>
<tr><td colspan="3">Conclusion: Repeated intramuscular injection of AS03 adjuvant, 15µg of H3N2 antigen (Dresden or Quebec source) adjuvanted with AS03 was generally well tolerated in male and female New Zealand White rabbits. Treatment with H3N2 antigen (Dresden or Quebec source) adjuvanted with AS03 had a transient effect on food consumption in the males following SD 15 dose administration. Changes in the selected clinical pathology parameters and histopathologic findings in the injection sites, in perineural connective tissue surrounding the sciatic nerves and skeletal muscle (biceps femoris) were consistent with an inflammatory response to the adjuvant and/or vaccine administration.</td></tr>
</table>

Note to the reviewer: In this study, the adjuvant was, historically, named AS03B. Its composition is, however, the same as the current AS03 adjuvant.

Table 4 Study Design of Repeat-dose Toxicity ([redacted] Study No. 1536-06194)

Group	Treatment		Dose Level		Days of Dosing		Number of Animals	
	Priming	Boost	Priming	Boost	Priming	Boost	Male	Female
1	PBS	PBS	0.5mL	0.5mL	SD1, 15, and 29	SD 71 and 85	10	10
2	AS03	AS03	0.25mL AS03 full human dose with PBS	0.25mL AS03 full human dose with PBS	SD1, 15, and 29	SD 71 and 85	16	16
3	Q-H3N2* + AS03	Q-H3N2* + AS03	15µg H3N2 with full human dose AS03	15µg H3N2 with full human dose AS03	SD1, 15, and 29	SD 71 and 85	16	16
4	D-H3N2** + AS03	Q-H3N2* + AS03	15µg H3N2 with full human dose AS03	15µg H3N2 with full human dose AS03	SD1 and 15	SD 71 and 85	16	16

Note: SD = Study day, SD 29 dosing was only for animals that were sacrificed on SD 32 and 43.
*Q-H3N2: A/Wisconsin/67/2005
**D-H3N2: A/New York/55/2004

Table 5 Summary of Results ([redacted] Study No. 1536-06194)

Group	**1**	**2**	**3**	**4**
Dosage Priming **Boost**	**PBS (**0.5mL) **PBS (**0.5mL)	**AS03 (**0.25mL in PBS) **AS03 (**0.25mL in PBS)	**15µg Q-H3N2* with AS03** **15µg Q-H3N2* with AS03**	**15µg D-H3N2** with AS03** **15µg Q-H3N2* with AS03**
Number of Animals	10 Females/10 Males	16 Females/16 Males	16 Females/16 Males	16 Females/16 Males
Toxicokinetics:	ND	ND	ND	ND
Noteworthy Findings				
Died or Sacrificed Moribund	0	0	0	0
Body Weight	No effect	No effect	No effect	No effect
Food Consumption	No effect	No effect	Transient decrease	Transient decrease
Water Consumption	ND	ND	ND	ND
Ophthalmoscopy	No ocular effect	No ocular effect	No ocular effect	No ocular effect
Electrocardiography	ND	ND	ND	ND
Clinical Observations	-			
Serum Chemistry				
Organ Weights	No effect	No effect	No effect	No effect
Dermal Draize Observation:				
- Injection Site Erythema	minimal erythema	Minimal erythema	Minimal erythema	Minimal erythema
- Injection Site Edema	minimal edema	Minimal edema	Minimal edema	Minimal edema

*Q-H3N2: A/Wisconsin/67/2005,
**D-H3N2: A/New York/55/2004

Table 6 Summary of Repeat-dose Toxicity Study ([redacted] Study No. 1990/956)

<table>
<tr><td colspan="3">Report Title: Pandemic Influenza Candidate Vaccines (split H5N1 and split H5N1/AS03): Repeated (4 Occasions) Intramuscular Administration Toxicity Study in the Rabbit with a 4-Week Recovery Period</td></tr>
<tr><td colspan="2">Duration of Dosing: 4 occasions (days 1, 15, 29, and 43)
Duration of Recovery: 4 weeks</td><td>Test Articles (Batch No.):
Adjuvant, AS03, DA3AA001A
Candidate Vaccine 1, split H5N1, DFLUA016A
Candidate Vaccine 2, split H5N1/AS03 (batches as above)</td></tr>
<tr><td>Species/Strain: New Zealand White rabbits</td><td>Route/Frequency: Intramuscular injection on days 1, 15, 29 and 43</td><td>Date of First Dose: [redacted] 20[redacted] (Males)
[redacted] 20[redacted] (Females)</td></tr>
<tr><td>Initial Age: 10 to 12 weeks</td><td>Vehicle/Formulation: Sterile physiological saline</td><td>Study in Compliance with GLP: Yes</td></tr>
<tr><td colspan="3">Data Collected: Clinical observations, skin irritation observations, body weight, food consumption, ophthalmoscopy, haematology, clinical chemistry, organ weights, macroscopic and microscopic pathology</td></tr>
<tr><td colspan="3">Conclusion: The local and systemic toxic effects of the candidate vaccines (split H5N1 and split H5N1/AS03) and the adjuvant (AS03) were assessed in male and female rabbits after four intramuscular injections. The reversibility of any changes over a 4-week recovery period was also assessed.

Local effects were restricted to erythema and/or edema noted at the injection sites of some animals administered AS03 or split H5N1/AS03 up to 48 hours after dosing, suggestive of an effect from the adjuvant, rather than the vaccine as it was not apparent in the split H5N1 treatment group.

There were no systemic effects due to treatment with AS03, split H5N1 or split H5N1/AS03.</td></tr>
</table>

Table 7 Study Design of Repeat-dose Toxicity ([redacted] Study Number 1990/956)

Group	Treatment	Dose Level	Days of Dosing	Number of Animals	
				Male	Female
1	Saline	0.5mL	1, 15, 29, and 43	10	10
2	AS03 full human dose	0.5mL	1, 15, 29, and 43	10	10
3	30µg H5N1 antigen full human dose	0.5mL	1, 15, 29, and 43	10	10
4	30µg H5N1 antigen with AS03 full human dose	1.0mL	1, 15, 29, and 43	10	10

Table 8 Summary of Results ([redacted] Study Number 1990/956)

Tabular summary - continued								
	Male				Female			
Daily Dose	0	AS03	split H5N1	split H5N1/AS03	0	AS03	split H5N1	split H5N1/AS03
Numbers of Animals: Main	5	5	5	5	5	5	5	5
(Recovery)	5	5	5	5	5	5	5	5
Noteworthy Findings								
Unscheduled Deaths	0	0	0	0	0	0	0	1
	The death was considered not to be related to local or systemic effects of split H5N1 pandemic influenza candidate vaccine and/or Adjuvant (AS03).							
Clinical Observations	Erythema (very slight to slight) was noted in some animals administered AS03 and split H5N1/AS03 after dosing on Day 1, 29 and 43. Oedema was of a low incidence and was only present after the last dosing occasion (Day 43) after treatment with AS03, split H5N1 or split H5N1/AS03.							
Haematology								
Day 2 Fibrinogen(g/L)	1.9	68 ***	16	74 ***	1.8	50 *	-	56 *
White blood cells (1000/cmm)	8.0	16	-14	21	7.3	19	14	56 ***
Neutrophils (1000/cmm)	1.4	3-fold ***	-14	3-fold ***	1.0	3-fold ***	10	4-fold ***
Day 4 Fibrinogen (g/L)	2.6	27 **	-15	12	2.0	45 ***	-10	30 *
Day 44 Fibrinogen (g/L)	2.3	30	-9	17	1.8	28	0	33 *
White blood cells (1000/cmm)	7.2	26	-10	38 *	6.4	41 *	8	45 *
Neutrophils (1000/cmm)	1.0	4-fold ***	10	5-fold ***	0.9	3.7-fold ***	22	3.4-fold ***
Day 46 Fibrinogen (g/L)	2.4	38 ***	4	25 ***	1.8	28 *	6	22 *
Neutrophils (1000/cmm)	1.1	55 **	9	27	1.1	36	-9	45 *
Day 71								
White blood cells (1000/cmm)	7.9	-33 *	-16	-16	6.6	11	18	33 *
Adjusted Organ Weights [c](x control mean)								
Day 46 kill Spleen (g)	1.007	41 *	34	25	1.392	24	24	7

Table 8 (Cont'd) Summary of Results: [redacted] Study Number 1990/956 (Continued)

Tabular summary - continued									
		Male				Female			
Daily Dose		0	AS03	split H5N1	split H5N1/AS03	0	AS03	split H5N1	split H5N1/AS03
Day 71 kill	Spleen (g)	1.497	-25	-16	-33 *	1.792	5	1	-10
	Testes (g)	8.027	8	12	23 *	-	-	-	-
Microscopic Examination – Day 46 (number of animals)		5	5	5	5	5	5	5	5
Right anterior thigh									
Granulomatous/needle track myositis		3	3	3	4	5	4	5	4
Fasciitis	Grade -	5	0	4	0	5	1	3	1
	Grade 1	0	1	1	2	0	3	2	3
	Grade 2	0	4	0	3	0	1	0	1
Perivascular cuffing		0	0	5	5	0	0	4	5
Sciatic nerve									
Fasciitis/perivascular cuffing		0	1	0	5	0	0	1	4
Spleen									
Lymphoid hyperplasia		1	3	4	2	0	3	3	3
Microscopic Examination – Day 71 (number of animals)		5	5	5	5	5	5	5	4
Right anterior thigh									
Chronic fasciitis	Grade -	5	1	5	3	5	0	5	2

Table 8 (Cont'd) Summary of Results: [redacted] Study Number 1990/956 (Continued)

Tabular summary - continued								
	Male				Female			
Daily Dose	0	AS03	split H5N1	split H5N1/AS03	0	AS03	split H5N1	split H5N1/AS03
Grade 1	0	3	0	2	0	5	0	2
Grade 2	0	1	0	0	0	0	0	0
Chronic needle track/myositis/fibrosis	0	0	1	0	2	2	0	2
Perivascular cuffing	0	0	1	0	0	0	1	0

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$
\- Not applicable
Grade key: "-" = unremarkable, 1 = minimal, 2 = slight
For controls, group means are shown, for treated groups x of the mean control or ratios are shown. [Statistical significance is based on actual data not x control differences.]
Absolute and/or relative weights different from controls in the direction indicated. Numbers indicate x control difference for absolute organ weights.

2.6.7.7 Genotoxicity: In Vitro

As noted in EMEA/CPMP Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95), this is not required for vaccines.

However two genotoxicity studies were performed *in vitro* with the AS03 adjuvant alone:

- AS03 was tested for mutagenic potential in a bacterial reverse mutation (Ames) assay ([redacted] study number 21354)
- AS03 was tested for mutagenic potential in an *in vitro* mammalian cell mutation assay based on the detection and of forwards mutations in mouse lymphoma L5178Y cells ([redacted] study number [redacted] 785/052587).

Table 9 Study Design: [redacted] Study No. 21354

Genotoxicity: *In Vitro*	**Report Title:** AS03, Batches 1011 and 1017 Cupac1: Testing for Mutagenic Activity with *Salmonella typhimurium* TA 1535, TA 1537, TA 98 and TA 100 and *Escherichia coli* WP2*uvr*A	**Test Article:** AS03 **Batch Number:** Batches 1011 and 1017 Cupac1
Test for Induction of: Reverse Mutation in Bacterial Cells	**No. of Independent Assays:** 2 per test article	**Study No/Ref. No.:** 21354
Strains *Salmonella typhimurium* and *Escherichia coli* WP2*uvr*A	**No. of Replicate Cultures:** 3	**Location in CTD: 4.2.3.3.1.1**
Metabolizing System: Aroclor-induced rat liver S9 mix (9 parts cofactor with 1 part S9 (v/v))		
Vehicles for: • **Test Article:** Phosphate buffered saline, pH 6.8 • **Positive Controls:** Sodium azide (NaN_3) = water; 2-aminoanthracene (2AAN), N-ethyl-N-nitrosoguanidine (ENNG), 9-aminoacridine (9AA), 2-nitrofluorene (2NF) = DMSO		**GLP Compliance:** Yes
Treatment: Plate incorporation method (1st Assays) and pre-incubation method (2nd Assays) with and without metabolic activation		**Date of Treatment(s):** [redacted] 20[redacted]
Cytotoxic Effects: No toxicity to the background lawn of bacteria was observed. Dense precipitation prevented assessment of lawns at higher dose levels.		
Genotoxic Effects: None		

Table 10 First Mutation Assay: Mean Number of Revertant Colonies per Plate

Metabolic Activation	Test Article	Concentration (µL/plate)	Revertant Colonies (Mean ± SD)				
			TA 1535	TA 1537	TA 98	TA 100	WP2*uvr*A
Without Activation	PBS pH 6.8	100	8±2	9±4	12±3‡	81±22	10±2
	AS03, batch 1011	1.7	9±4	6±2	13±2‡	89±11	14±2
		5	5±4	5±4	12±1‡	89±6	12±2
		17 (P)	13±4	5±3	16±3‡	94±20	15±6
		50 (P#)	7±3	5±2	12±4‡	107±7	11±3
		167 (P#)	5±3	4±1	11±5‡	105±5	13±2
		500 (P#)	6±3	6±1	15±5‡	119±8	16±3
	NaN_3	1	283±22			861±8	
	9AA	80		4183±157			
	2NF	1			502±54		
	ENNG	2					314±11
With Activation	PBS pH 6.8	100	10±1	14±2	22±3	105±15	8±1
	AS03, batch 1011	1.7	14±5	10±4	17±4	112±13	8±3
		5	17±5	9±2	22±4	109±13	13±2
		17	11±6	3±2	20±7	80±12	11±3
		50 (P)	10±5	11±4	21±3	77±52	13±6
		167 (P#)	14±4	9±2	22±7	96±12	8±5
		500 (P#)	13±1	7±0	21±3	106±10	14±6
	2AAN	2	366±36	331±9			
		0.5			271±27	515±48	
		20					721±29

SD = Standard Deviation.
P = Precipitation.
P# = Precipitation too dense to access toxicity.
‡ = Results from retest, conducted independently of main assay.

Table 10 (cont'd) First Mutation Assay: Mean Number of Revertant Colonies per Plate

Metabolic Activation	Test Article	Concentration (µL/plate)	Revertant Colonies (Mean ± SD)				
			TA 1535	TA 1537	TA 98	TA 100	WP2*uvr*A
Without Activation	PBS pH 6.8	100	8±2	9±4	12±3‡	81±22	10±2
	AS03, batch 1017 Cupac1	1.7	9±6	4±3	11±2‡	86±17	15±1
		5	8±6	6±2	14±2‡	88±16	12±7
		17 (P)	9±6	3±3	9±1‡	68±20	12±5
		50 (P#)	8±6	7±3	11±2‡	101±10	13±6
		167 (P#)	8±2	4±1	12±2‡	102±13	18±2
		500 (P#)	9±4	5±2	12±4‡	102±8	12±4
	NaN_3	1	283±22			861±8	
	9AA	80		4183±157			
	2NF	1			502±54		
	ENNG	2					314±11
With Activation	PBS pH 6.8	100	10±1	14±2	22±3	105±15	8±1
	AS03, batch 1017 Cupac1	1.7	7±5	9±1	27±4	115±12	12±5
		5	11±6	13±2	23±3	105±5	8±3
		17	13±2	7±3	17±4	94±11	7±3
		50 (P)	8±3	10±1	22±1	100±4	12±3
		167 (P#)	12±3	11±5	21±2	100±6	12±3
		500 (P#)	9±3	8±1	21±4	86±6	15±5
	2AAN	2	366±36	331±9			
		0.5			271±27	515±48	
		20					721±29

SD = Standard Deviation.
P = Precipitation.
P# = Precipitation too dense to access toxicity.
‡ = Results from retest, conducted independently of main assay.

Table 11 Second Mutation Assay: Mean Number of Revertant Colonies per Plate

Metabolic Activation	Test Article	Concentration (µL/plate)	Revertant Colonies (Mean ± SD)				
			TA 1535	TA 1537	TA 98	TA 100	WP2*uvr*A
Without Activation	PBS pH 6.8	100	10±4	8±4	16±5	108±15	5±1
	AS03, batch 1011	1.7	17±3	8±4	11±3	100±7	7±2
		5	10±5	6±2	13±2	101±9	8±3
		17 (P)	11±3	7±4	9±4	87±5	10±5
		50 (P#)	11±4	6±2	15±6	113±10	10±2
		167 (P#)	9±6	6±2	9±3	104±6	9±3
		500 (P#)	9±2	8±3	17±4	120±11	8±1
	NaN_3	1	387±18			997±14	
	9AA	80		3333±579			
	2NF	1			401±12		
	ENNG	2					677±14
With Activation	PBS pH 6.8	100	19±5	16±4	14±5	129±16	23±5
	AS03, batch 1011	1.7	16±5	14±5	23±5	132±12	26±3
		5	15±11	11±1	19±6	113±11	30±1
		17	15±4	6±3	25±3	115±11	36±3
		50 (P)	13±1	9±4	23±2	116±8	37±1
		167 (P#)	16±4	11±2	19±5	119±9	32±5
		500 (P#)	14±3	8±1	21±4	124±5	30±6
	2AAN	2	237±14	255±21			
		0.5			362±26	1162±34	
		20					709±68

SD = Standard Deviation.
P = Precipitation.
P# = Precipitation too dense to access toxicity.

Table 11 (cont'd) Second Mutation Assay: Mean Number of Revertant Colonies per Plate

Metabolic Activation	Test Article	Concentration (µL/plate)	Revertant Colonies (Mean ± SD)				
			TA 1535	TA 1537	TA 98	TA 100	WP2*uvr*A
Without Activation	PBS pH 6.8	100	10±4	8±4	16±5	108±15	5±1
	AS03, batch 1017 Cupac	1.7	8±4	7±2	12±3	90±14	10±4
		5	11±3	7±1	12±1	87±8	7±5
		17 (P)	8±3	5±1	14±3	89±4	7±5
		50 (P#)	12±1	8±4	15±4	103±12	9±2
		167 (P#)	10±2	5±2	16±1	97±11	6±3
		500 (P#)	8±4	7±2	14±4	109±15	6±4
	NaN_3	1	387±18			997±14	
	9AA	80		3333±579			
	2NF	1			401±12		
	ENNG	2					677±14
With Activation	PBS pH 6.8	100	19±5	16±4	14±5	129±16	23±5
	AS03, batch 1017 Cupac	1.7	17±6	10±3	26±3	146±8	32±8
		5	20±6	16±1	22±2	130±14	31±4
		17	13±6	8±2	20±2	106±8	32±9
		50 (P)	21±1	12±1	23±5	117±4	30±8
		167 (P#)	13±2	7±2	25±4	116±1	35±3
		500 (P#)	15±8	4±1	18±5	119±18	32±3
	2AAN	2	237±14	255±21			
		0.5			362±26	1162±34	
		20					709±68

SD = Standard Deviation.
P = Precipitation.
P# = Precipitation too dense to access toxicity.

Table 12 Study Design: [redacted] Study No. [redacted] 785/052587

Genotoxicity: *In Vitro*	**Report Title:** AS03 new, AS03 old and AS03/alpha tocopheryl quinone: *In vitro* Mutation Assay with L5178Y Mouse Lymphoma Cells at the TK Locus	**Test Article:** AS03 New[1] **Batch Number:** DSBAAPA001/TOX **Test Article:** AS03 old[2] **Batch Number:** 1017(cupac2)/TOX **Test Article:** AS03 alpha-tocopheryl quinone[3] **Batch Number:** FAS0501/02/TOX
Test for Induction of: Forward mutation at the TK$^{\pm}$ locus	**No. of Independent Assays:** 7	**Study No./Ref. No.:** [redacted] 785/052587
Cell Type: L5178Y Mouse Lymphoma Cells	**No. of Replicate Cultures:** 4 (vehicle); 2 (treatment and positives)	**Location in CTD: 4.2.3.3.1.2**
Metabolizing System: Aroclor-induced rat liver S9-mix containing 2% v/v S9-fraction (final)		
Vehicles **Test Article:** PBS **Positive Controls:** DMSO		**GLP Compliance:** Yes
Treatment: 3h treatment with and without S9-mix; treatment for 24h without S9-mix		**Date of Treatment(s):** [redacted] 20[redacted]
Cytotoxic Effects: Reduction in RTG		
Genotoxic Effects: Negative		

[1]AS03 new = Oil-in-water emulsion containing less than 0.05mg/mL tocopherylquinone
[2]AS03 old = Oil-in-water emulsion containing 0.25mg/mL tocopherylquinone
[3]AS03/alpha tocopherylquinone = Oil-in-water emulsion containing 4.94mg/mL tocopherylquinone

Table 13 Results of Main Mutation Test with AS03 New (Oil-in-Water Emulsion Containing <0.05mg/mL Tocopheryl Quinone)

Test Article	Dose Level[1] (µg/mL)	3h Treatment -S9-mix		3h Treatment +S9-mix	
		Mean RTG (%)	Pooled Mutant Freq. ($x10^{-6}$)	Mean RTG (%)	Pooled Mutant Freq. ($x10^{-6}$)
PBS[2]	-	100	133	100	153
AS03 New	500	96	99	111	94
AS03 New	1000	113	107	75	95
AS03 New	2000	90	95	52	107
AS03 New	3000	70	106	25	233
AS03 New	4000	62	129	21	200[3]
AS03 New	4500	60	139	15	192[3]
AS03 New	5000	66	105	11	258[3]
MMS	10	57	829	-	-
3MC	2.5	-	-	40	1123

[1]Expressed in terms of active moiety
[2]Vehicle control dosed at 10% v/v
[3]Precipitate observed post-wash
- = Not treated

Table 13 (cont'd) Results of Main Mutation Test with AS03 New (Oil-in-Water Emulsion Containing <0.05mg/mL Tocopheryl Quinone)

Test Article	Dose Level[1] (µg/mL)	24h Treatment -S9 Mix	
		Mean RTG (%)	Pooled Mutant Freq. ($x10^{-6}$)
PBS[2]	-	100	125
AS03 New	2	109	167
AS03 New	50	119	126
AS03 New	100	94	113
AS03 New	150	63	148
AS03 New	200	45	178
AS03 New	250	26	184
AS03 New	275	22	172
AS03 New	300	23	184
AS03 New	325	25	158
AS03 New	400	12	196
MMS	5	42	1700

[1]Expressed in terms of active moiety
[2]Vehicle control dosed at 10%v/v

Table 14 Results of Main Mutation Test with AS03 Old (Oil-in-Water Emulsion Containing 0.25mg/mL Tocopheryl Quinone)

Test Article	Dose Level[1] (µg/mL)	3h Treatment -S9-mix		3h Treatment +S9-mix		24h Treatment -S9-mix	
		Mean RTG (%)	Pooled Mutant Freq. ($x10^{-6}$)	Mean RTG (%)	Pooled Mutant Freq. ($x10^{-6}$)	Mean RTG (%)	Pooled Mutant Freq. ($x10^{-6}$)
PBS[2]	-	100	114	100	116	100	132
AS03 Old	2	-	-	-	-	70	102
AS03 Old	50	100	114	105	128	67	203
AS03 Old	100	-	-	-	-	131	155
AS03 Old	200	-	-	-	-	54	158
AS03 Old	300	-	-	-	-	38	162
AS03 Old	325	-	-	-	-	31	153
AS03 Old	350	-	-	-	-	26	164
AS03 Old	375	-	-	-	-	16	224
AS03 Old	500	95	111	68	113	-	-
AS03 Old	1000	64	110	53	162	-	-
AS03 Old	1250	50	139	33	192	-	-
AS03 Old	1500	41	128[3]	28	153	-	-
AS03 Old	1750	27	145[3]	25	173[3]	-	-
AS03 Old	2000	-	-	15	151[3]	-	-
MMS	5	-	-	-	-	51	1171
MMS	10	55	957	-	-	-	-
3MC	2.5	-	-	46	1180	-	-

[1]Expressed in terms of active moiety
[2]Vehicle control dosed at 10% v/v
[3]Precipitate observed post-wash
- = Not treated

Table 15 Results of Main Mutation Test with AS03/α-Tocopheryl Quinone (Oil-in-Water Emulsion Containing 4.94mg/mL Tocopheryl Quinone)

Test Article	Dose Level[1] (µg/mL)	3h Treatment -S9-mix		3h Treatment +S9-mix		3h Treatment +S9-mix, Additional Test	
		Mean RTG (%)	Pooled Mutant Freq. (x10^{-6})	Mean RTG (%)	Pooled Mutant Freq. (x10^{-6})	Mean RTG (%)	Pooled Mutant Freq. (x10^{-6})
PBS[2]	-	100	95	100	81	100	75
AS03/a t-q[3]	100	108	100	96	74	-	-
AS03/a t-q[3]	500	106	98	82	101	-	-
AS03/a t-q[3]	1000	94	84	96	71	-	-
AS03/a t-q[3]	2000	-	-	56	111	-	-
AS03/a t-q[3]	2250	-	-	57	97	-	-
AS03/a t-q[3]	2500	34	140[4]	38	113	-	-
AS03/a t-q[3]	3000	-	-	30	137	34	106
AS03/a t-q[3]	3200	-	-	-	-	29	124
AS03/a t-q[3]	3400	-	-	-	-	28	151
AS03/a t-q[3]	3500	-	-	28	180	26	175
AS03/a t-q[3]	3550	-	-	-	-	29	121
AS03/a t-q[3]	3600	-	-	-	-	27	166
AS03/a t-q[3]	3650	-	-	-	-	31	163
AS03/a t-q[3]	3700	-	-	-	-	26	196[4]
AS03/a t-q[3]	3850	-	-	-	-	25	157[4]
AS03/a t-q[3]	4000	18	138[4]	-	-	19	179[4]
MMS	10	43	1137	-	-	-	-
3MC	2.5	-	-	58	964	55	662

[1]Expressed in terms of active moiety
[2]Vehicle control dosed at 10% v/v
[3]AS03/a t-q = AS03/alpha tocopheryl-quinone
[4]Precipitate observed post-wash - - Not treated

Table 15 (cont'd) Results of Main Mutation Test with AS03/α-Tocopheryl Quinone (Oil-in-Water Emulsion Containing 4.94mg/mL Tocopheryl Quinone)

Test Article	Dose Level[1] (µg/mL)	24h Treatment -S9-mix	
		Mean RTG (%)	Pooled Mutant Freq. ($x10^{-6}$)
PBS[2]	-	100	169
AS03/a t-q[3]	2	52	136
AS03/a t-q[3]	50	35	149
AS03/a t-q[3]	100	54	140
AS03/a t-q[3]	200	19	225
AS03/a t-q[3]	300	16	178
AS03/a t-q[3]	400	16	138
AS03/a t-q[3]	500	20	120
AS03/a t-q[3]	525	18	151
AS03/a t-q[3]	550	14	168
AS03/a t-q[3]	575	16	147
AS03/a t-q[3]	600	18	161
MMS	5	7[4]	2481

[1]Expressed in terms of active moiety.
[2]Vehicle control dosed at 10% v/v.
[3]AS03/a t-q = AS03/alpha tocopheryl-quinone
[4]While the RTG for the positive control is marginally outside the 10% stated in the acceptance criteria, the positive controls are still considered to be valid and acceptable as an absolute increase in MF over the concurrent vehicle control was obtained.
- = Not treated

2.6.7.8 Genotoxicity: In Vivo

As noted in EMEA/CPMP Note for Guidance on Preclinical Pharmacological and Toxicological testing of Vaccines (CPMP/SWP/465/95) this is not required for vaccines.

However, one genotoxicity study ([redacted] study number [redacted] 0002/062069) with the adjuvant AS03 was performed *in vivo* in a rat micronucleus study to assess the potential of AS03 to induce an increase in micronuclei in bone marrow cells of Sprague-Dawley (CD) rats. This study is summarized in Table 16.

Table 16 Tabulated Summary of [redacted] Study No.: [redacted] 0002/062069

Genetic Toxicology: *In Vivo*	**Report Title:** AS03 New, AS03 Old, AS03/alpha tocopheryl quinone: Intravenous Bone Marrow Micronucleus Assay in Rats	**Test Articles and Batch Numbers:** AS03 new: (DSBAAPA001/TOX) AS03 old: (1017 (cupac2/TOX)) AS03/alpha tocopheryl quinone: (FAS0501/02/TOX)
Test for Induction of: Structural chromosomal damage and/or aneuploidy	**Treatment Schedule:** Two doses 24 hours apart (vehicle & test compound). Positive control dosed once only.	**[redacted] Study No:** [redacted] 0002/062069 **GSK Reference No:** N/A **GSK Document No:** N/A
Species/Strain: Rat: (Crl:CD(SD))	**Sampling Time**: 24h after final dose	**Location in CTD:** 4.2.3.3.1.3
Approximate Age: 9 weeks at time of dosing	**Method of Administration:** Intravenous by bolus injection in the lateral tail vein **Dose volume:** 2mL/kg/day	
Cells Evaluated: Immature erythrocytes (ie)	**Vehicle/Formulation:** As a solution in phosphate buffered saline	**GLP Compliance:** Yes
No. of Cells Analyzed/Animal: 2000 ie		**Date of Dosing:** [redacted] 20[redacted]
Special Features: Each test substance administered as supplied		
Toxic/Cytotoxic Effects: No clinical signs observed in any dose group		
Genotoxic Effects: Negative		
Evidence of Exposure:		

Test Compound	Dose (mg/kg/day)	Dose Volume (mg/kg/day)	No. of Animals Analysed	Group Mean (%) ie[1]	Group Mean mie[2]
Vehicle	-	2	7M	39	1.1
AS03new	As supplied	2	7M	39	0.6
AS03 old	As supplied	2	7M	34	2.3
AS03/alpha tocopheryl quinone	As supplied	2	7M	36	1.3
Cyclophosphamide	20	10	5M	35	21.0**

All test substance doses expressed as supplied

Vehicle: Phosphate buffered saline

[1] ie = Immature (polychromatic) erythrocytes

[2] Group mean number of micronucleated immature erythrocytes (mie) per 2000 ie analysed.

** P<0.01 considered significant, otherwise P >0.01.

2.6.7.9 Carcinogenicity

As noted in EMEA/CPMP Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95), this is not required for vaccines.

2.6.7.10 Reproductive and Developmental Toxicity – Non-pivotal Studies

No non-pivotal Reproductive and Developmental Toxicity studies were performed.

2.6.7.11 Reproductive and Developmental Toxicity: Fertility and Early Embryonic Development to Implantation (Pivotal)

Please refer to m2.6.7.12, Reproductive and Developmental Toxicity.

2.6.7.12 Reproductive and Developmental Toxicity (Pivotal)

Absence of potential reproductive effect of the AS03-adjuvanted Quebec H5N1 influenza vaccine is supported by data from the reproductive toxicity studies conducted with each individual component of the vaccine candidates, namely the reproductive toxicity studies with the FluLaval seasonal influenza vaccine ([redacted] study no. [redacted] 0009/064374) and with the AS03-adjuvanted Dresden manufacturing H5N1 (A/Vietnam/1194/04) influenza vaccine ([redacted] study no 1536-08129).

These two studies assessed the effect of (1) both Fluarix and FluLaval influenza vaccines, and (2) both AS03 alone and AS03 with H5N1 antigen produced with the Fluarix-process on embryo-fetal and peri- and post-natal development in naïve or pre-immunized Crl:CD® (SD) IGS BR rats following intramuscular administration.

In order to confirm this absence of reproductive toxicity with the AS03-adjuvanted Quebec H5N1 (A/Indonesia/5/2005) influenza vaccine, the Company has initiated a new reproductive toxicity study (1536-08129).

Reproductive toxicity of AS03 and AS03-adjuvanted Dresden H5N1 antigen influenza vaccine (Fluarix process)

GSK has conducted a reproductive toxicology study ([redacted]-007) to evaluate the effect of AS03-adjuvanted Dresden split H5N1 influenza vaccine, AS03 alone, and aluminum-adjuvanted Dresden whole H5N1 influenza vaccine on embryo-fetal, pre- and post-natal development in the CD rat. A control group has received saline. Forty-eight animals were allocated to each group in order to obtain approximately 44 females with positive indication of mating in each group for treatment during gestation.

During the study, clinical condition, body weight, food consumption, gestation length and parturition observations, and macroscopic pathology investigations were undertaken on F0 females. Fetuses on the embryo-fetal phase of the study were examined

macroscopically at necropsy and subsequently by detailed internal visceral examination or skeletal examination. For offspring on the littering phase of the study, clinical condition and survival, sex ratio, body weight and pre-weaning reflex development were assessed.

The results are summarized in the Table 17 and Table 18.

Table 17 Study Design

Reproductive and Developmental Toxicity Effects on Embryo-fetal, Pre- and Postnatal Development, Including Maternal Function		
Report Title: Pandemic Influenza Candidate Vaccines (Whole H5N1/AI and Split H5N1/AS03) :Study of Effects on Embryo-foetal, Pre-and Post-natal Development Study in CD Rats by Intramuscular Administration (Including Pre-mating Immunisation Phase)		**Test Article:** Whole H5N1/AI Split H5N1/AS03 (Dresden) **Controls:** AS03 Saline
Design Similar to ICH 4.1.2: Yes	**Days of Dosing**: 30PM, G6, G8, G11, G15 **Day of Mating**: G0	**Study Number:** [redacted]/0007
Species/Strain: CD rats **Initial age**: 38- 42 days	**Day of C-Section**: G20 **Method of Administration**: Intramuscular	**Location in CTD**: 4.2.3.5
Date of First Dose: [redacted] 20[redacted] **Special Features**: None	**Litters Culled/Not Culled**: Culled to 5/sex/litter	**GLP Compliance**: Yes

Table 18 Summary of Results

Group	1	2	3	4	5	6
Compound	**Saline**	**AS03 Adjuvant**	**Saline (PM) split H5N1/AS03**	**Split H5N1/AS03**	**Saline (PM) whole H5N1/AI**	**Whole H5N1/AI**
Dosage (µL) per dosing occasion	**200**	**200**	**200**	**200**	**100**	**100**
F0 Females						
No. Premating	48	48	48	48	48	48
No. Died or Sacrificed Moribund	0	1[b]	0	0	0	0
Clinical Observations	-	-	-	-	-	-
Necropsy Observations (Incidence)						
- Injection Site - Pale Area(s)	0/44	5/43	5/44	1/44	8/44	14/44
- Injection Site - Raised Area(s)	0/44	1/43	1/44	3/44	5/44	9/44
Premating Body Weight (%)[c]	248g	-3	-1	-4[a]	-1	-2
Gestation Body Weight (%)[c]	414g	0	0	-3	1	-1
Lactation Body Weight (%)[c]	331g	-2	-1	-4	-1	-2
Premating Food Consumption (%)[c]	23g/animal/day	-4[a]	-4	-9[a]	-4	-4
Gestation Food Consumption(%)[c]	34g/animal/day	3	3	0	3	0
Lactation Food Consumption (%)[c]	118g/animal/day	3	3	3	2	1
No. of Females Sperm-Positive	48	48	47	48	48	48
No. of Pregnant Females	48	48	47	48	48	48
F0 Females - Embryo-foetal Phase						
Mean No. Corpora Lutea	17.2	17.0	16.2	16.6	17.0	16.7
Mean No. Implantations	15.0	15.9	15.3	15.3	16.0	15.7
Mean % Pre-implantation Loss	12.3	6.3	6.1	7.4	5.3[a]	6.3
Mean No. Live Fetuses	14.4	15.4	14.8	14.3	15.3	15.1

- No Noteworthy findings. PM= Pre-mating; G = Gestation day; L = Lactation day

[a] Lipsitz test - $p<0.05$

[b] Fetuses died in-utero

[c] At the end of the specified phase. For controls, group means are shown. For treated groups, percent differences from control are shown. Statistical significance is based on actual data (not on the percent differences).

Table 18 (cont'd) Summary of Results

Group	1	2	3	4	5	6
Compound	**Saline**	**AS03 Adjuvant**	**Saline (PM) Split H5N1/AS03**	**Split H5N1/AS03**	**Saline (PM) Whole H5N1/AI**	**Whole H5N1/AI**
Dosage (µL) per Dosing Occasion	**200**	**200**	**200**	**200**	**100**	**100**
Litters - Embryo-feetal Phase						
No. of Litters Evaluated	22	21	22	22	22	22
No. Live Fetuses	316	324	326	314	336	333
Mean No. Resorptions	0.6	0.4	0.5	1.0	0.7	0.5
Mean % Post-implantation Loss	4.4	3.0	2.8	6.6	4.5	3.5
Mean Fetal Body Weight (g)	3.82	3.82	3.88	3.82	3.95	3.86
Fetal Sex Ratios	54.2	53.6	50.1	48.5	50.0	54.2
Fetal Anomalies:						
- Gross External	-	-	-	-	-	-
- Visceral Anomalies	-	-	-	-	-	-
- Skeletal Anomalies	-	-	-	-	-	-
F0 Females - Littering Phase						
Mean Duration of Gestation (Days)	22.3	22.3	22.3	22.2	22.3	22.3
Abnormal Parturition	-	-	-	-	-	-

No Noteworthy findings. PM= Pre-mating, G = Gestation day, L = Lactation day

Table 18 (cont'd) Summary of Results

Group	1	2	3	4	5	6
Compound	**Saline**	**AS03 Adjuvant**	**Saline (PM) Split H5N1/AS03**	**Split H5N1/AS03**	**Saline (PM) Whole H5N1/AI**	**Whole H5N1/AI**
Dosage (µL) per Dosing Occasion	**200**	**200**	**200**	**200**	**100**	**100**
F1 Litters - Littering Phase						
No. Litters Evaluated	22	22	22	22	22	22
Mean No. Implantations	16.3	15.5	15.5	15.5	16.4	16.1
Mean No. Pups/Litter[e]	15.8	14.4	14.1[a]	14.3	15.4	14.8
Mean No. Liveborn Pups/Litter[e]	15.7	14.2	14.1[a]	14.2	15.3	14.3
No. of Litters with Dead Pups prior to L 1						
Post-natal Survival to L 4 (%)	97.2	99.1	96.3	97.7	98.1	98.8
Post-natal Survival to L 4-25 (%)	98.1	97.7	100.0	97.7	95.5	99.5
Change Male Pup Body Weights L 1-25 (g)[f]	59.7	60.9	63.0[a]	63.1	62.3	61.6
Change Female Pup Body Weights L 1-25 (g)[e]	56.8	58.1	60.6[a]	60.4	59.6	59.1
Pup Sex Ratios (Total L 1)[g]	55.3	46.2[a]	53.8	49.1	45.3[a]	54.1
Pup Clinical Signs	-	-	-	-	-	-
Pup Necropsy Obs.	-	-	-	-	-	-
Pre-weaning Behavioural Assessment						
- Surface Righting (Day of Age)	4.3	4.5	4.4	4.2	4.4	4.1
- Air Righting (Day of Age)[e]	18.1	17.7	17.6	17.7	17.8	17.5[b]
- Air Righting (No. of Pups Which Did Not Pass Prior to Day 21)	2	13	0	0	5	0
-Startle Response (%) Pass	100.0	100.0	100.0	100.0	100.0	100.0
-Pupil Response (%) Pass	99.5	100.0	100.0	99.1	100.0	100.0

- No Noteworthy findings. PM= Pre-mating G = Gestation day L = Lactation day

[e]T-test, [f]Wilcoxon test, [g] Lipsitz test

[a] - $p<0.05$

[b]- $p<0.01$

Reproductive toxicity of Fluarix and FluLaval seasonal influenza vaccines

A reproductive toxicity study ([redacted]-0009/064374) was conducted to assess the effect of both Fluarix and FluLaval seasonal influenza vaccines on embryo-fetal and peri- and post-natal development in naïve or pre-immunized Crl:CD® (SD) IGS BR rats following intramuscular administration. A control group has received saline. Forty-eight animals were allocated to each group in order to obtain approximately 44 females with positive indication of mating in each group for treatment during gestation.

During the study, clinical condition, body weight, food consumption, gestation length and parturition observations, and macroscopic pathology investigations were undertaken on F0 females. Fetuses on the embryo-fetal phase of the study were examined macroscopically at necropsy, and subsequently by detailed internal visceral examination or skeletal examination. For offspring on the littering phase of the study, clinical condition and survival, sex ratio, body weight and pre-weaning reflex development were assessed.

The results are summarized in Table 19 and Table 20.

Table 19 Study Design

Reproductive and Developmental Toxicity - Effects on Embryo-fetal, Pre- and Post-natal Development, Including Maternal Function		
Report Title: Influenza Vaccines (Fluarix and FluLaval): Study of Effects on Embryo-foetal, Pre-and Post-natal Development Study in CD Rats by Intramuscular Administration (Including Pre-mating Immunisation Phase)		**Test Article:** Fluarix and FluLaval **Control** Saline
Design Similar to ICH 4.1.2: Yes, modified for testing of vaccines	**Days of Dosing**: 28PM, G6, G8, G11, G15 **Day of Mating**: G0	**Study Number:** [redacted] 0009/064374
Species/Strain: CD rats **Initial Age**: 43 Days	**Day of C-Section**: G20 **Method of Administration**: Intramuscular	**Location in CTD**: 4.2.3.5
Date of First Dose: [redacted] 20[redacted] **Special Features**: None	**Litters Culled/Not Culled:** Culled to 5/sex/litter	**GLP Compliance**: Yes

Table 20 Summary of Results

Group	1	2	3
Compound	**Saline**	**Fluarix**	**FluLaval**
Dosage (µL) per Dosing Occasion	**100**	**100**	**100**
F0 Females			
No. Pre-mating	48	48	48
No. Died or Sacrificed Moribund	0	0	0
Clinical Observations	-	-	-
Necropsy Observations	-	-	-
Pre-mating Body Weight (%)[a]	229g	0	-2
Gestation Body Weight (%)[a]	402g	-1	-3
Lactation Body Weight (%)[a]	248g	1	-3
Pre-mating Food Consumption (%)[a]	22 g/animal/day	0	0
Gestation Food Consumption (%)[a]	32 g/animal/day	0	0
Lactation Food Consumption (%)[a]	116g/animal/day	3	-2
No. of Females Sperm-Positive	48	46	46
No. of Pregnant Females	48	46	46
F0 Females - Embryo-fetal Phase			
Mean No. Corpora Lutea	15.8	16.0	15.3
Mean No. Implantations	15.5	14.2	14.8
Mean % Pre-implantation Loss	3.1	10.5*	3.4
Mean No. Live Fetuses	14.5	13.4	14.1

- No Noteworthy findings. PM= Pre-mating G = Gestation day L = Lactation day

[a] At the end of the specified phase. For controls, group means are shown. For treated groups, percent differences from control are shown. Statistical significance is based on actual data (not on the percent differences).

*Lipsitz test - $p<0.05$

Table 20 (cont'd) Summary of Results

Group	1	2	3
Compound	**Saline**	**Fluarix**	**FluLaval**
Dosage (µL) per Dosing Occasion	**100**	**100**	**100**
Litters - Embryo-fetal Phase			
No. of Litters Evaluated	22	22	22
No. Live Fetuses	320	295	311
Mean No. Resorptions	0.9	0.8	0.7
Mean % Post-implantation Loss	5.6	7.6	4.4
Mean Fetal Body Weight (g)	4.02	3.97	3.90
Fetal Sex Ratios (% males)	49.3	50.6	52.2
Adverse Fetal Anomalies:			
- Gross External	-	-	-
- Visceral Anomalies	-	-	-
- Skeletal Anomalies	-	-	-
F0 Females - Littering Phase			
Mean Duration of Gestation (Days)	22.1	22.2	22.1
Abnormal Parturition	-	-	-

- No Noteworthy findings. PM= Pre-mating, G = Gestation day, L = Lactation day

Table 20 (cont'd) Summary of Results

Group	1	2	3
Compound	**Saline**	**Fluarix**	**FluLaval**
Dosage (µL) per Dosing Occasion	**100**	**100**	**100**
F1 Litters -Littering Phase			
No. Litters Evaluated	22	22	22
No of Total Litter Losses	0	0	1
Mean No. Implantations	15.5	16.1	14.7
Mean No. Pups/Litter	14.1	15.2	14.1
Mean No. Liveborn Pups/Litter	13.7	15.0	14.0
No. of Litters with Dead Pups Prior to L 1	3	4	2
Postnatal Survival to L 4 (%)	95.5	97.0	94.5
Postnatal Survival to L 4-25 (%)	95.2	96.4	98.6
Change Male Pup Body Weights L 1-25 (g)	60.8	59.6	61.4
Change Female Pup Body Weights L 1-25 (g)[e]	57.9	55.8	56.5
Pup Sex Ratios (Total L 1)[g]	53.8	47.0	50.5
Pup Clinical Signs	-	-	-
Pup Necropsy Obs.	-	-	-
Pre-weaning Behavioural Assessment			
- Surface Righting (Day of Age)	4.2	4.6	4.3
- Air Righting (Day of Age)[e]	17.3	17.2	17.2
- Startle Response (%) Pass	100.0	100.0	100.0
- Pupil Response (%) Pass	100.0	100.0	100.0

- No Noteworthy findings. PM= Pre-mating G = Gestation day L = Lactation day

[e]T-test, [f]Wilcoxon test, [g] Lipsitz test

2.6.7.13 Studies in Juvenile Animals

As noted in EMEA/CPMP Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95), this is not required for vaccines.

2.6.7.14 Local Tolerance Study

A local tolerance study ([redacted] Study Number 1536-06196) with AS03 alone and Quebec-manufactured A/Wisconsin/67/2005 antigen at a dose containing 15µg of HA (i.e. approximately 20-fold higher than the intended human dose on a body weight basis) and combined with a full human dose of AS03 has been performed. Design and results for this study are summarized in Table 21 to Table 25.

A second local tolerance study ([redacted] study number 2990/355) was conducted using the Quebec H5N1 antigen with AS03 adjuvant. In this study, the candidate vaccine was compared to a saline control and a developmental quadrivalent seasonal vaccine with and without AS03 adjuvant. The Quebec H5N1 antigen was dosed at 30µg HA in 0.5mL, the equivalent of approximately 8 times the dose intended for human use (3.8µg HA/dose). Design and results for this study are presented in Table 26 to Table 30.

Table 21 Study Design – Local Tolerance, [redacted] Study No. 1536-06196

Report Title: Pandemic Influenza Candidate Vaccine with AS03 Adjuvant (Q-Flu/AS03): A Local Tolerance Study in New Zealand White Rabbits		
Duration of Dosing: 1 Occasion (day 1) **Duration of Recovery**: 2 days		**Test Articles and Batch No**.: - Adjuvant, AS03B, DA3BA004A - Candidate Vaccine A/Wisconsin (Q-Pan) Antigen, 1M6071WC - Control, Sterile Phosphate Buffered Saline with 100µg Thimerosal (PBS),S-0037-089
Species/Strain: New Zealand White rabbit	**Route/Frequency**: Intramuscular injection, once	**Date of Dosing**: [redacted] 20[redacted]
Initial Age: Approximately 12 weeks	**Vehicle/Formulation**: Sterile physiological saline	**Study in Compliance with GLP**: Yes
Data Collected: Cageside observations (included observation for mortality, moribundity, general health, and signs of toxicity), clinical observations (included evaluation of skin and fur characteristics, injection site, eye and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor and behavior patterns), body weight and dermal Draize observation.		
Conclusion: All animals survived until scheduled sacrifice and there were no adverse clinical observations or dermal responses. There were no adverse observations noted at necropsy. Minimal or mild subacute inflammation of the subcutaneous and/or epimysial tissues was noted in animals receiving the adjuvant. There were no microscopic findings associated with the injection of the vaccine antigen. In conclusion, a single intramuscular injection of 15µg HA of Quebec antigen adjuvanted with AS03 to male and female New Zealand White rabbits was tolerated well and resulted in no adverse effects.		

Note to the reviewer: In this study, the adjuvant was, historically, named AS03B. Its composition is the same as the current AS03 adjuvant.

Table 22 Local Tolerance [redacted] Study No. 1536-06196*:* Summary of Animal Disposition and Clinical Observation

Table 1
Summary of Animal Disposition and Clinical Observations
Pandemic Influenza Candidate Vaccine with AS03B Adjuvant [Pan2 (Q-Flu/AS03B)]: A Local Tolerance Study in New Zealand White Rabbits

Sex: Male

	Group 1	Group 2	Group 3
Animal Disposition:			
Terminal Kill			
Number of Animals	3	3	3
Days from - to	4 4	4 4	4 4
Clinical Observations:			
Abrasion			
Number of Observations	.	.	2
Number of Animals	.	.	1
Days from - to	.	.	1 4

Sex: Female

	Group 1	Group 2	Group 3
Animal Disposition:			
Terminal Kill			
Number of Animals	3	3	3
Days from - to	4 4	4 4	4 4
Clinical Observations:			
None			

. - Not applicable

Nominal Dose: Group 1 - 0 ug Group 2 - 0.25 mL AS03B Group 3 - 15 ug Q-Flu

Table 23 Local Tolerance [redacted] Study No. 1536-06196*:* Summary of Cageside Observations

Table 2
Summary of Cageside Observations
Pandemic Influenza Candidate Vaccine with AS03B Adjuvant [Pan2 (Q-Flu/AS03B)]: A Local Tolerance Study in New Zealand White Rabbits

Cageside observations were performed twice daily. No abnormalities were detected.

Table 24 Local Tolerance [redacted] Study No. 1536-06196: Summary of Dermal Draize Observation

Table 3
Summary of Dermal Draize Observations
Pandemic Influenza Candidate Vaccine with AS03B Adjuvant [Pan2 (Q-Flu/AS03B)]: A Local Tolerance Study in New Zealand White Rabbits

Group Sex	Clinical Sign	Severity	Day Numbers Relative to Start Date: 1 Predose	1 3 HR	2 24 HR	3 48 HR	4 72 HR
1m	NO. OF ANIMALS EXAMINED		3	3	3	3	0
	Edema (treated site - 1)	none; no swelling	2	3	3	3	3
	Erythema (treated site - 1)	none; normal color	2	1	1	3	3
		minimal;light pink	.	2	2	.	.
2m	NO. OF ANIMALS EXAMINED		3	3	3	3	0
	Edema (treated site - 1)	none; no swelling	3	2	2	3	3
		minimal; slight swelling	.	1	1	.	.
	Erythema (treated site - 1)	none; normal color	3	3	3	3	3
3m	NO. OF ANIMALS EXAMINED		3	3	3	3	0
	Edema (treated site - 1)	none; no swelling	3	3	3	3	3
	Erythema (treated site - 1)	none; normal color	3	3	3	3	3
1f	NO. OF ANIMALS EXAMINED		3	3	3	3	0
	Edema (treated site - 1)	none; no swelling	3	2	3	3	3
		minimal; slight swelling	.	1	.	.	.
	Erythema (treated site - 1)	none; normal color	3	3	3	3	3
2f	NO. OF ANIMALS EXAMINED		3	3	3	3	0
	Edema (treated site - 1)	none; no swelling	3	3	3	3	3
	Erythema (treated site - 1)	none; normal color	3	3	3	3	3
3f	NO. OF ANIMALS EXAMINED		3	3	3	3	0
	Edema (treated site - 1)	none; no swelling	3	1	2	3	3
		minimal; slight swelling	.	2	1	.	.
	Erythema (treated site - 1)	none; normal color	3	3	3	3	3

m - Male f - Female . - Not applicable HR - Hours postdose

Nominal Dose: Group 1 - 0 ug Group 2 - 0.25 mL AS03B Group 3 - 15 ug Q-Flu

Table 25 Local Tolerance [redacted] Study No. 1536-06196*:* Summary of Gross Pathology Findings

Table 4
Summary of Gross Pathology Findings
Pandemic Influenza Candidate Vaccine with AS03B Adjuvant [Pan2 (Q-Flu/AS03B)]: A Local Tolerance Study in New Zealand White Rabbits

Sex:	MALES			FEMALES		
Group:	1	2	3	1	2	3
Number of Animals:	(3)	(3)	(3)	(3)	(3)	(3)
injection site;						
Submitted	(3)	(3)	(2)	(3)	(3)	(3)
No Visible Lesions	3	3	0	1	3	2
discoloration; red	0	0	1	2	0	1
skin;						
Submitted	(0)	(0)	(1)	(0)	(0)	(0)
abrasion	0	0	1	0	0	0

Nominal Dose: Group 1 - 0 ug Group 2 - 0.25 mL AS03B Group 3 - 15 ug Q-Flu

Table 26 Study Design – Local Tolerance, [redacted] Study No. 2990/355

Report Title: Quebec Seasonal and Pandemic Influenza Candidate Vaccines: Intramuscular Single-dose Toxicity and Local Tolerance Study in the Rabbit		
Duration of Dosing: 1 Occasion (day 1) **Duration of Recovery**: 3 days	**Test Articles and Batch No**.: Group 1: Saline (Baxter) BN08B18BE Group 2: QIV60[a] EFLAA002 + AS03½ adjuvant (half-dose) DA3AA007A Group 3: QIV60 (unadjuvanted) EFLAA001 Group 4: Quebec H5N1 antigen EFLPA001 + AS03 adjuvant (full human dose) AA03A125A	
Species/Strain: New Zealand White rabbit	**Route/Frequency**: Intramuscular injection, once	**Date of Dosing**: [redacted] 20[redacted]
Initial Age: Approximately 20-21 weeks	**Vehicle/Formulation**: Sterile physiological saline	**Study in Compliance with GLP**: Yes
Data Collected: In-life animal observations, injection site reactions, body weight, macroscopic observations and microscopic observations (injections site only)		
Conclusion: Minor inflammation observed in all groups. Adjuvanted vaccines (Group 2 and 4) were associated with fasciitis, cellulitis, and, in males, granulomatous myositis. There was no clear difference in severity between the two adjuvanted vaccines, indicating that the inflammation may be caused by the adjuvant and not the vaccine.		

[a]QIV60 is a developmental quadrivalent seasonal influenza vaccine.

Table 27 Local Tolerance, [redacted] Study No. 2990/355: Group Mean Body Weights

Test Article		Influenza Vaccines			
Group		1	2	3	4
Description		Control Saline	Candidate Vaccine 1 FluQIV60/AS03H1	Candidate Vaccine 2 Flu QIV60 unadjuvanted	Candidate Vaccine 3 Flu Q-PAN30/AS03

Day		Mean body weights (kg) for Group: 1M	2M	3M	4M
-7	Mean	2.87	2.74	2.61	2.87
	SD	0.063	0.109	0.074	0.207
1	Mean	3.19	3.02	2.88	3.16
	SD	0.096	0.198	0.090	0.186
4	Mean	3.27	3.08	2.98	3.26
	SD	0.093	0.188	0.103	0.197
Day		1F	2F	3F	4F
-7	Mean	2.65	2.68	2.72	2.75
	SD	0.270	0.031	0.150	0.032
1	Mean	2.96	2.97	3.04	3.03
	SD	0.377	0.064	0.165	0.090
4	Mean	3.07	3.09	3.09	3.12
	SD	0.413	0.084	0.180	0.085

Table 28 Local Tolerance, [redacted] Study No. 2990/355: Group incidence Macroscopic Data

	Test Article Group Description	1 Control Saline	2 Candidate Vaccine 1 FluQIV60/A903%	Influenza Vaccines 3 Candidate Vaccine 2 Flu QIV60 unadjuvanted	4 Candidate Vaccine 3 Flu Q-PAN30/A903

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TABLE INCLUDES:
SEX=ALL;GROUP=ALL;WEEKS=ALL
DEATH=ALL;SUBSET=ALL

--- N U M B E R - O F - A N I M A L S - A F F E C T E D ---

ORGAN AND KEYWORD(S) OR PHRASE		SEX:	-------MALE-------				------FEMALE------			
		GROUP:	-1-	-2-	-3-	-4-	-1-	-2-	-3-	-4-
		NUMBER:	3	3	3	3	3	3	3	3
** TOP OF LIST **										
LIVER	NUMBER EXAMINED:		3	3	3	3	3	3	3	3
KIDNEY	NUMBER EXAMINED:		3	3	3	3	3	3	3	3
CYST			0	0	0	1	0	0	0	0
PALE			0	0	1	0	0	0	0	0
ONE NOT PRESENT			0	0	0	0	0	1	0	0
LARGE			0	0	0	0	0	1	0	0
HEART	NUMBER EXAMINED:		3	3	3	3	3	3	3	3
LUNG	NUMBER EXAMINED:		3	3	3	3	3	3	3	3
PALE			0	0	0	0	0	1	0	0
DARK FOCUS			0	0	0	0	0	1	0	0
R ANTERIOR THIGH	NUMBER EXAMINED:		3	3	3	3	3	3	3	3
ANIMAL	NUMBER EXAMINED:		3	3	3	3	3	3	3	3
NOT REMARKABLE			3	2	2	2	3	2	2	3
TESTIS	NUMBER EXAMINED:		3	3	3	3	0	0	0	0
SMALL			0	0	1	0	0	0	0	0
GALL BLADDER	NUMBER EXAMINED:		3	3	3	3	3	3	3	3
SMALL			0	1	0	0	0	0	1	0
** END OF LIST **										

Table 29 Local Tolerance, [redacted] Study No. 2990/355: Group Incidence Microscopic Data

Test Article Group		Influenza Vaccines		
	1	2	3	4
Description	Control Saline	Candidate Vaccine 1 FluQIV60/AS03B+	Candidate Vaccine 2 Flu QIV60 unadjuvanted	Candidate Vaccine 3 Flu Q-PAN30/AS03

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TABLE INCLUDES:
SEX=ALL;GROUP=ALL;WEEKS=ALL
DEATH=ALL;FIND=ALL;SUBSET=ALL

		--- NUMBER-OF-ANIMALS-AFFECTED ---							
	SEX:	-------MALE-------				------FEMALE------			
	GROUP:	-1-	-2-	-3-	-4-	-1-	-2-	-3-	-4-
ORGAN AND FINDING DESCRIPTION	NUMBER:	3	3	3	3	3	3	3	3
** TOP OF LIST **									
R ANTERIOR THIGH	NUMBER EXAMINED:	3	3	3	3	3	3	3	3
--GRANULOMATOUS MYOSITIS		1	3	1	3	1	2	2	3
--FASCIITIS		0	3	1	3	1	3	1	3
--CELLULITIS		0	1	0	1	0	1	0	0
--DERMATITIS		0	0	0	0	2	1	0	2
** END OF LIST **									

Table 30 Local Tolerance, [redacted] Study No. 2990/355: Group Incidence Microscopic Data by Severity

Test Article			Influenza Vaccines	
Group	1	2	3	4
Description	Control Saline	Candidate Vaccine 1 FluQIV60/AS03₄	Candidate Vaccine 2 Flu QIV60 unadjuvanted	Candidate Vaccine 3 Flu Q-PAN30/AS03

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TABLE INCLUDES:
SEX=ALL;GROUP=ALL;WEEKS=ALL
DEATH=ALL;FIND=ALL;SUBSET=ALL

		--- NUMBER-OF-ANIMALS-AFFECTED ---							
	SEX:	-------MALE-------				------FEMALE------			
	GROUP:	-1-	-2-	-3-	-4-	-1-	-2-	-3-	-4-
ORGAN/TISSUE EXAMINED	NUMBER:	3	3	3	3	3	3	3	3
** TOP OF LIST **									
R ANTERIOR THIGH (IJ1)	NUMBER EXAMINED:	3	3	3	3	3	3	3	3
--GRANULOMATOUS MYOSITIS	->	2	0	2	0	2	1	1	0
	1>	0	2	0	2	0	1	1	2
	2>	1	1	1	1	1	1	1	1
	TL>	3	3	3	3	3	3	3	3
--FASCIITIS	->	3	0	2	0	2	0	2	0
	1>	0	0	1	1	1	2	1	1
	2>	0	3	0	2	0	1	0	2
	TL>	3	3	3	3	3	3	3	3
--CELLULITIS	->	3	2	3	2	3	2	3	3
	1>	0	1	0	0	0	0	0	0
	2>	0	0	0	1	0	1	0	0
	TL>	3	3	3	3	3	3	3	3
--GRANULOMA	->	3	3	3	3	3	3	3	3
	TL>	3	3	3	3	3	3	3	3
--DERMATITIS	->	3	3	3	3	1	2	3	1
	1>	0	0	0	0	2	1	0	2
	TL>	3	3	3	3	3	3	3	3
** END OF LIST **									

2.6.7.15 Other Toxicity Studies

Not applicable.